Enhanced Biodiesel Production from *Botryococcus braunii* Using Zn-Doped Eggshell-Based CaO Nanocatalyst

Fekraddis Molla Yalew

A Thesis Submitted to the Department of Applied Biology

School of Applied Natural Science

Presented in Partial Fulfillment of the Requirement for the Degree of

Master's in Applied Biology (Specialization in Biotechnology)

Office of Graduate Studies

Adama Science and Technology University

December 2022

Adama, Ethiopia

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Co-advisor: Dr. Yedilfana Setarge (PhD)

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DECLARATION

I hereby declare that this MSc Thesis is my original work and has not been presented for a degree in any other university, and all sources of material used for this thesis have been duly acknowledged.

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Name of Student	Signature	Date

This MSc Thesis has been submitted for examination with my approval as thesis advisor.

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Advisor	Signature	Date
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APPROVAL SHEET

This is to certify that the thesis entitled "**Enhanced Biodiesel Production from *Botryococcus braunii* Using Zn-Doped Eggshell-Based CaO Nanocatalyst**" is submitted in partial fulfillment of the requirements for the degree of Masters of Science in Biotechnology, the Graduate program of the department of Biology (Biotechnology), and has been carried out by Fekraddis Molla Yalew Id. No PGR/19133/12, under our supervision. Therefore, we recommend that the student has fulfilled the requirements and hence hereby she can submit the thesis to the department.

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We, the undersigned, members of the Board of Examiners of the final open defense by Fekraddis Molla Yalew have read and evaluated her thesis entitled "**Enhanced Biodiesel Production from *Botryococcus braunii* Using Zn-Doped Eggshell-Based CaO Nanocatalyst**" and examined the candidate. This is, therefore, to certify that the thesis has been accepted in partial fulfillment of the requirement of the Degree of Masters of Science in Biotechnology."

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ACKNOWLEDGEMENT

First, I would like to express my deep gratitude to my advisor Dr. Yeshimebet Major, for her suggestion, guidance, and assistance in this research development. I would also like to express my sincere gratitude to my research co-advisor Dr. Yedilfana Setarge for giving me the opportunity to do research and providing invaluable advices, guidance and financial support throughout this research.

My acknowledgement goes to Dr. Demeke Kifle for his kind laboratory, material and technical cooperation during culturing of microalgae. Additionally, I would like to extend my deepest appreciation to Mr. Solomon Melaku, PhD candidate at Addis Ababa University, for his guidance that helped me in all the time of this research work. Also, I would like to recognize the help I received from Mr. Temesgen Aragaw, Dr. Mekonnen Abebayehu, and Leta Guta. I am extending my thanks to all staffs of the Center for environmental sciences and Chemistry department of Addis Ababa University for their technical support and laboratory facilities.

I would also like to forward my appreciation to University of Gondar, Ethiopia, for sponsoring my MSc program and to Adama Science and Technology University department of Applied Biology (Biotechnology).

I am extremely grateful to my parents for their love, prayers, caring and sacrifices for educating and preparing me for my future. I am very much thankful to my friends Abere Asnakew, Naod Hailu, Muktar Ahmed, Natnael Alemayehu, and Yordanos Getachew for their understanding and continuing support to complete this research work. My special thanks go to my brother Yohannes Molla for his love and support during this research work.

Finally, my thanks go to all the people who have supported me to complete the research work directly or indirectly.

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LIST OF ACRONYMS AND ABBREVIATIONS

ANOVA	Analysis of variance
ASTM	American standards for testing and materials
AV	Acid value
BBM	Bold basal medium
BET	Brunauer–Emmett–Teller
BJH	Barrett–Joyner–Hlenda
CP	Cloud point
CPE	Cationic polyelectrolytes
CSN	Czech Republic
CVD	Chemical vapor deposition
DOE	Design of experiments
DSC	Differential Scanning Calorimetry
DSNR	Difference in maximum SNRL to minimum SNRL of particular parameter
dw	Dry cell weight
EN	European standard
FA	Fatty acid
FAME	Fatty acid methyl esters
FFA	Free fatty acid
FTIR	Fourier transform infrared spectroscopy
FWHM	Full-width at half-maximum or half-width
GHG	Greenhouse gas
ILs	Ionic liquids

IUPAC	International Union of Pure and Applied Chemistry
LTB	Larger-the-Better
MUFAs	Monounsaturated fatty acids
MW	Molecular weight
NTB	Nominal-the-Better
OA	Orthogonal array
PBRs	Photobioreactors
PP	Pour point
PUFAs	Polyunsaturated fatty acids
SC	Specific conductivity
SEM	Scanning electron microscopy
SNR	Signal-to-noise ratio
STB	Smaller-the-Better
SV	Saponification value
TAGs	Triacylglycerols
TEM	Transmission electron microscopy
TGA	Thermogravimetric analysis
TPD	Temperature-programmed desorption
XRD	X-ray diffraction

ABSTRACT

Botryococcus braunii is a microalga that is regarded as a potential source of renewable fuel because of its ability to produce large amounts of lipids that can be converted into biodiesel. This study aims to produce biodiesel from B. braunii microalgae oil with methanol using efficient and low-cost heterogeneous Zn-doped eggshell-based CaO nanocatalyst. The B. braunii species were isolated and cultured in a modified CHU-13 medium. The experiment was performed using white fluorescent light (2500 lux) at a photoperiod of 16:8hour light and dark cycle. The microwave-assisted solvent extraction method was used to extract microalgae oil. Synthesized nanocatalysts were characterized using X-ray diffraction (XRD), scanning electron microscopy (SEM), and Fourier transform infrared spectroscopy (FTIR) techniques. The results showed that synthesized eggshell-based CaO calcinated at 900 ℃ and impregnated Zn doped CaO exhibited an average crystalline size of 39.12 nm and 44.74nm, respectively with a regular shape, porous in structure by possessing a greater number of active sites. The total lipid content of B. braunii biomass in this study was found to be 37.7%. Taguchi's experimental design was used for the production of microalgae oil methyl ester using process parameter optimization; four factors (i.e. alcohol to oil molar ratio, catalyst concentration, reaction temperature, and reaction time) were optimized. Signal/noise (S/N) ratio and analysis of variances (ANOVA) were employed to find out the optimal process parameters. Upon optimization, maximum biodiesel conversion of 96.21% was recorded under the reaction conditions of 16:1 methanol to oil molar ratio, 5 wt% catalyst loading, 50 °C reaction temperature and 120 min of reaction time. Taguchi's model described the accuracy of the statistical approach for fatty acid methyl esters yield with R-sq: 96.18% and R-sq (adj): 92.37%. The fuel properties of the produced biodiesel were analyzed according to ASTM D6751 standards and were also good in comparison to conventional diesel. The results of this study indicated that B. braunii species is a promising source of feedstock for biodiesel production as an alternative source of fuel via microwave-assisted oil extraction and the use of eggshell-based Zn-doped CaO as a nanocatalyst.

***Key Words**: Biodiesel, B. braunii, Microwave digester, Taguchi, Transesterification, Zn-doped CaO nanocatalyst*

CHAPTER ONE

1. INTRODUCTION

1.1. Background of the Study

Bioenergy is one of the most significant components to mitigate greenhouse gas emissions and replace fossil fuels. Because of the rise in industrialization and population, the need for energy is constantly increasing. Petroleum, natural gas, coal, hydro and nuclear are the basic sources of this energy (Kulkarni & Dalai, 2006). The main drawback of using petroleum-based fuels is the atmospheric pollution created by the use of petroleum diesel. The combustion of petroleum diesel is a major source of greenhouse gas (GHG). Apart from these emissions, petroleum diesel is also a significant source of other air contaminants such as CO, NOx, SOx, particulate matter, and volatile organic compounds (Kulkarni & Dalai, 2006; Nazari & Raheb, 2015).

Renewable energy sources such as biofuels are regarded as critical to reducing dependence on petroleum fuels and lowering the impact of greenhouse gas emissions, especially carbon dioxide and methane, as well as achieving rural development goals (Abdala et al., 2020). Biomass is one of the most efficient energy sources. The introduction of biomass energy on a large scale could help to promote sustainable development on several fronts, including environmental, social, and economic. Biodiesel (monoalkyl esters) is an alternative fuel, which is obtained by the transesterification of triglyceride oil with monohydric alcohols in the presence of a suitable homogeneous or heterogeneous catalyst (acid or base) to form biodiesel (fatty acid methyl ester (FAME)) and glycerol. Biodiesel obtained from canola and soybean, palm, sunflower oil, and algal oil has been widely reported as a diesel fuel substitute (Spolaore et al., 2006). Biodiesel has proven to be a suitable substitute for petroleum-based diesel. This fuel has properties that are comparable to diesel oil and it can be used directly in diesel engines without modification to achieve similar performance. Additional advantages also include renewability and biodegradability (Abdala et al., 2020).

Biodiesel is a biodegradable and non-toxic alternative fuel that is derived from renewable sources. Biodiesel fuel can be prepared from algae, waste cooking oil, such as canola, chicken fat, coconut, corn oil, fish oil, palm, rice bran, soybean, and sunflower which would partly reduce the dependency on petroleum-based fuel. The burning of massive amounts of fossil fuel has increased

the CO_2 levels in the atmosphere, resulting in global warming (Sharif & Salleh, 2008). Since it is a renewable resource and it fixes CO_2 in the atmosphere through photosynthesis, biomass has been focused on as an alternative energy source. Since the CO_2 emitted by the burning of biomass is offset by the CO_2 fixed by Photosynthesis, its combustion has no impact on the CO_2 balance in the atmosphere, if the biomass is grown sustainably. Among biomass, algae (macro and microalgae) usually have a higher photosynthetic efficiency than other biomass (Sharif & Salleh, 2008; Nazari & Raheb, 2015). Algae are a particularly promising lipid source because of their high lipid content and ability to fix atmospheric CO_2 through photosynthetic capture. They can also be cultivated in aquatic environments using wastewater as a nutrient source or on non-agricultural land without competing with food production or impacting land use. Industrial production of algal biodiesel is still far from commercially viable, but it appears to be a potentially suitable (Tasić et al., 2016).

According to Nazari & Raheb (2015), algae are one of the best sources of biodiesel. Algae are the highest-yielding biodiesel feedstock. It has the potential to produce up to 250 times the amount of oil per acre as soybeans. Producing biodiesel from algae may be the only way to generate enough automotive fuel to replace current gasoline usage. Algae yield 7 to 31 times greater oil than palm oil. Extracting oil from algae is a simple process. Microalgae are the best algae for the production of biodiesel. They are an organism capable of photosynthesis with a diameter of less than 2 mm. Macroalgae, such as seaweed, are not as commonly used in the production of biodiesel. Microalgae produce much more oil than macroalgae and it is much faster and easier to grow (Spolaore et al., 2006; Nazari & Raheb, 2015). Microalgae can be used to produce a variety of renewable biofuels such as methane is produced by anaerobic digestion of the algal biomass, biodiesel derived from microalgal oil, and bio-hydrogen produced photobiological. The concept of using microalgae as a source of fuel is not new, but it is now gaining attention as a result of the rising price of petroleum and, more significantly, the emerging concern about global warming caused by the burning of fossil fuels (Spolaore et al., 2006; Thomas, 2006).

Microalgae are single-celled photosynthetic organisms that use carbon dioxide (CO_2) and light energy, with relatively higher photosynthetic efficiency. Microalgae cultivation needs no arable land as crops and it can grow much faster than other biofuel crops and has more energy per unit weight. Additionally, they can yield up to 40 times more oil per acre than other plants used for biofuels. In comparison to most plant-based feedstocks, microalgae have recently shown to be a

promising attribute due to their high growth rates and lipid productivities. Several microalgae including *Chlorella vulgaris, Nannochloropsis sp, Scenedesmus sp,* and others have the potential for accumulation of intracellular lipids. *B. brauni* and *Schrzochytrium sp* are microalgae contain up to 80% of lipids (Kalsum et al., 2017).

Biodiesel is typically obtained by the transesterification method, which involves a reaction between triglyceride and alcohol and a catalyst. CaO is a promising catalyst in transesterification for the production of biodiesel due to its availability, low cost, ease of preparation, and non-corrosiveness, and because of its high basic resistance, it causes less environmental impact. Thus, this solid catalyst is commonly used because of its long-life usage, high catalytic activity, and the fact that it only needs moderate conditions for the reaction to occur. CaO can be obtained from a variety of sources such as chicken eggs, ostrich eggs, bones, oyster shells, snail shells, crab shells, and calcite. CaO from eggshells, has recently been studied by researchers, which is considered a green catalyst and is promising in the biodiesel production (Da Silva Castro et al., 2018).

Waste eggshells contain large amounts of calcium compounds ($CaCO_3$) that can be easily transformed into CaO by calcination at high temperatures. Moreover, CaO is an inexpensive, non-corrosive, and environmentally friendly material that can be recycled as well as reused for further transesterification. Due to these advantages, many researchers are interested in further explorations of CaO in enhancing its overall properties. A way to improve the CaO catalytic activity is to prepare it in nanocrystalline form. Currently, the application of heterogeneous nanocatalysts has received a lot of attention due to their unique physical and chemical properties. Due to the increased surface-to-volume ratio the high catalytic activity of nanocatalysts is formed and hence greater concentrations of highly reactive sites. In addition, they can be reused again for several cycles of the reaction (Borah et al., 2019).

Zinc oxide can be used as a catalyst because it is inexpensive, commercially available, stable, reusable, and environmentally benign. However, it can be used as a support with alkaline metals or doped with other metal oxides to produce a suitable basic solid catalyst and to enhance the catalytic activity for the transesterification of vegetable oils due to its low basicity and catalytic activity. Calcium oxide, on the other hand possesses high catalytic activity and basic strength. Previous studies have demonstrated the use of a combination of CaO with other metal oxides such as Zn to provide a higher yield of FAME (Abdala et al., 2020).

In this study, a nanocatalyst was synthesized from waste chicken eggshell and enhanced catalytic activity by zinc doping techniques and characterized by various instrumentation techniques such as X-ray diffraction (XRD), Fourier transforms infrared spectroscopy (FTIR), and Scanning electron microscope (SEM). The main objective of this study was to produce biodiesel from *B. braunii* microalgae and to check the feasibility of enhanced waste chicken eggshells as a precursor for the development of a nanocatalyst and its use in the synthesis of biodiesel using *B. braunii* microalgal oil. The effects of the four parameters namely methanol to oil ratio, catalyst concentration, temperature, and time were studied on biodiesel yield. The optimization of reaction parameters was carried out via Taguchi experimental design. The produced biodiesel was also characterized according to American Standards for Testing and Materials (ASTM D6751) protocols to determine fuel properties.

1.2. Statement of the Problem

The most serious global environmental problem is climate change. The potential threat of global climate change has increased, and much of the risk has been attributed to greenhouse gas (GHG) emissions by fossil fuel usage. It has become critical to establish techniques and adopt policies to reduce the impacts of global warming that result from the increase in anthropogenic GHG emissions (Wuebbles & Jain, 2001). Another concern is a future energy crisis as a result of the depletion of fossil fuels. It is widely recognized that the continuous use of fossil fuels as a primary source of energy is unsustainable. Therefore, it is necessary to ensure new energy resources before the world is confronted with an energy crisis (Takisawa et al., 2014).

Biodiesel is attractive renewable energy for several reasons: it is biodegradable and has low toxicity; it can be used in current diesel engines with little or no modification; and it can be blended in any ratio with traditional petroleum-based diesel fuel (Demirbaş, 2002). However, there are some drawbacks to the existing biodiesel production technology: the area of crops for biodiesel production is growing, potentially destroying natural habitats and competing with food production. To meet the demand, therefore, new feedstocks such as microalgae are needed (Takisawa et al., 2014).

Agro-wastes are agricultural residues or post-harvest products that are generated through various agricultural activities and have diverse scopes for various applications. Reutilization of agro-waste such as eggshells is an environmentally friendly and cost-effective process of waste management. One of the major challenges for human society nowadays is the optimal and proper utilization of these waste by-products (Basumatary et al., 2018). For instance, eggshell is a solid waste, with the production of several tons per day. Eggshell is mostly disposed of in landfills with a high management cost. It is economical to transform the eggshell waste to create new values from these waste materials (Faridi & Arabhosseini, 2018). Thus, the utilization of waste chicken eggshells as a heterogeneous base catalyst in biodiesel synthesis will help to enhance the clean and clear environment in the society (Basumatary et al., 2018).

The catalyst used in transesterification is usually homogeneous acid or base. A faster rate of reaction is reported when using base catalysts rather than acid catalysts. Homogeneous basic catalysts provide much faster reaction rates, but the overall cost is not competitive due to separation and purification of the catalysts from the reaction mixture, and moreover it leads to soap formation,

toxic, highly flammable and corrosive (Abdala et al., 2020; Da Silva Castro et al., 2018). In the recent years, great attention has been paid on the development of heterogeneous catalyst because of its excellent properties such as environmental friendly, reusability, non-corrosiveness as well as easy catalyst separation from the reaction mixture, thus reducing the generation of pollutants (Borah et al., 2019).

Heterogeneous catalysts, such as alkaline earth metal oxides, have been investigated for biodiesel production. Among the alkaline earth metal oxides is calcium oxide (CaO) (Da Silva Castro et al., 2018). One of the most used solid catalysts for the transesterification of various feedstocks into biodiesel is calcium oxide (CaO), which has already been shown to be a very effective catalyst for biodiesel synthesis. In this regard, waste egg shells could be a potential candidate to be used as a low cost biodiesel production catalyst. Waste egg shells contains large amount of calcium compounds ($CaCO_3$) which can be easily converted to CaO by calcination at high temperature. Moreover CaO is noncorrosive, environmental friendly, and cheap material that can be regenerated as well as reused for further transesterification. These advantages draw the attention of many researchers for further explorations of CaO in improving its overall properties. A way to enhance the catalytic activity of CaO is to prepare it in nanocrystalline form (Abdala et al., 2020; Borah et al., 2019).

Currently, the application of heterogeneous nanocatalysts has received much attention owing to its unique physical and chemical properties. The high catalytic activity of nanocatalyts is due to the increased surface to volume ratio and hence greater concentrations of highly reactive sites. Moreover, they can be reused for several cycles of reaction (Borah et al., 2019). Heterogeneous nanostructure metal oxide catalysts have become more preferable for transesterification reactions due to their high catalytic activity, high stability, ease of separation from products, and high yield of the reaction product. Zinc oxide can be used as a catalyst since it is cheap, commercially available, environmentally benign, stable, and reusable. However, due to its low basicity and catalytic activity, it can be used as a support with alkaline metals or doped with other metal oxides to generate a good basic solid catalyst and to improve the catalytic activity for the transesterification of oils. On the other hand, calcium oxide possesses high basic strength and catalytic activity. Previous studies have demonstrated the use of a combination of CaO with other metal oxides to provide a higher yield of FAME (Abdala et al., 2020; Borah et al., 2019).

Currently, limited literature is reported on biodiesel production using calcium oxide doped with different metals for the transesterification reaction of algae oil. At present, there is an essential need to produce an effective and inexpensive nanocatalyst with an environmentally benign process. Thus, it is important to investigate the effect of the synthesis conditions on the catalytic performance for biodiesel production from algae oil (Abdala et al., 2020).

Usually, the optimization of biodiesel production process were carried out with the variation of one factor at a time and the response is a function of a single parameter which is time consuming and expensive. This technique cannot determine the interactive effects of all the variables and as such the overall effects of the variables on the response cannot be predicted. To reduce these shortcomings multivariate statistical methods were introduced. Taguchi Methodology, a statistical experimental design can be used to eliminate such restrictions by optimizing all the process parameters collectively (Saravanakumar et al., 2016; Sathish Kumar et al., 2015). Therefore, this study focuses on the isolation of *B. braunii* from natural sources to produce biodiesel by transesterification of *B. braunii* oil using enhanced Zn-doped eggshell-based calcium oxide nanocatalyst with Taguchi methodology.

1.3. Objectives of the Study

1.3.1. General Objective

- ✓ To produce biodiesel from *B. braunii* with methanol using enhanced heterogeneous Zn-doped eggshell-based CaO nanocatalyst and to optimize process parameters using the Taguchi methodology.

1.3.2. Specific Objectives

- ✓ To isolate, culture and harvest *B. braunii* microalgae.
- ✓ To extract *B. braunii* microalgal oil using a microwave digester and characterize the physicochemical properties of the oil.
- ✓ To synthesize and characterize eggshell-based CaO and Zn-doped CaO nanocatalyst.
- ✓ To optimize the process of production of biodiesel from *B. braunii* microalgal oil using the Taguchi methodology.
- ✓ To characterize the quality of the biodiesel produced in this study with ASTM standards.

1.4. Significance of the Study

According to their production technologies, biodiesel can be categorized as first, second, and third-generation biofuels. First-generation biofuels are unsustainable because they are produced from edible oils such as soybean, corn, and rapeseed. Although raw materials of second-generation biofuels such as jatropha, jojoba, etc. are nonedible, they need arable land, and need for heavy fertilization and irrigation. Also difficulty in obtaining raw materials of the first and second-generation, in the collection and high costs; for example, canola oil accounts for about 80% of the total operating costs; mass production and commercial application of biodiesel are still limited. For those reasons, the first and second-generation biofuel production processes are not considered sustainable (Çakırca et al., 2019). As a result, studies on biofuel production are constantly being developed to improve its sustainability and reliability as a green energy resource. Since the production cost of biodiesel is directly related to the type of oil sources, using microalgae oil as biological feedstock in the third generation could be an alternative for economic and environmental benefits.

Ashes derived from agro-wastes have recently been extensively studied as the heterogeneous base catalysts in biodiesel synthesis which is gaining increasing attention around the world. Catalysts

derived from agro-wastes are easily available, simple to prepare, easy to handle, biodegradable, non-toxic, more environmentally benign, and more cost-effective (Basumatary et al., 2018). As compared to commercial catalysts the agro-waste-derived heterogeneous base catalysts such as catalysts from eggshells are highly active for catalyzing transesterification of oil to biodiesel with a shorter reaction time and higher conversion. In general, the eggshell waste can be used in biodiesel production as a solid base catalyst used for minimizing biodiesel pollutants, reducing biodiesel production costs and making the biodiesel production process fully ecological and environment-friendly (Basumatary et al., 2018; Faridi & Arabhosseini, 2018). Furthermore, agro-waste-derived heterogeneous base catalysts perform better and more efficiently than that of the metal source-loaded ash catalysts. Moreover, efficient utilization of waste materials will help to reduce global warming and enhance the clean and clear environment in society (Basumatary et al., 2018).

1.5. Scope of the Study

The study is designed to investigate the production of biodiesel from *B. braunii* microalgae oil with methanol using an enhanced eggshell-based CaO nanocatalyst. There were different major steps to meet the research objectives these are; microalgae cultivation, harvesting, oil extraction and characterization, synthesizing CaO and Zn/CaO nanocatalyst from waste eggshell by calcination and enhancing the CaO by zinc doping wet impregnation method, characterization of the synthesized nanocatalyst, transesterification of microalgal oil with methanol using Zn-doped CaO nanocatalyst, optimizing transesterification parameters, and characterization of the physicochemical properties of produced biodiesel according to ASTM.

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Biofuels

Biofuels are solid, liquid, or gaseous fuels predominantly produced from biomass or agricultural, commercial, and/or industrial wastes. The production of renewable biofuels generally involves carbon fixation, as occurs in plants or microalgae through the process of photosynthesis. Biofuels can be broadly classified as primary and secondary (Nigam & Singh, 2011). Primary biofuels, such as wood chips, are used in their unprocessed form to supply cooking fuel, heating, or electricity production needs in small and large-scale applications. Secondary biofuels are modified primary biofuels that have been processed and produced in the form of solids (charcoal), liquids (ethanol, biodiesel, and bio-oil), or gases (hydrogen and methane). They can be used for a range of applications including transportation and industrial processes. Using renewable sources to produce biofuels along with microalgae as raw materials can minimize the conflict between food and fuel. Biofuels produced from microalgae generate low greenhouse gas (GHG) emissions, which reduce environmental impacts (Hoekman, 2009).

2.2. Different Generations of Biofuels

Current concerns about climate change as a result of greenhouse gas emissions and energy crises have prompted the need to transition from unsustainable fossil-derived energies to renewable and sustainable energies. Renewable carbon sources must be used in the development of sustainable and economically viable bio-refinery processes for the biofuel production (Gaurav et al., 2017).

Biofuels produced from food-based crops such as starch- and sugar-based substrates, for example, corn and sugarcane, are considered first-generation biofuels. Nevertheless, when it comes to using feedstocks for first-generation fuels, there is a food-versus-fuel debate. Thus, next-generation biofuels were introduced and are regarded as critical for meeting the world's energy demand in the transportation sector (Dellomonaco et al., 2010).

Second-generation biofuels are produced from lignocellulosic biomass, and they can minimize carbon emissions, improve energy efficiency, and reduce nations' energy dependency. Non-food lignocellulosic substrates are abundant and potentially inexpensive organic sources for the production of renewable chemicals and fuels. Lignocellulosic wastes can be originated from

agricultural residues (e.g., straws, stover, and non-food seeds), forestry wastes (i.e., hardwoods and softwoods), industrial wastes (e.g., sawdust, paper mill discards, and food industry wastes), domestic wastes (e.g., kitchen wastes, sewage, and waste papers), and municipal solid wastes (Behera et al., 2014).

Third-generation biofuels are produced from algae. Biofuel production from algal species, such as *Chaetoceros calcitrans, B. braunii,* several *Chlorella* spp, *Schizochytrium limacinum, Nannochloropsis, Isochrysis galbana* and *Scenedesmus* spp, is a promising technology because algae are fast-growing, compared to many terrestrial plants, with no soil needed, while they have high capturing ability for CO_2 and other greenhouse gases. Algae contain significant amounts of carbohydrates and lipids (up to 70%), which makes them attractive feedstocks for conversion to biofuels. Different technologies have been reported to produce biodiesel, bioethanol, bio-hydrogen, and biogas from micro- and macro-algae (Kumar et al., 2016).

Engineered algae are used in fourth-generation biofuels for biofuel production from oxygenic photosynthetic organisms. This technology is reported to be capable of producing gaseous biofuels, algal ethanol, algal butanol, four carbon alcohols, and algal biodiesel (Lü et al., 2011).

2.3. Algae: Potential Feedstock for Biofuel Production

Algae are photosynthetic organisms that derive their biomass from solar energy and carbon dioxide from the atmosphere. They range from unicellular to multicellular, microscopic to gigantic kelps, and vary in shape from spherical to filamentous. Algae are regarded as a good candidate for biodiesel production because of several advantages such as high photosynthetic effectiveness, rapid growth rate, and high biomass productivity. The algae biomass contains carbohydrates, lipids, proteins and nucleic acid. The lipids are present in form of triacylglycerides (TAGs) that are utilized for biodiesel production (Amaro et al., 2011).

They can grow on non-arable land, even in brackish and saline waters; they can tolerate the desert, arid, or semiarid lands; and require less amount of water for their growth and survival as compared to other terrestrial crops. As compared to other crops that are normally harvested once or twice a year algae can be harvested all year round. Based on their size they are subdivided into two major categories as Micro and Macroalgae. Microalgae are easily distinguished from their larger photosynthetic cousins, the macroalgae, which have evolved defined anatomical structures resembling leaves, stems, and roots of higher plants (Pienkos et al., 2010).

2.4. Microalgae

Microalgae are microscopic algae existing as unicellular organisms. They partake in a process known as photosynthesis (Equation 1), by which carbon dioxide gets converted to glucose as a stored energy source.

$$6CO_2 + 6H_2O + \text{sunlight} \longrightarrow C_6H_{12}O_6 + 6O_2 \quad \text{.....Equation (1)}$$

Microalgae are responsible for fixing more than 40% of fixed CO_2 globally. Furthermore, microalgae grow in a higher density than traditional feedstock, have a relatively fast growth, and have the ability to accumulate a higher quantity of cellular lipids and carbohydrates than conventional feedstocks. The lipid and carbohydrate productivity of microalgae is strongly linked to microalgal strain (e.g. marine or freshwater species) and climatic conditions (e.g. light intensity, temperature). Some microalgae have been reported to produce up to 50% of dry cell weight (dw) of triacylglycerols, as lipid storage, under photo-oxidative stress or other adverse environmental conditions (Ge et al., 2016).

In addition, microalgae can be produced year-round and have oil productivity (12,000 l/ha) that greatly exceeds that of oilseed crops, such as rapeseed (1190 l/ha). They can be grown on marginal or infertile land with adequate moisture, CO_2, and sunlight, which minimizes competition with land for agricultural food sources, and in wastewater, whereby they participate in municipal wastewater remediation. This remediation involves the removal of nitrogen and phosphorus from the wastewater, diminishing the potential for eutrophication. Microalgae can also be used to produce valuable co-products, such as proteins and residual biomass after lipid extraction, which may be used as fertilizer or animal feed, or bioethanol or biomethane through fermentation processes. For these reasons, microalgae are currently considered to be one of the most promising feedstocks for renewable energy (Ge et al., 2016; Halim et al., 2012).

2.5. Biofuels Derived from Microalgae

A variety of biofuels are derived from microalgae because of their unique potential. The biofuels include alcohols, which are produced via fermentation, algal biomass processing through dual approach of fermentation and hydrolysis, conventional method of transesterification, gasification of biomass or Fischer-Tropsch synthesis.

2.5.1. Biodiesel

Biodiesel has equivalent engine performance to petroleum diesel fuel, while reducing sulfur and particulate matter emissions. Biodiesel is a non-toxic, biodegradable alternative fuel derived from renewable sources. Triacylglycerols (TAGs) are transesterified with an acid or alkali catalyst to produce biodiesel and glycerol during the manufacturing process (Johnson & Wen, 2009).

Fatty acid methyl esters (FAME) are processed in algal biodiesel production. The biodiesel chemical composition is usually produced by transesterification of algal oil in the presence of an acid or alkali as a catalyst. The biodiesel from algae can be derived directly from algal biomass through transesterification. Alternately, it can also be produced by a two-step process in which the lipids are extracted initially and then transesterified, though either of the processes involves lipid extraction through solvents and alcohols like isopropanol, methanol, and petroleum ether (Johnson & Wen, 2009). Biodiesel produced from microalgae can be a great solution to the existing diesel crisis, but strains with a high growth rate and oil content must have to be chosen to efficiently produce biodiesel from microalgae (Li et al., 2017).

2.5.2. Biogas

The anaerobic digestion of organic matter leads to the formation of fuel called biomethane or biogas. Biogas is mainly formed by CO_2 (25–45%) and methane (55–75%) (Oncel, 2013). Biopolymer hydrolysis to monosaccharides mediated by hydrolytic bacteria, conversion of monosaccharides to acids via fermentation, the action of acetogenic bacteria leading to the formation of acetate and methane, and carbon dioxide formation by methanogenic bacteria are the four stages of anaerobic digestion (Oncel, 2013).

Microalgae have been reported to generate biogas as a source of fuel, although the yield of biogas formation is quite low due to the low carbon and nitrogen (C:N) ratio and sensitivity of algal cells to bacterial degradation, which leads to the formation of inhibitor (ammonia). Residual biomass free from lipids and amino acids was investigated for biogas production in *Scenedesmus* spp., and results exhibited that residual biomass gives better biogas yield compared to raw biomass (Ramos-Suárez & Carreras, 2014).

2.5.3. Hydrocarbons

Hydrocarbons can be produced by microalgae species, which can then be converted to diesel, gasoline, and kerosene. For example, the microalgae, *B. braunii*, has been reported to produce hydrocarbons with excellent oil yield. *B. braunii* lives in freshwater, which can be one of the factors leading to its adaptation to a diverse range of salt concentrations. Furthermore, the hydrocarbons from *B. braunii* get deposited outside the cell, thus the extraction becomes relatively simpler and more convenient (Ranga Rao et al., 2007).

2.5.4. Hydrogen

Microalgae can directly generate hydrogen from sunlight and water, only in the complete absence of oxygen. Since hydrogen does not emit greenhouse gases and releases water as a by-product then, it is a promising future energy source. There are some limitations existing regarding the large-scale production of hydrogen as fuel.

Hydrogen is produced by steam reformation, photofermentation, and photolysis of water-mediated by photosynthetic algae. Green sulfur bacteria get hydrogen gas from hydrogen sulfide (H_2S), while purple non-sulfur bacteria derive hydrogen from diverse substrates. Other microalgae can directly make hydrogen from sunlight and water, but only in the complete absence of oxygen. Searching new organisms for hydrogen production, optimization of growth conditions and use of biotechnological techniques can open new doors to making hydrogen a viable fuel for future fields (Melis & Happe, 2001).

2.5.5. Biosyngas

Biosyngas are generated by biomass gasification in presence of oxygen, water vapor, or air, which produces hydrogen, methane, carbon monoxide, water, other hydrocarbons, and ashes. For gasification, a high temperature (800–1200°C) is needed, and the biomass feedstock must contain no more than 20% water content. Burning in boilers and turbines can produce electricity and subsequently, naphtha, wax, kerosene, and gasoline can be obtained (Ghasemi et al., 2012).

2.5.6. Ethanol

Ethanol generation from or by microalgae has a lot of potentials, but it is still only in the preliminary phase of research. Bioethanol can be used as a biofuel, which can substitute part of the fossil-derived petrol. To analyze a full-scale production system, more development is needed.

Bioethanol is currently produced by fermenting sugars, which in the case of corn are derived from hydrolyzing starch. There have been reports of microalgae species with a starch content of more than 50%. Using new technologies, cellulose and hemicellulose can be hydrolyzed into sugars, thereby allowing for the formation of ethanol from a major portion of dry algal biomass (Hamelinck et al., 2005). In comparison to the conventional use of woody biomass, microalgae provide several advantages, including (Deng & Coleman, 1999): microalgae lack lignin so the processing becomes easier; the microalgal cellular composition is very simple and biomass can be utilized readily; microalgal cells consist of copious amounts of polysaccharides, which can be then converted to sugar and microalgae can be genetically engineered to produce ethanol.

2.5.7. Advantages and Disadvantages of Biodiesels

Table 1: Advantages and disadvantages of the biodiesel (Firoz, 2017).

Advantages of biodiesel fuel	**Disadvantages of biodiesel fuel**
• Simple to use: No vehicle modification or any fueling apparatus is needed.	• Currently, Biodiesel fuel is about one and a half times more expensive than petroleum diesel fuel.
• Power, Performance, and Economy: Biodiesel is a beneficial fuel due to its proven power generation, performance and cost effectiveness.	• Soybean crops requires energy to be planted, fertilized, and harvested in addition to the energy needed to make biodiesel fuel from them.
• Impact on the environment: Biodiesel aids in reducing pollution and enhancing health by lowering CO_2 emission which reduces the impact of global warming.	• As Biodiesel cleans the dirt from the engine, this dirt can get collected in the fuel filter and causing clogging. Therefore, filters should be changed regularly.
• The use of foreign oils is reduced via biodiesel.	• Another drawback of the biodiesel fuel is that biodiesel fuel distribute on infrastructure needs improvement.
• Compared to petroleum, biodiesel is less toxic and easier to store and this makes it safer to handle.	

Table 1 Continued

• By keeping energy dollars at home, biodiesel benefits the communities.

2.6. Biodiesel Production from Microalgae

Biodiesel is a liquid fuel made up of mono alkyl esters (ethyl or methyl) of long-chain fatty acids derived from animal fats or vegetables, as well as micro and macroalgal oil. Biodiesel is the name given to fuel for diesel engines that is made by chemically converting animal fats or vegetable oils. It can also be described as a biofuel made up of mono-alkyl esters of long-chain fatty acids produced by transesterification of renewable bio-lipids. It is a non-toxic, biodegradable, clean-burning, and eco-friendly fuel produced primarily from plant oils and animal fats (Indhumathi et al., 2014). Biodiesel can be produced from lipid sources such as waste oil, oil crops, animal fat, and microalgae. Among all the feedstock, microalgae, which grow on sludge, saltwater, contaminated or wastewater on non-arable or marginal lands, have been suggested to be more viable and rich source of lipids for biodiesel production because it does not compete with food production neither fresh water nor farmland in any way. Microalgae can also be grown in arid and semi-arid regions that have poor cultivation conditions for common plants (Chen et al., 2012).

2.6.1. Lipid Content and Fatty Acid of Microalgae

In general, the fatty acid composition and lipid content of every biodiesel feedstock are significant factors to consider in the biodiesel production process. Table 2 shows the lipid content of some species of microalgae and also it shows that some algae species have more than 50% lipid content. The fatty acid composition and lipid content have important effects on the yield and quality of biodiesel produced (Atabani et al., 2013). The structure of fatty esters determines the very vital properties of biofuel such as cetane number (ignition quality), oxidative stability, cold-flow properties, and iodine value.

Furthermore, the fatty ester properties are determined by the length of the carbon chain, its degree of unsaturation, and the alcohol moieties that comprise a fatty ester. Therefore, the microalgae species that will be suitable for biodiesel production must have a suitable fatty acid (FA) composition and high lipid productivity. Microalgae fatty acid composition can be either saturated or unsaturated. Unsaturated fatty acids may vary in the position and number of double bonds on

the carbon chain backbone. MUFAs (Monounsaturated fatty acids) contains one double bond while PUFAs (polyunsaturated fatty acids) contain two or more double bonds. Individual fatty acids are called dienoic, trienoic, tetraenoic, pentaenoic, and hexaenoic fatty acids based on the number of double bonds. A fatty acid may also be either x3 PUFA or x6 PUFAs depending on the position of the first double bond from the terminal methyl end (x) of the carbon chain, (x3 implies the third carbon from the end of the fatty acid, while x6 implies the sixth carbon from the end of the fatty acid) (Basova, 2005; Hu et al., 2008). Some algae have the ability to synthesize medium-chain fatty acids such as C10, C12, and C14 as predominant species, whereas others produce very-long-chain fatty acids (>C20).

Biodiesel generated from saturated fat has superior oxidative stability, higher cetane, poor low-temperature properties and they are more likely to gel at ambient temperatures, while biodiesel produced from feedstocks that are high in PUFAs has good cold flow properties but they are susceptible to oxidation which could result to instability problems during prolonged storage. For satisfactory low-temperature operability and oxidative stability, research suggests that quality biodiesel should contain relatively low concentrations of both long chain saturated fatty acid methyl esters (FAME) and polyunsaturated FAME (Basova, 2005; Hu et al., 2008).

Table 2: Lipid content of some microalgae species (Takisawa et al., 2014).

Microalgae species	**Lipid content (% dry wt)**
Anabaena cylindrical	4–7
Ankistrodesmus sp.	24–41
B. braunii	25–80
Chlamydomonas reinhardtii	21
Chlorella emersonii	28–32
Chlorella minutissima	57
Chlorella protothecoides	57.9
Chlorella pyrenoidosa	2
Chlorella sp	28–32
Chlorella vulgaris	14–22
Crypthecodinium cohnii	20–51

Table 2 Continued

Cylindrotheca sp	16–37
Dunaliella bioculata	8
Dunaliella primolecta	23
Dunaliella salina	6
Dunaliella tertiolect	35.6
Euglena gracilis	14–20
Hormidium sp.	38
Isochrysis sp	25–33
Monallanthus salina	>20
Nannochloris sp.	30–50
Nannochloropsis sp	31–68
Nanochloris sp	20–35
Neochloris oleoabundans	35–54
Nitzschia sp	45–47
Phaeodactylum tricornutum	20–30
Pleurochrysis carterae	30–50
Porphyridium cruentum	9–14
Prymnesium parvum	22–38
Scenedesmus dimorphus	16–40
Scenedesmus obliquus	12–14
Schizochytrium sp	50–77
Spirogyra sp	11–21
Spirulina maxima	6–7
Spirulina platensis	4–9
Synechoccus sp.	11
Tetraselmis maculate	8
Tetraselmis suecia	15–23
Zitzschia sp.	45–47

Table 3: Comparison of microalgae with other biodiesel feedstocks (Takisawa et al., 2014).

Plant source	Oil content (% dry weight biomass)	Oil yield (L oil/ha year)	Land use (m^2 year/kg biodiesel)
Corn	44	172	66
Hemp	33	363	31
Soybean	18	636	18
Jatropha	28	741	15
Camelina	42	915	12
Canola/Rapeseed	41	974	12
Sunflower	40	1070	11
Castor	48	1307	9
Palm oil	36	5366	2
Microalgae (low oil content)	30	58,700	0.2
Microalgae (medium oil content)	50	97,800	0.1
Microalgae (high oil content)	70	136,900	0.1

2.7. *Botryococcus braunii*

B. braunii is a green pyriform shaped planktonic microalga, with colonies growing in a group and they can be found in temperate or tropical oligotrophic lakes and estuaries, widely found in freshwater and brackish lakes, ponds, reservoirs, or even ephemeral lakes. In all climate zones, *B. braunii* strains can be found except in Antarctica. Cell size (length-width) is in the range of (8–95) mm to (137–9) mm (Liu et al., 2016). *B. braunii* has been identified as the most promising for biofuel production among various micro and macro algae species, because of its excellent lipid production capability (content up to 80%, see Table 2). Even though this microalga is known for high amounts of hydrocarbons and ether lipids, its high content of saturated and monounsaturated FAs as well as TAGs makes it suitable for biodiesel production. Saturated, monounsaturated and polyunsaturated FAs percentages in *B. braunii* dry biomass revolve around 9.85%, 79.61% and 10.54%, respectively (Nascimento et al., 2013). During the steady-state phase of algae growth, most of the FAs are synthesized with the highest content of palmitic and oleic acids located intracellularly, but also in small amounts in the extracellular medium.

Since all known species of microalgae have cytoplasmic lipids, lipid secretion in the extracellular medium is an unusual phenomenon of *B. braunii*. Biosynthesis of FAs lipids by *B. braunii*, for instance, starts in the plastid, elongates within the endoplasmic reticulum, transfers to the endoplasmic reticulum plasma membrane or via Golgi bodies and then secretes to the cell surface. On the surface of the cell apex the lipid droplets are seen. The yield and lipids composition vary with the strain, growth conditions and cell aging (Hirose et al., 2013).

Table 4: Classification of *B. braunii*

Microalgae	***Botryococcus braunii***
Domain	Eukaryota
Kingdom	Plantae
Phylum	Chlorophyta
Class	Trebouxiophyceae
Order	Trebouxiales
Family	Botryococcaceae
Genus	Botryococcus
Species	*B. braunii*

2.8. Microalgal Biomass Production Systems

Microalgae cultivation can be done in open-culture systems (Figure 1) such as ponds or lakes or in highly controlled closed-culture systems called photobioreactors (PBRs, Figure 2).

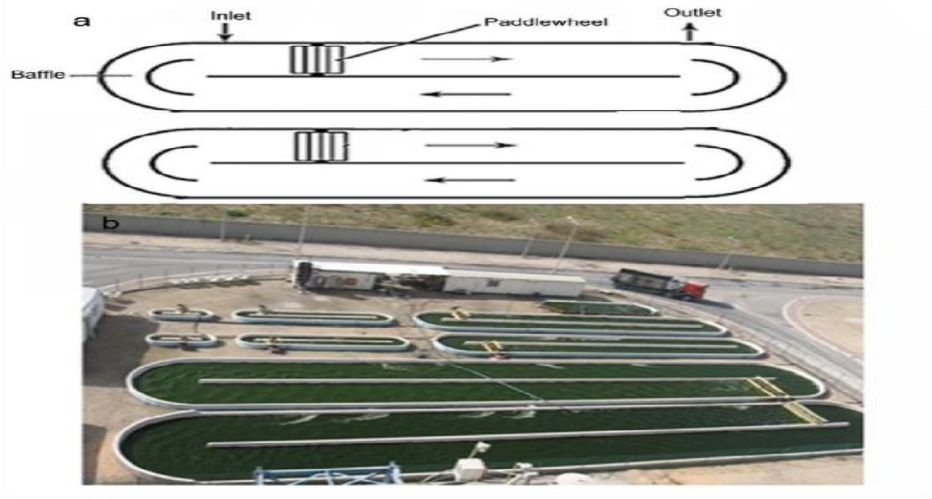


Figure 1. Open cultivation system for growing algae: a) diagram shows the constituents of the open pond, b) Picture of race-track style open-pond (El-Sheekh & Abomohra, 2016).

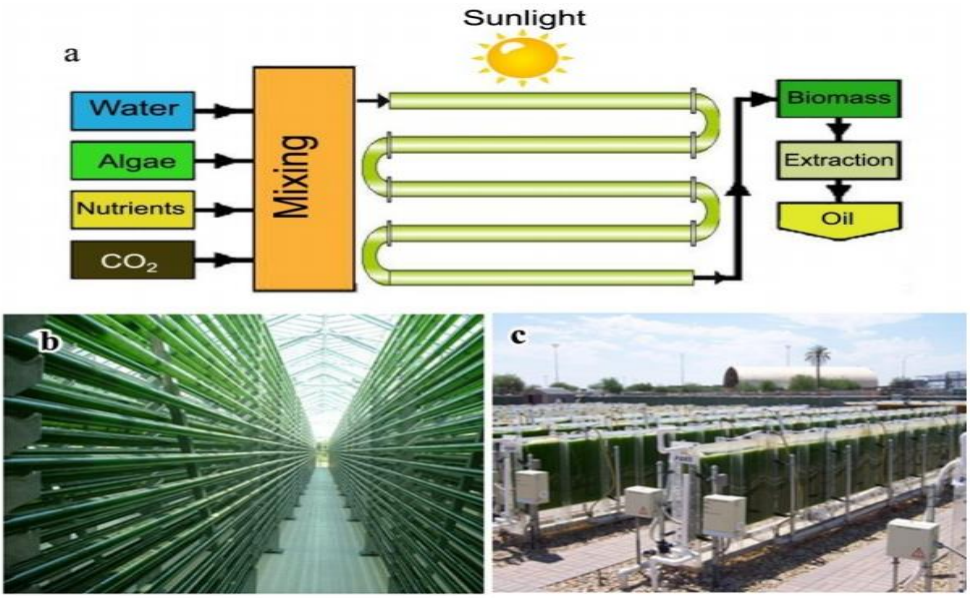


Figure 2. Photobioreactor system for growing algae: a) the constituents of a tubular photobioreactor, b) tubular vertical photobioreactor, and c) flat plate photobioreactor (El-Sheekh & Abomohra, 2016).

Open-culture systems (Figure 1) are normally less expensive and more durable than photobioreactors. However, according to (Richmond, 2004), ponds require more energy to homogenize nutrients and the water level cannot be held much lower than 15 cm (150 L m^{-2}) for

the microalgae to obtain enough solar energy to grow. Due to the lack of control over water temperature, evaporation, and lighting, open ponds are more susceptible to weather conditions. Also, they may produce large quantities of microalgae, but occupy a more extensive land area and are more susceptible to contaminations from other microalgae or bacteria (Mata et al., 2010).

PBRs (Figure 2) on the other hand, are more controlled systems and can be optimized according to the physiological and biological characteristics of the cultivated algal species, enabling the cultivation of algal species that cannot be grown in open ponds. PBRs are considered to have several advantages over open ponds; they offer better control over culture conditions and growth parameters (temperature, pH, mixing, CO_2, and O_2), reduce CO_2 losses, prevent evaporation, allow to attain higher microalgae densities or cell concentrations, higher volumetric productivities, provide a more safe and protected environment, preventing contamination or minimizing invasion by competing microorganisms (Mata et al., 2010). Despite their benefits, PBRs have a number of drawbacks that need to be considered and solved. Their main limitations include: overheating, oxygen accumulation, bio-fouling, the high cost of building and operating algal biomass cultivation, difficulty in scaling up, cell damage by shear stress and deterioration of material used for the photo-stage (Richmond, 2004).

2.9. Harvesting Methods

Biomass harvesting is the process of separation of biomass from the culture medium. 20%-30% expenditure was recorded due to the harvesting process but in some cases, it was increased up to 50% and up to 90% (Milledge & Heaven, 2013). The challenges of harvesting include the small size of the microalgae cells, similarity in the density of the algae cells and the medium, formation of stable suspensions due to presence of negative charge on the surface of algae cell requirement of frequent harvesting of biomass due to high growth rate (Milledge & Heaven, 2013). To meet these challenges, several different methods for harvesting algae biomass have been tested.

2.9.1. Sedimentation: Sedimentation is the process in which the liquid or solid particles are separated from the liquid medium (with different densities) with the help of a gravitational force (Milledge & Heaven, 2013). *Chlorella*, a common round-shaped alga, has achieved a settlement velocity of 0.1 m/day in fresh water. However, the settlement rate varies greatly depending on factors such as the type of microalgae, age of the cell, nutrient deficiency, and light intensity.

Therefore, sedimentation is not widely used to harvest algae biomass. The recovery of harvesting was also low i.e. 60%-65% of biomass (Shen et al., 2009).

2.9.2. Flocculation: Flocculation is the aggregation of microalgae cells by the use of flocculating agents. It increases the rate of sedimentation. Flocculation is considered as most reliable but expensive method. There are two types of flocculation these are: auto-flocculation and induced flocculation (Mata et al., 2010). Due to various environmental factors such as stress, changes in nitrogen, pH, and dissolved oxygen, the cells of microalgae get flocculated. But it is a slow and unreliable process. Flocculation can be induced physically, biologically, or chemically. The flocculants should be less expensive, non-toxic, highly effective in low concentration, derived from non-fossil fuel, sustainable and renewable sources. Chemical flocculants may be organic or inorganic. Inorganic flocculants include lime, aluminum chloride (alum), ferric sulfate and ferric chloride. In case of *Chlorella* and *Scenedesmus*, alum has been found more effective flocculent (Molina et al., 2003). These inorganic flocculent can be toxic, causing algal biomass to be damaged. However, inorganic flocculants are less expensive than organic flocculants. So, some new inorganic flocculents must be tested which don't have any adverse effects (Milledge & Heaven, 2013; Molina et al., 2003).

Organic flocculants like cationic polyelectrolytes (CPE), at a concentration of 2 mg/l-25 mg/l provide 35 times higher results. Chitosan is a renewable organic flocculant obtained from crustacean shells that are used to treat wastewater in the food industry. It is non-toxic in nature, but its dosage is very high ranging from 20 mg/l-150 mg/l (Harith et al., 2009; Molina et al., 2003). Another category of flocculent that is used to harvest microalgae is starch and modified starch. These modified starches can be used more effectively than both synthetic organic and inorganic flocculants, but they are more expensive (Vandamme et al., 2010). Microorganisms such as bacteria can be utilized as a flocculent and also reported in case of *Chlorella* and *Pleurochrysis carterae* but they need a good amount of carbon source to grow which made this process less cost-effective. Thus, there is no single or universal flocculent that can be used to harvest all types of microalgae, even though flocculation is a potential method of harvesting due to the input of energy is very less (Milledge & Heaven, 2013; Molina et al., 2003).

2.9.3. Flotation: Flotation is commercially done by introducing air bubbles into the culture. Flotation may be classified as dissolved air flotation, electrolytic flotation and dispersed air

flotation. At a gas pressure of 3 atmospheres, a sparger is used to prepare bubbles. The range of size of bubbles is 10 to 100 micrometers. However, a lot of energy is needed for the production of a small bubble, which is large in comparison to centrifugation that made this process less economic.

2.9.4. Filtration: At a very large scale, filtration gives satisfactory results. The filters used in the filtration process have a range and can be categorized according to pore size as micro filtration (0.1-10 µm), ultra-filtration (0.02-0.2 µm), macro filtration (>10 µm), reverse osmosis (is less economic than centrifugation at commercial scale) (Molina et al., 2003). Ultra-filtration is used for fragile cells and microalgae can't be processed with it as the operating and maintenance costs are very high (Mata et al., 2010; Molina et al., 2003). Filter press is also a method of filtration. Filter presses have a number of advantages such as flexibility, simple design, and the ability to handle a wide range of slurries, and the equipment is relatively cheap. However, the labor costs can be high. For the filtration of large microalgae, rotary vacuum filters with a simple filter design are used, but they are not effective for smaller species. Two extensive reviews have also concluded that filtration is good for large cells of algae but not very effective for cells with a diameter of less than ten micrometers (Molina et al., 2003; Uduman et al., 2010).

2.9.5. Centrifugation: In centrifugation, gravity is replaced as the force driving separation at a much greater rate. Almost all types of microalgae can be reliably and easily separated by centrifugation. The most common industrial centrifuge is the disc stacking centrifuges, which are widely used in commercial plants for high-value algal products and in algal biofuels pilot plants (Molina et al., 2003). They are ideally suitable for separating particles of the size ranging from 3 to 30 micrometers and concentrations ranging from 0.02%-0.05% of the algal cells in a growth medium. On a continuous basis they can separate liquid/solid, liquid/liquid, or solid/liquid. Disc stacking centrifuges generally have a high energy consumption (Uduman et al., 2010).

2.10. Algal Oil Extraction

2.10.1. Mechanical Methods of Oil Extraction

2.10.1.1. Expeller Press

The expeller press, also known as an oil press, is one of the simplest and oldest methods for extracting oil from oil seeds. The mechanical crushing method is often used to extract oil from

algal biomass, which is a simple and effective method. Oil content is retained in dried algal biomass, which can then be pressed out using an oil press. The idea underlying this technique is to apply high mechanical pressure to the cells in order to crush and break them and squeeze out the oil from the algal biomass. Pressure applied in a particular range improves extraction efficiency, but too much pressure will reduce lipid recovery, increases heat generation, and causes choking problems (Ramesh, 2013).

2.10.1.2. Bead Beating

Bead beating is a mechanical method for cell disruption in which the concept of high-speed spinning of the biomass slurry with fine beads causes direct damage to the cells (Geciova et al., 2002). In bead mills, the impact of grinding beads against the cells disrupts the cells. The above method can be used to process all types of cells including those of microalgae. The two common types of bead mills are shaking vessels and agitated beads. In the shaking vessel type, shaking the entire culture vessel damages the cells. In most cases, multiple vessels are shaken on a vibrating platform and this type of bead mill is best for samples that need similar disruption treatment conditions. As a result, this setup can only be used on a laboratory scale. The second type, in which the beads are agitated along with the cell culture, achieves better disruption and extraction efficiencies. Since the rotating agitator within the culture vessel produces heat, the vessels are provided with cooling jackets to protect the heat-sensitive biomolecules. The combined effect of agitation, collision, and grinding of the beads unquestionably produces a more effective disruption process (Lee et al., 2012).

2.10.1.3. Ultrasonic-Assisted Extraction

Ultrasound-assisted lipid extraction is an alternative method that avoids the difficulties that come with traditional mechanical disruption methods. The process is simple with easy working set-up conditions, resulting in a higher purity final product and eliminating the need to treat wastewater produced during the process. Furthermore, the technique is more cost-effective and environmentally friendly and it can be completed in a very short time with high reproducibility. When compared to that in conventional methods, the energy input is very little and can be operated at lower temperatures (Chemat et al., 2011). When using liquid cultures, there are two main mechanisms by which ultrasound can damage the cells. These are: cavitation and acoustic streaming. Cavitation is the formation of microbubbles as a result of applied ultrasound, which in

turn can cause pressure on the cells to break up, and acoustic streaming allows the algal culture to be mixed more easily. Because of the rapid compression/decompression cycles that occur during the treatment, the ultrasonic waves produce transient and stable cavitation. Transient cavitation will result from unsteady oscillations, which will eventually implode. The microalgal cells are disrupted by a cavitation implosion, which causes extremely localized heat shock waves. Thus, due to the cavitation effect, sonication cracks the cell wall and membrane. The two critical steps in determining the lipid yield extraction efficiency are microstreaming and heightened mass transfer resulting from cavitation and bubble collapse (Adam et al., 2012; Chemat et al., 2011).

2.10.1.4. Microwave-Assisted Extraction

Previously, the applications of microwave radiation were limited to the digestion of samples for the measurement of trace metals and the extraction of organic contaminants. In the mid-1980s, the feasibility of extracting lipids using microwave irradiation was first reported. They developed a microwave extraction technique that was more efficient than the traditional methods for isolating lipids and pesticides from seeds, foods, feeds, and soil. Therefore, microwave technology has enabled the development of rapid, safe, and cost-effective lipid extraction methods that do not require the dewatering of algal biomass. Similarly, among the other tested methods for microalgal lipid extraction, the use of microwave remains the most simple and most effective method (Lee et al., 2010). Because of the frictional forces arising from inter- and intra-molecular movements, a dielectric or polar material introduced in a rapidly oscillating electric field, such as that generated by microwaves, will produce heat. Intracellular heating causes the formation of water vapor, which causes internal cell disruption. This causes the electroporation effect, which further opens up the cell membrane, thereby allowing for efficient intracellular metabolite extraction. Thus, rapid generation of pressure and heat within the biological system drives out compounds from the cell matrix, resulting in the production of high-quality extracts with a better target compound recovery (Hemwimon et al., 2007).

According to (Šoštarič et al., 2012), microwave-pretreated microalgae have higher bio-oil yields due to the presence of several micro-cracks in the cell wall. The oils can also be extracted and transesterified into biodiesel using microwaves. Microwaves are the pick of the options at the current due to the economics involved in the above process; it is supposed to be attractive because of their short reaction time, low-operating costs, and efficient extraction of algal oils. The recovery

of biodiesel from the reaction mixture in a microwave-assisted process takes about 15–20 minutes, which is much faster than the 6-hour cycle required by the traditional heating method (Refaat et al., 2008). The microwave-assisted method, on the other hand, has a disadvantage in terms of the maintenance costs involved, particularly on a commercial scale.

2.10.2. Chemical Methods for Extraction of Oil

2.10.2.1. Solvent Extraction Method

The concept of chemistry 'like dissolving like' is the basic principle behind the extraction of lipids from microalgae using solvents. An ideal solvent requires a high degree of specificity for lipids mainly acylglycerols and the solvent must be sufficiently volatile to ensure low energy distillation to separate the lipid from solvents. Non-polar solvents including hexane, diethyl ether, chloroform, benzene, toluene, and polar solvents like methanol, ethyl acetate, acetone, and ethanol can be used to extract lipids from algal biomass. The hydrophobic interactions between non-polar and neutral lipids present in the algae biomass are disrupted by non-polar solvents. The solvents used for lipid extraction from microalgae biomass are n-hexane, ethanol, dimethyl ether, and mixtures of chloroform/methanol, n-hexane/ethanol, n-hexane isopropanol, n-hexane/2-propanol, methanol/1-ethyl-3-methyl imidazolium methyl sulfate, 1-butanol, methylene chloride/methanol, DBU (1,8-diazabicyclo-[5.4.0]-undec-7-ene), DBU/octanol, DBU/ethanol, dichloroethane/methanol, dichloroethane/ethanol, and acetone/dichloromethane (Mubarak et al., 2015). For extracting lipids such as from animal tissues, the Folch method used a mixture of chloroform: methanol 2:1 by volume, and called it the Folch method. For total lipid extraction and purification the Bligh and Dyer method used chloroform and methanol as solvents, and water as co-solvent. The Bligh and Dyer method of lipid extraction has been employed in a number of studies, and modified forms of this method have also been used (Lee et al., 2021; Mubarak et al., 2015).

2.10.2.2. Accelerated Solvent Extraction

(Richter et al., 1996) proposed a method known as accelerated solvent extraction (ASE), which uses the organic solvent at increased pressure (500–3000 psi) and temperature above its boiling point (50–200 °C) in an effort to increase the efficiency of organic solvent extraction. When the organic solvent is at the temperature above its boiling point, sufficient pressure is maintained in the system to keep it in a liquid state. There are a number of benefits related to this method. Since solvents become significantly less viscous at high temperatures, they diffuse into the cell matrix

more readily. When the temperature of the solvent increased from 25 to 150 °C, the diffusion rate increased from two to tenfold. Additionally, moderate or high pressure in the system makes it easier for the solvent to enter the cell matrix (Lee et al., 2021).

2.10.2.3. Supercritical Fluids

A volatile and flammable organic solvent system is unlikely to be viable for the commercial-scale extraction of microalgal lipids from an environmental and safety perspective. In this regard, supercritical fluids have become green solvents to replace organic solvents. Carbon dioxide is the most commonly used supercritical solvent, which has a moderate critical pressure of 72 bar and a low critical temperature of 32 °C. SC-CO_2 shows high selectivity toward neutral lipids. Due to its low viscosity (like that of gas) and high diffusivity (like that of a liquid), SC-CO_2 can quickly penetrate solid matrices to extract lipids. Additionally, a rapid method for recovering the extracted lipids is to evaporate SC-CO_2 to the gas phase by a simple depressurizing step following the extraction procedure, leaving a solvent-free product (Lee et al., 2021; Mubarak et al., 2015).

2.10.2.4. Ionic Liquids

Ionic liquids (ILs) are salts that are only comprised of ions, and they are characterized by having low melting temperatures below 100 °C. ILs have emerged as promising extraction solvents for microalgal lipids because of their well-known solvation capability for highly recalcitrant biopolymers such as lignin or cellulose. They have a strong tendency to break down the hydrogen bonding networks that connect the microfibrils of microalgae structure. ILs disrupt the microalgae cell walls by dissolving the cellulose and polysaccharides, which are the main constituents of the microalgae cell wall. They have also been taken in to consideration to replace harmful and volatile organic solvents. The high thermal stability and non-volatility of ILs increase their recycling ability and process operational safety. ILs are referred to be designer solvents because their physicochemical and thermal characteristics may be tailored by the careful selection of cationic and anionic ingredients (Lee et al., 2021).

2.11. Biodiesel Production Technologies

Direct use or blending of oils, micro-emulsion, pyrolysis, and transesterification are the most dominant technologies that enable us to use oil and fat feedstock types as fuel in diesel engines. Various researchers are currently mentioning transesterification as the most preferred method due to the higher quality of fuel obtained.

2.11.1. Pyrolysis

Pyrolysis is a chemical change that occurs when thermal energy is applied in the absence of air or oxygen, or when heat is applied in the presence of a catalyst, resulting in bond cleavage and the formation of a variety of small molecules. Pyrolysis takes place at temperatures ranging from 400 and 600℃. Depending on the rate of pyrolysis, the process produces gases, bio-oil, and char. According to (Mahanta & Shrivastava, 2012), product of pyrolysis (pyrolyzate) from any feedstock type has a lower viscosity, pour point, and flash point than petroleum diesel fuel, while having comparable calorific values. Furthermore, the cetane number of the pyrolyzate is lower. According to them, the pyrolyzed vegetable oils have acceptable amounts of sulfur, water and sediments, and give acceptable copper corrosion values but unacceptable quantities of ash, pour point, and carbon residual.

2.11.2. Micro-emulsification

The viscosity of raw vegetable oil is one of the physical properties that prevent it from being used as a fuel. According to (Ma & Hanna, 1999), micro-emulsion formation is one of the possible solutions to solve the problem of vegetable oil viscosity. According to the IUPAC definition, micro-emulsion is dispersion made of oil, water, and surfactant(s) that is an isotropic and thermodynamically stable system with dispersed domain diameter ranging from 1 to 100 nm, usually 10 to 50 nm. Diesel fuel, vegetable oil, alcohol, surfactant and cetane improver in suitable proportions are the components of a biodiesel micro-emulsion. Higher alcohols are used as surfactants, and alkyl nitrates are used as cetane improvers, alcohols such as ethanol and methanol are used as viscosity lowering additives (Chiaramonti et al., 2003). According to (Mahanta & Shrivastava, 2012), micro-emulsion can be made of vegetable oils with an ester and dispersant (co-solvent), or of vegetable oils, and alcohol and a surfactant and a cetane improver, with or without diesel fuels. The maximum viscosity requirement for diesel fuel was met by all micro-emulsions containing butanol, hexanol, and octanol.

2.11.3. Transesterification

Transesterification is the most convenient method to generate biodiesel from oil and fat feedstock types, which is chemically similar to petroleum diesel. Oils and fats (triglycerides) are converted through this method to their alkyl esters with reduced viscosity to near diesel fuel levels. Therefore, this product is a fuel with properties similar to petroleum based diesel fuel, allowing it to be used

without modifications in existing petroleum diesel engines. In general, transesterification is a reversible reaction that is carried out by mixing the reactants usually under heat and/or pressure. If some kind of catalyst is added to the reaction, however, it will be accelerated (Hoekman et al., 2012). Figure 3 shows the simplest chemical reaction for the transesterification of triglycerides.

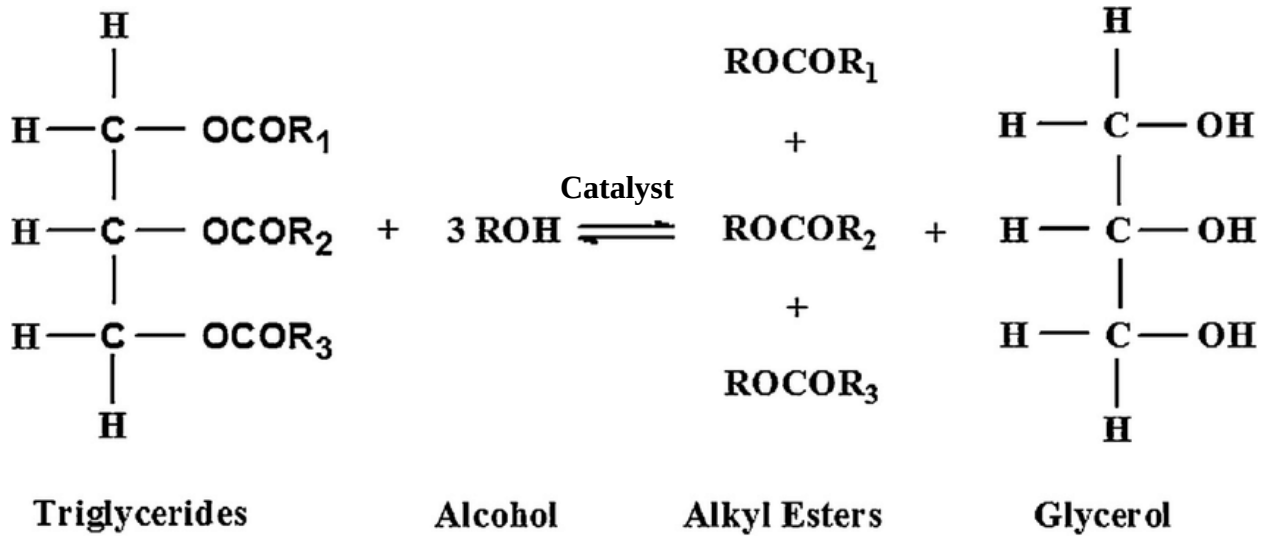


Figure 3. The general chemical reaction depicting transesterification of triglycerides (Koh & Tinia, 2011).

The reaction of a triglyceride (fat or oil) with an alcohol in the presence of some catalyst produces esters and glycerol in all catalytic transesterification processes. Even though biodiesel fuel formed from transesterification of triglycerides contains numerous individual FAME species, a particular fuel is generally dominated by only a few species; typically five of them dominate the composition of FAME derived from animal fats, and vegetable oils: linolenic acid (18:3), linoleic acid (18:2), oleic acid (18:1), stearic acid (18:0), and palmitic acid (16:0). Some algal-derived lipids are dominated by these same fatty acid groups, while other algae are more diverse in their composition, containing significant amounts of several other FA groups (Hoekman et al., 2012).

2.12. Catalysts in Transesterification

The reaction of a fat or oil with an alcohol in the presence of a catalyst to form esters and glycerol is known as transesterification. The yield of biodiesel, the reaction duration length, waste minimization and separation process easiness are all determined by the type of catalyst used. Generally, there are four types of catalysts used for biodiesel production: homogeneous catalyst, heterogeneous catalyst, biocatalyst and nanocatalyst (Adeyemi et al .,2011).

2.12.1. Homogeneous Catalysts

Homogeneous catalysis includes a sequence of reactions that are catalyzed by a chemical that is in the same phase as the reaction system. The homogeneous catalyst is the most preferred catalyst used for the production of biodiesel, as they are easy to use and take less time to achieve a complete reaction. This category includes both acidic and basic catalysts. In certain cases, homogeneous catalysts are dissolved in a solvent that is in the same phase as all reactants.

2.12.1.1. Homogeneous Base Catalyst

Homogeneous base catalysts are alkaline liquids such as alkali metal-based oxides such as sodium and potassium methoxides; carbonates and alkali metal-based hydroxides, namely, sodium or potassium hydroxides. Base catalysts have a high transesterification activity. Because of their lower prices, metallic hydroxides are frequently used as catalysts, but generally possess lower activity than alkoxides. According to reports, the rate of a base-catalyzed reaction is 4,000 times faster than that of an acid-catalyzed reaction. One well-known disadvantage is that oil containing significant amounts of FFA cannot be completely converted into biodiesels but remains as soap in vast quantities. The reaction can still be catalyzed with an alkali catalyst up to ∼5% FFAs, but an additional amount of catalyst is required to compensate for the catalyst lost to the soap (De Lima et al., 2016). For biodiesel production using the homogeneous catalyst, most studies recommend that the FFA content should be < 2 wt.%.

2.12.1.2. Homogeneous Acid Catalyst

Brønsted acids, preferably sulfonic and sulfuric acids as well as hydrochloric acid catalyze the esterification process. These catalysts generate very high yields in alkyl esters. However, the reactions are slower than alkali catalyzed reactions, making the process economically challenging because of the increased energy requirements. Homogeneous acid catalysis is insensitive to FFA content and can catalyze both transesterification and esterification reactions. Despite these benefits, homogeneous acid catalysis has the same separation problems as homogeneous base catalysis (Silitonga et al., 2020).

2.12.2. Heterogeneous Catalysts

Heterogeneous catalysts exist in a different phase or state from those of the reactants. These are the catalysts that form active sites with their reactants on a regular basis during a reaction. The heterogeneously catalyzed methanolysis reaction is extremely complex because it takes place in a

three-phase system consisting of a solid (heterogeneous catalyst) and two immiscible liquid phases (oil and methanol). Some side reactions such as glycerides and methyl esters saponification and FFAs neutralization by catalyst also occur concurrently with methanolysis. The main drawbacks of this catalysis are higher temperatures and higher oil/alcohol ratios than that of homogeneous catalysis. Other advantages include ease in separation and purification, as well as superior reusability of the catalyst, etc. Acid and base catalysts are the two types of Heterogeneous catalysts (Di Serio et al., 2008).

2.12.2.1. Heterogeneous Base Catalyst

A heterogeneous base catalyst aims to overcome constraints like saponification which hinders the separation of glycerol from the methyl ester layer, associated with the usage of a homogeneous base catalyst. Under mild conditions, these catalysts also show superior catalytic activity. These catalysts have a number of other advantages, including environmental friendliness, non-corrosiveness, and fewer disposal problems. Furthermore, they are easily separated from the reaction environment and can be designed to provide increased activity, selectivity, and a longer catalyst lifetime (Calero et al., 2014). Metal-based oxides, for example, are the most commonly used heterogeneous catalysts for transesterification. Zhang and his collogues used temperature-programmed desorption (TPD) of adsorbed carbon dioxide analysis to demonstrate the basic properties of alkaline earth metal oxides (G. Zhang et al., 1988). They discovered that the number of basic sites per unit weight approximately corresponds to that of the surface area and follows the sequence: CaO > MgO > SrO > BaO. Stronger basic site oxides promote the reaction more effectively. CaO has been the most studied metal-based catalyst material for biodiesel production because it has a number of advantages, including: high activity, relatively high basic strength, long catalyst life, and low solubility in methanol, as well as requiring only moderate reaction conditions (Latchubugata et al., 2018).

2.12.2.2. Heterogeneous Acid Catalysts

As compared to homogeneous acid catalysts, heterogeneous acid catalysts have a less toxic and corrosive effect, and they cause less environmental problems. These catalysts contain a range of acidic sites with different Brønsted or Lewis acidity strengths. Although these catalysts show encouraging results in moderate reaction conditions, they react much more slowly than solid base

catalysts. Furthermore, high temperature, high catalyst loading, and long reaction time are required to employ this type of catalysts (Mansir et al., 2017).

2.12.2.3. Waste-Derived Heterogeneous Catalyst

The use of heterogeneous catalysts has the potential to reduce the current high production costs of biodiesel, rendering it competitive with petro-diesel fuels. As a result, research is being focused on the development of a cost-effective and environmentally friendly heterogeneous catalyst for biodiesel production. The utilization of waste heterogeneous catalysts has become recent interest as a way to make the biodiesel production more sustainable (Talha & Sulaiman, 2016).

Waste materials such as eggshells, mollusk shells, snail shells, oyster shells, animal bones, slag, dolomite rocks, ash, rice husk, lime mud, red mud, shrimp, and fish scales have been used for the production of sustainable and environmentally friendly heterogeneous catalysts. The high catalytic activity of calcium found in various types of wastes makes them ideal candidates for use as heterogeneous catalysts. Additionally, various researchers found that solid base catalysts made of calcium oxide are the most active and efficient catalysts. It has a large surface area, nontoxic nature, low cost, high basicity, and effective yields of fatty acid methyl ester (Jambhulkar et al., 2022).

MgO, CaO, and SrO are alkaline earth metal oxides with high activity for use in the typical processes that take place at low temperatures and under atmospheric pressure conditions. Among the alkaline earth metal oxides, CaO is close to the environmental material. In general, $CaCO_3$, $Ca(NO_3)_2$, or $Ca(OH)_2$ are the most known raw materials to produce CaO catalysts. There are several natural calcium sources from wastes as an alternative way to synthesize CaO catalysts such as eggshell, mollusk shell, and bone. For the biodiesel industry, not only eliminating waste management cost, but also the catalysts with high cost-effectiveness can be simultaneously achieved (Adeyemi et al., 2011).

To obtain 100 mesh particle size, the raw carbide slag from chemical industries is grounded and sieved. The obtained powder is dried at a temperature of around 105 °C and then further calcinated in a muffle furnace for 4 hrs at a temperature of more than 700 °C to prepare CaO. A similar procedure is carried out to obtain CaO from different other waste products such as eggshells, bones, etc. It is often found that the CaO generated from waste shell materials is quite suitable for

catalysis applications due to their high calcium carbonate content which imparts characteristics comparable to those imparted by ordinary limestone (Jambhulkar et al., 2022).

2.12.3. Biocatalysts

Chemical catalysis for biodiesel production is energy consuming and creates undesirable byproducts such as soaps and polymeric pigments, which hinder the separation of product from glycerol and di- and monoacylglycerols. These impediments can be eliminated by using biocatalysts. Biocatalysts, also known as enzymes, are obtained from living organisms that stimulate chemical reactions without affecting themselves chemically. In biodiesel production, two types of enzymatic biocatalysts are usually used, namely, intracellular lipases and extracellular lipases. Extracellular lipases are the enzymes that have been recovered from the microorganism broth and then purified. Intracellular lipase, on the other hand, remains either inside the cell or in the cell-producing walls (Amini et al., 2017).

2.12.4. Nanocatalysts

With the development of nanotechnology, nanocatalysts have become increasingly important in enhancing biodiesel production quality and yield along with the reduction in reaction time. The involvement of a nanocatalyst also leads to a reduction in alcohol-to-oil ratio, catalyst weight, and reaction temperatures. It was found that nanocatalysts significantly contribute in increasing the rate of reaction without being self-deteriorated. Additionally, it also lowers the activation energy of the reaction profiles. In addition to having excellent properties for withstanding reaction conditions, these highly developed catalysts also provide a large surface area to volume ratio (Bano et al., 2020).

The development of nanocatalysts consisting of polymer, zeolite, and carbon-based nanomaterials has recently attracted many researchers by mainly presenting attractive properties of high catalytic activity, lowering the reaction temperature and time. Thus, the synthesis and modification of nanocatalysts will offer a significant increase in biofuel production. Many possible methods such as precipitation, impregnation, chemical vapor deposition (CVD), and electrochemical deposition techniques can be used to synthesize the newly developed nanocatalyst (Bano et al., 2020).

Before it can be used for biodiesel production, characterization of these catalysts is critical. To date, different methods have been used to achieve this. XRD is the most commonly used method for the characterization of the crystallinity and composition of these catalysts. SEM is used to

determine the morphology of prepared nanocatalysts and their precursors. Transmission electron microscopy (TEM) is used to analyze the particle diameter and morphology of the catalysts. Other characterization methods include FTIR spectroscopy that is used for determining assimilation of phases, thermogravimetric analysis (TGA) for examining the decomposition nature of catalyst samples, Barrett–Joyner–Hlenda (BJH) and Brunauer–Emmett–Teller (BET) methods for specific surface area calculation, etc. (Baskar et al., 2018).

2.12.4.1. Preparation Method of Heterogeneous Nanocatalysts

Catalysts are prepared using a variety of methods such as precipitation method, impregnation method, precipitation impregnation method, sol-gel method, chemical deposition method, etc.

Table 5. Classification of preparation methods of heterogeneous catalysts (Zhang, 2020).

No.	Category	Principle
1	Precipitation	In order to form a crystal or gel of hydrated oxides, carbonates, or gels a precipitating agent is added to an aqueous solution of a metallic salt.
2	Impregnation method	The carrier is immersed in a solution containing active components. After reaching equilibrium, the solution is immersed in solid (the remaining liquid is removed), and the catalyst is obtained after drying, calcining and activation.
3	Precipitation-impregnation method	A new technique developed based on the impregnation method and the uniform precipitation method involves preparing the precipitant matrix in the impregnation solution in advance, and then the precipitant component is deposited on the surface of the carrier by heating up, after the operation of the impregnation unit is completed.
4	Sol-gel method	The solid catalyst of nanoparticles or block inorganic materials is obtained after the metal alcohols or inorganic salts are hydrolyzed to generate sol directly or lysed to form sol. The solute is then aggregated to gel, made into a film or dried directly and heat treatment to remove organic components.

Table 5 Continued

5	Chemical deposition method	It is a technique that involves employing one or more compounds or elemental materials that include film elements to produce films by chemical reactions on the substrate surface.

2.12.4.2.1 Impregnation Method

The simplest and easiest catalyst preparation method for the synthesis of supported catalysts is the wet impregnation method. This system has the ability to distribute the catalyst active site on the surface which is very well used in the esterification and transesterification reactions. The impregnation method involves mixing solid support with a solution of metal acetate, sulfate, or nitrate salts of metal oxides used. For 5 to 6 hours the mixture is stirred. After being shaped like a slurry the solid is dried for 24 hours in an oven at 110℃ and then, the catalyst is calcined (Widiarti et al., 2019).

To obtain doped CaO catalysts, the impregnation method of an active catalyst precursor was usually used. When alkali metal ions are impregnated onto nano CaO particles the basic strength of CaO catalysts is enhanced. In a typical preparation, first CaO solid carrier is suspended in water, then the aqueous solution of the precursor is added and finally, the catalyst is calcined at the temperature between 500 °C and 900 °C in order to convert the precursor to the active form. High calcination temperature encourages the interaction between the carrier and the active component to produce new crystals, such as new active sites, but overheating causes surface sintering and specific surface area reduction, i.e. the catalytic activity (Widiarti et al., 2019). The type of metal ion, available basic sites in CaO and interaction of the metal ions with CaO all affect the activity of a doped CaO catalyst. As an impregnation precursor, ions of alkali metals (Li^+, K^+ and Na^+) with various concentrations are used. According to (Kumar & Ali, 2013) compared to neat CaO, nano CaO impregnated with alkali metal ions showed a high specific surface area; the highest specific surface area was found in the case of lithium–impregnated CaO. With increasing the ion size, the basic strength of the alkali metal ion impregnating CaO and the catalytic activity toward transesterification decreases. However, alkali metal doped nano CaO showed poor stability and suffered from leaching of active species into the reaction medium. As a result, they experimented the use transition–metal ions, such as Cu, Ni, Zn, Mn, Fe, Co, and Cd, for the CaO activation and

demonstrated that due to its highest basic strength the produced Zn/CaO catalyst showed the best activity toward transesterification under the optimized reaction conditions among the prepared catalysts. In addition, the nano-Zn/CaO catalyst demonstrated superior moisture resistance compared to neat CaO, even after exposure to the atmospheric moisture (Banković-Ilić et al., 2017; Widiarti et al., 2019).

2.13. Physico-Chemical Studies of Biodiesel Production

In biofuel production, physico-chemical properties test methods for free fatty acids (FFA) content and FAME (fatty acid methyl esters) play a very important role. The quality of biodiesel is mainly based on standardized conditions specified by ASTM (American Standard for Testing and Materials), Czech Republic (CSN), and European standards (EN). The biodiesel quality must fulfill the given specifications. The properties such as density, oxidation stability, kinematic viscosity, cloud point, flash point, and fire point of the biodiesel generated by utilizing different catalysts were reported and compared with the EN 14214 and ASTM (D6751) standards of the biodiesel (Hoang, 2018). Estimation of acid value and saponification value of the feedstock are also important because they correspondingly determine the purity and yield of biodiesel.

2.13.1. Density Measurement

The density, which is defined as weight per unit volume, is important for the mass-to-volume conversion process needed in biofuel production. It has a significant impact on the quality of fuel. Density measurements are used to estimate blends of biodiesel fuel and the correct formulation of blends. The density of biodiesel primarily depends on the types of oil and the treatment process used. It is also affected by molecular weight of the components such as fatty acids. A high molecular weight correlates to a high density which affects the quality of the fuel. It has been widely reported that low density is better for good quality of fuel (Hoang, 2018).

2.13.2. Kinematic Viscosity

The viscosity of biodiesel is an important parameter in determining the quality of biodiesel. Viscosity refers to the fuel-flowing ability as it affects the operation of fuel injection equipment especially at low temperatures. During the atomization process, fuel with too low viscosity will cause leakage and increased wear; hence it significantly affects engine performance, while high viscosity fuel causes large droplets formation, therefore creating operational problems, increased carbonization eventually leads to increased emission and smoke. As compared to diesel fuel, the

influence of the high viscosity of biodiesel leads to concern in process equipment and design. A viscometer is used to measure experimental viscosity at a temperature of 40°C. According to ASTM D6751 and EN 14214, the kinematic viscosity of biodiesel is 1.9–6 and 3.5–5.0 mm^2/s, respectively (Huang et al., 2020).

2.13.3. Acid Values

The acid value is a critical parameter because it affects the quality of particular biodiesel. It is defined as the presence of free acids in the given sample. Since an excess of free fatty acid leads to catalyst deactivation and soap formation, it can be measured by neutralizing the sample using a specific amount of KOH. In general, the acid value of feedstock should range between 1.86-3.31 mg KOH/g oil (Ismail & Ali, 2015).

2.13.4. Iodine Value

The iodine value is defined as the measurement of the degree of unsaturation. It also depends on the type and nature of feedstock and extremely influences the fuel's oxidative ability. The iodine value of the diesel fuel must be less than 120 g I2/100 g of the given sample. It will determine the degree of unsaturation in a given mixture of fatty acids. Lower oxidative stability is associated with higher iodine value. It will also disclose information about the sludge formation in fuel, affect lubricant quality, and also cause corrosion (Bassam et al., 2019).

2.13.5. Saponification Value

The saponification value is explained as milligrams of KOH used to saponify 1 g of oil or fat under a given condition. It is a measurement of molecular weight and length of fatty acids. The saponification value of crude esters is higher than that of oil, and ranges from 199 to 207 mg KOH/g oil. As the number of free carboxylic acid groups decreases per unit of fat or oil mass, longer fatty acid chains have low saponification values. It is primarily used to measure the average molecular weight of the oil or fats contained in a given sample. The saponification value of biodiesel and crude feedstock, on the other hand, does not vary significantly as the average molecular weight undergoes small changes. The differences in saponification values are almost identical (Gopinath et al., 2009).

2.13.6. Cetane Number

The determination of the cetane number is an important parameter in quantifying the ignition ability of the fuel. It corresponds to the fuel's ability to auto-ignite quickly after being injected into

the fuel line, as well as shows the ignition quality of the fuel. A low cetane number primarily affects engine parameters with increased emission of smoke, combustion instability, also excessive deposition of unburnt materials in the engine, while a higher cetane number corresponds to a better ignition quality (Ismail & Ali, 2015). The number of cetane increase with the increasing length of the fatty acids chain. When compared to diesel fuel, biodiesel possesses a higher cetane number.

2.13.7. Cloud and Pour Point

Cloud point (CP) is explained as the temperature at which wax crystals become visible when the fuel is cooled. It allows fuel to solidify at lower temperatures which could cause blockage in fuel lines. Cold point is estimated from ASTM Standards D2500, D5771, D5772 and D5773. The formation of crystallization biodiesel can be easily examined using a Differential Scanning Calorimetry (DSC) curve. An increase in crystallization would eventually lead to agglomeration in the fuel, therefore, deteriorating the engine performance. Due to thickening, it can also cause blockages in the fuel filters of the engine, which eventually ceases the fuel flow capability. The presence of a high amount of saturated fatty acids contributes to the easy crystallization of the fuel (Sierra-Cantor & Guerrero-Fajardo, 2017).

The lowest temperature at which fuel starts flowing is known as the Pour point. Biodiesel with high saturated fatty acid content possesses a high value of pour point which indicates poor fuel properties. In order to provide better quality fuel, the transesterification process significantly reduces the value of the pour point. High fatty acid content after catalysis shows significant viscosity which also hampers fuel properties.

2.13.8. Flash Point

The temperature at which fuel will ignite when exposed to flame is known as a flash point. Biodiesel fuels have higher flash points than petrochemical-derived fuels, and hence leading to safe transportation and storage of fuels. The low value of flash point implies high volatility, and it is also helpful in the classification of fuel to reduce the risk of handling and transportation. The flash point of biodiesel is greater than that of diesel fuel. The biodiesel value based on these specification standards ASTM 6751 and EN 14214 must be a minimum of 130 and 120°C respectively. It is also easily affected by the presence of impurities such as oil content, moisture content, and unreacted alcohol. Therefore, it indirectly assists in identifying the quality of the biodiesel fuels (Rasouli & Esmaeili, 2019).

2.14. Optimization Parameters of Raw Materials for Increasing Biodiesel Production

2.14.1. Methanol-to-Oil Ratio

The alcohol-to-oil molar ratio, as well as the type of alcohol and triglycerides used, are the most important factors in biofuel production. The most likely M:O ratio is 3:1, which is advantageous in terms of increasing the solubility of oil and contact time. Within a short span of time, a high molar ratio results in greater conversion of ester. Stoichiometrically, the esterification process requires 3 mol of alcohol and 1 mol of triglycerides in order to yield 3 mol of fatty acids and 1 mol of glycerol. Since the molar ratio is predominantly dependent on the nature of the oil and catalyst used, a high fatty acid content needs a large amount of alcohol in order to proceed with the reaction in the forward direction. Using an optimized amount of alcohol subsequently increases biofuel production. However, too much alcohol will eventually block the catalyst's active sites and increase backward reaction more favorably (Gashaw et al., 2015). In the transesterification process, various alcohols such as methanol, ethanol, propanol, and butanol are commonly used. As reported by many researchers, methanol is commonly used because of its low cost, but ethanol can also be used because of its production from agricultural waste products and its environmentally friendly nature (Hossain & Boyce, 2009).

2.14.2. Catalyst Weight

This is another important factor in the production of biodiesel. The amount and nature of catalyst are mainly dependent on the reaction conditions required, for instance: acidic or basic. Inadvertently, it is also dependent on the nature of feedstock used. The optimized amount of catalyst is enough in order to carry out the desired reaction, if the feedstock is pure with normal moisture and free fatty acid contents. However, if the feedstock has high moisture and free fatty acid content, the transesterification process that occurring is actually insignificant as it mainly results in soap formation and lowers the yield of biodiesel production. In the latter case, a higher amount of catalyst will certainly increase the production of biodiesel. Therefore, a proper feedstock pretreatment process is very significant in the controlled optimization of the catalyst (Gashaw et al., 2015).

In most of the studies, the average catalyst amount was reported in between 1–6 wt% for the optimum amount of the triglycerides content, but it also varied significantly depending on the scale of production. However, in some cases it has been reported to be high depending on the reaction conditions. High catalyst weight, in general, has been found to have a negative impact on catalytic

reaction because it causes mass transfer limitations and decreases active sites interaction caused by the agglomeration (Kazemifard et al., 2018). Further addition beyond the optimized amount will decrease production and it is not economically effective.

2.14.3. Reaction Temperature

Reaction temperature also plays a significant role in order to increase the yield. An increase in temperature increases the reaction rate and kinetics ultimately reducing the reaction time (Gashaw et al., 2015). Depending on the type of oil used, the transesterification process is usually carried out below the boiling temperature of alcohol within an optimum temperature range between 50–60°C. High temperature mainly corresponds to the evaporation of alcohol used. Many groups have reported that at the normal temperature a 78% conversion can be achieved within a 60–90 min time period (Hossain & Boyce, 2009).

2.14.4. Reaction Time

The reaction time plays a corresponding role in the transesterification of oils or fatty acid esters. The fatty acid conversion increases with an increase in time, as initially, the conversion is slow because of the dispersion and mixing of oil with methanol or alcohol. The conversion is usually faster owing to faster reaction kinetics, as the reaction proceeds towards the threshold. The catalyst's nature has a significant impact on reaction time as a mixed catalyst requires much less time for example, 1–2 hrs. It was reported that, maximum ester conversion was achieved within 90 min, however, due to the reversible the nature of reaction, an additional increase in time does not increase the yield, leading to more production of glycerol (Gashaw et al., 2015; Hossain & Boyce, 2009).

2.14.5. Effect of Doped Metal Ions on the Catalytic Activity

The type of metal ion, available basic sites in CaO and interaction of the metal ions with CaO all affect the activity of a doped CaO catalyst. According to (Kumar & Ali, 2013) alkali metal doped nano CaO demonstrated poor stability and suffered from leaching of active species into the reaction medium. As a result, they experimented the use transition–metal ions, such as Cu, Ni, Zn, Mn, Fe, Co, and Cd, for the CaO activation and demonstrated that due to its highest basic strength the produced Zn/CaO catalyst showed the best activity toward transesterification under the optimized reaction conditions among the prepared catalysts. In addition, the nano–Zn/CaO catalyst demonstrated superior moisture resistance compared to neat CaO, even after exposure to the atmospheric moisture (Banković-Ilić et al., 2017).

CHAPTER THREE

3. MATERIALS AND METHODS

In this chapter, the chemicals and equipment used and working procedures followed in this study were described. The method used to synthesize eggshell based zinc doped CaO nanocatalyst, isolation, culturing and harvesting of *B. braunii* microalgae, extraction and characterization of *B. braunii* microalgal oil, biodiesel production, and optimization of reaction parameters of transesterification are described.

3.1. Materials

3.1.1. Chemicals

The following chemicals were used to conduct the experimental work: KNO_3, K_2HPO_4, $MgSO_4.7H_2O$, $CaCl_2.6H_2O$, ferric citrate, citric acid, H_3BO_3, $MnCl_2.4H_2O$, $CUSO_4.5H_2O$, $CO(NO_3)_2.6H_2O$, $Na_2MoO_4.2H_2O$, $CoCl_2$, H_2SO4, $ZnSO_4$, HCl, $Al_2(SO_4)_3$, n-hexane, KOH, ethanol 99.5%, methanol 99.5%, diethylether, phenolphthalein. All the chemicals used in the production process were of analytical grade.

3.1.2 Equipment

The following equipment was used to conduct the experimental work: GX-65B oven drier, agate mortar equipment, grinder, Erlenmeyer flask, water bath, SX-2.3-10 muffle furnace, glass desiccators, crucible, microwave-assisted lipid extractor, separatory funnel, filter paper, condenser, glass rode, aerator, lux meter, beaker, graduated cylinder, distillation flask, thermometer, pH meter, conductivity meter, inverted microscope, digital balances, viscometer, magnetic stirrer, hot plate, three-necked round bottom flasks, 63 µm sieve, aluminum foil, UV/VIS spectrophotometer, XRD, SEM, and FTIR.

3.2. Methods

3.2.1. Description of the Sampling Area

Samples were collected from three different locations of Lake Hawassa (Figure 4) which is an endorheic basin, lies within the Ethiopian Rift Valley, and located in the northern section of the Great Rift of Africa. Lake Hawassa is located to the west of Hawassa town, which is the capital of the Sidama Region. The study site is situated 275km south of Addis Ababa and located between 7.0313° N, 38.4219° E, and at an altitude of 1685m above sea level. In terms of surface area, Lake

Hawasa is 90 km^2 and its maximum depth (Z_m) is 23m. The average annual rainfall in the region is 950mm, and has an average annual air temperature of 19.8°C (Adimasu Woldesenbet, 2015). Generally, the experiment was conducted at Addis Ababa University, College of Natural and Computational Sciences, Center for Environmental Science laboratory.

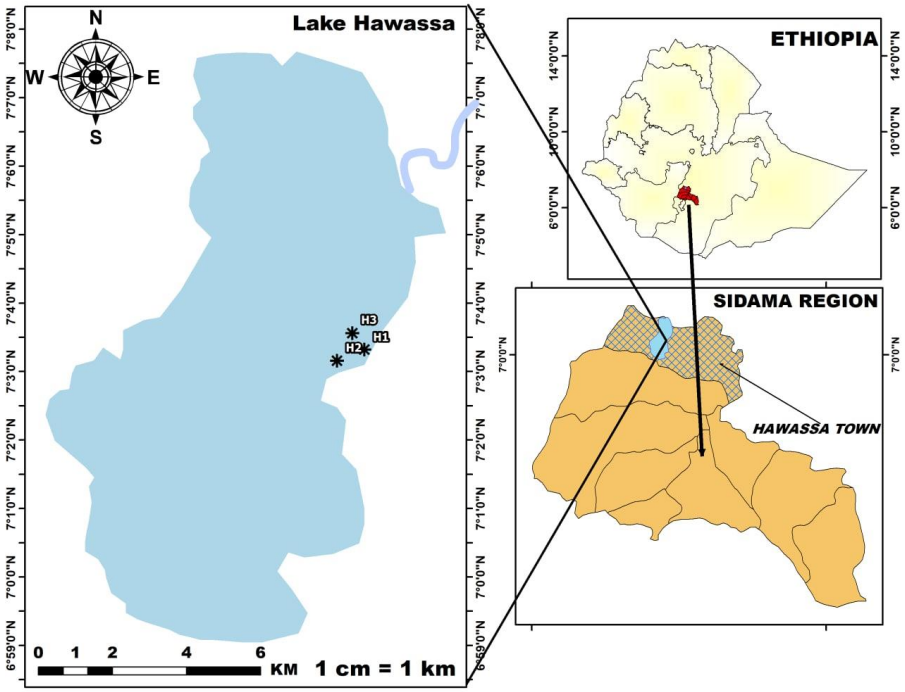


Figure 4. Map of Lake Hawassa

3.2.2. Study Design

The study was laboratory based on an experiment using appropriate methods to isolate *B. braunii* microalgae that have the potential application to produce biodiesel using enhanced eggshell-based heterogeneous nanocatalyst.

3.2.3. Measurement of Some Physicochemical Parameters of the Lake

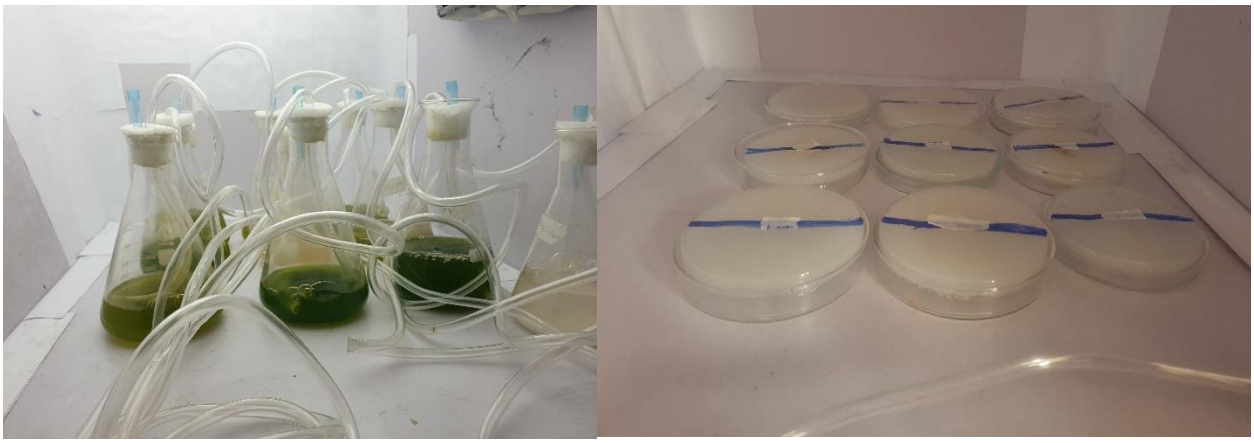
Surface water samples were collected in July, 2021 using a phytoplankton net (mesh size 15 µm) from three different locations of Lake Hawassa in polythene bottles of two litter capacity. Temperature, pH, and conductivity were measured in situ using a digital thermometer, pH meter, and conductivity meter respectively. Salinity was calculated from conductivity measurements according to UNESCO's handbook 'Algorithms for computation of fundamental properties of seawater (Fofonoff & Millard, 1983).

Twenty five ml water samples from each polythene bottles were taken and preserved immediately after collection by adding Lugol's iodine solution. Lugol's iodine solution was added until the sample was the color of weak tea/light beer (dark yellow/light brown) after preservation. The sample was agitated during addition of the Lugol's solution to ensure thorough mixing. Lugol's iodine is light sensitive, so samples were stored in the dark after they had been preserved. Observation and identification of species were conducted under an inverted microscope. Therefore, the availability and abundance of *B. braunii* species were checked.

3.2.4. Isolation, Purification and Culture Conditions of *Botryococcus braunii* and Scaling-Up its Cultures

The water samples collected from Lake Hawassa were initially cultured in modified Chu-13 medium and cells of *B. braunii* were isolated and the individual colony was microscopically observed for their colonial and morphological features (Kawamura et al., 2020). The microalgae were grown in both liquid and agar plates of modified Chu-13 medium, incubated at 25°C under light intensity 2500 lux and supply of sufficient CO_2 with continuous aeration to keep the homogeneity of the culture; with 16:8 hr light and dark cycle. The purity of the culture was ensured by repeated plating and by regular observation under microscope (Kong et al., 2012).

Cultivation of *B. braunii* was first carried out in triplicate in 250 and 500 ml Erlenmeyer flasks containing modified Chu-13 medium (pH 7.2) at 25^{o} C with light illumination (2500 lux; 16:8 hr light: dark cycle), supply of sufficient CO_2 in an illumination incubator for 21 days (Phukan et al., 2011; Kong et al., 2012); then subculturing and growth on modified chu-13 liquid and agar medium were followed (Figure 5). The microalgae on chu-13 solid agar medium were subjected to purification by plating followed by streaking. Then the individual colonies were isolated and inoculated into chu-13 liquid medium (Phukan et al., 2011). After that continuous subculturing on both liquid and the solid medium was followed until the purity of the culture confirmed. The pure cultures grown in 250 and 500 ml Erlenmeyer flasks were transferred into 5 L Erlenmeyer flasks and subsequently into three large aquariums for the cultivation of huge biomass (Figure 6). The culture volume was 80L for each of the three aquariums with similar growth conditions. Therefore, *B. braunii* was gradually scaled up in 250 and 500 ml, 1, 2 and 5 L flasks and then in three 100 L aquariums.



A B

Figure 5. Microalgae growing setup 1: (A) *B. braunii* microalgae growth in modified Chu-13 liquid medium and (B) *B. braunii* microalgae growth in modified Chu-13 agar medium

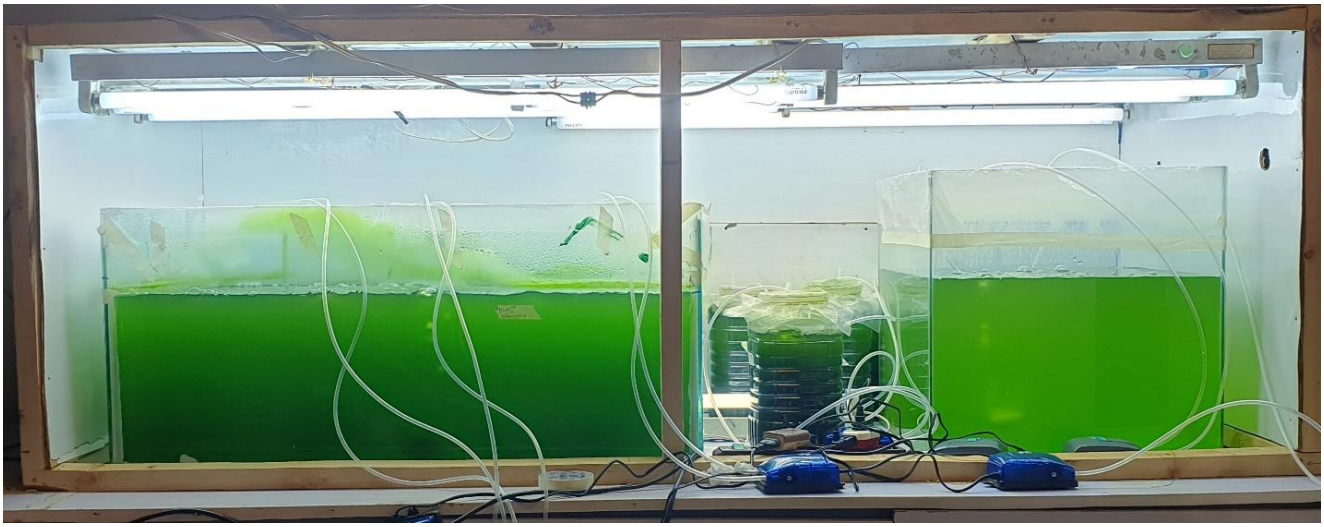


Figure 6. *B. braunii* microalgae growing setup 2

3.2.5. Morphological Profile Assay

The obtained colonies of *B. braunii* were observed for their morphological profile using an inverted microscope.

3.2.6. Growth Evaluation, Harvesting and Drying of *Botryococcus braunii*

The growth of *B. braunii* was monitored spectrophotometrically using UV/VIS spectrophotometer by measuring the optical density of algal suspension at 750 and 780 nm in every 24 hours (Phukan et al., 2011).

Experiments were carried out in triplicate (Appendix 2 and 3) and the specific growth rate of the microalgae was calculated using equation 2; the specific growth rate (μ) was determined as (Krzemińska et al., 2014):

$$\mu = \ln (N_2/N_1)/(t_2 - t_1), \quad \text{.....Equation (2)}$$

Where: μ is the specific growth rate, N_1 and N_2 are the biomass at time 1 (t_1) and time 2 (t_2), respectively.

The *B. braunii* cultures were harvested using the flocculation harvesting method. A 0.2 g of $Al_2(SO_4)_3$ was used for a litter of culture (Matter et al., 2019). The wet cell mass was kept for drying in a laboratory-grade hot air oven at 60 °C for 2 days then grinded by pestle and mortar as shown in Figure 7.

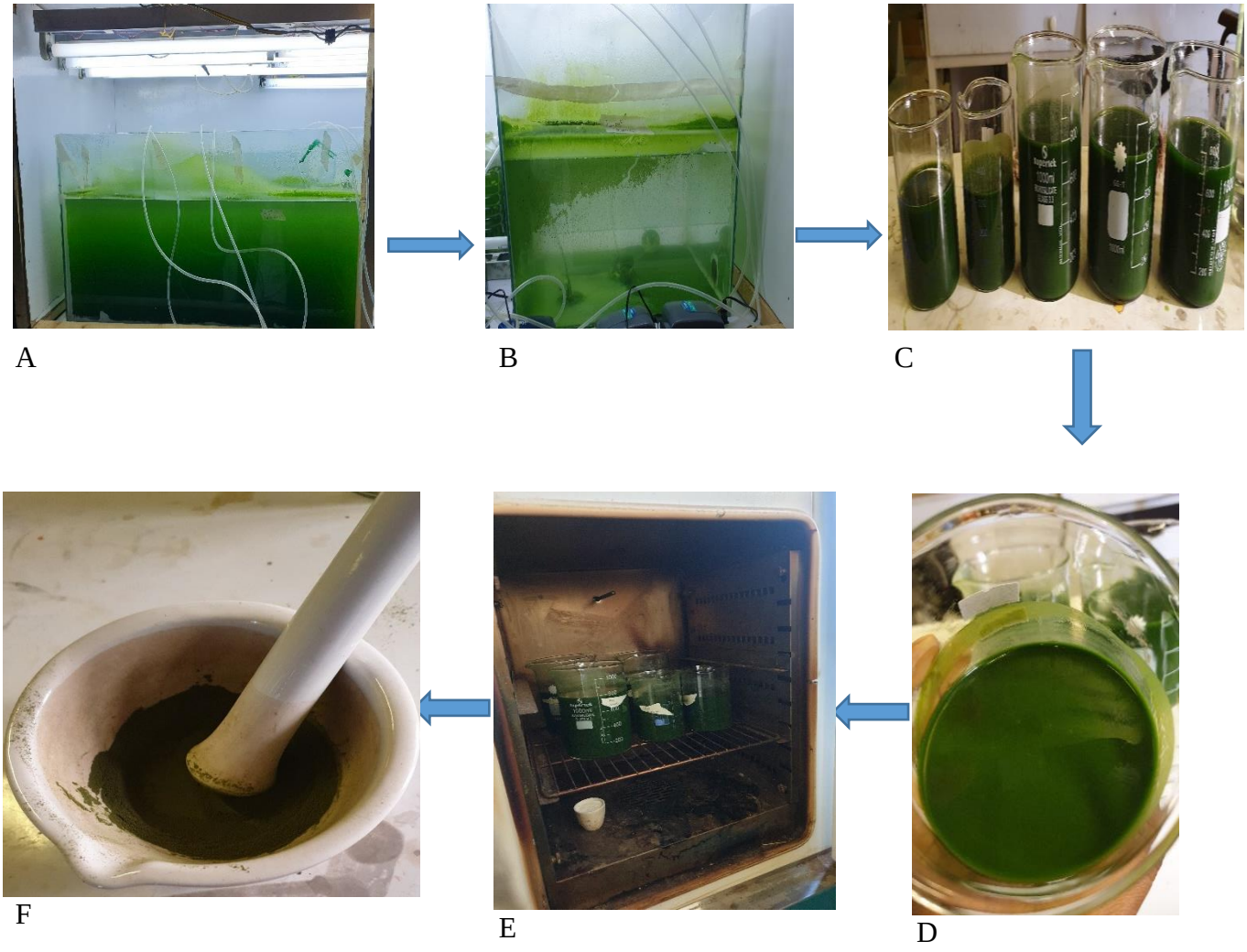


Figure 7. Microalgae biomass cultivation, harvesting and drying: (A) 18th day of culture, (B) harvesting of cultured microalgae after addition of $Al_2(SO_4)_3$ on 18th day of culture, (C & D) harvested microlgae (E) Oven drying of microalgae, and (F) grinded microalgae

3.2.7. Microwave-Assisted Lipid Extraction

The powder of microalgal biomass was used for oil extraction using the microwave-assisted extraction method (Dai et al., 2014). Ten grams of dried *B. braunii* microalgae powder was extracted using a mixture of methanol-hexane solvent 1:2 (v/v) at 600W of microwave power for a period of 40 min at 95 ^{o}C using Ethos One high-performance microwave digester. Following extraction, the mixture of biomass and solvent was filtered to separate filtrate and residue. Lipid from *B. braunii* was obtained after the remaining solvents were distilled and evaporated (Figure 8) (Kalsum et al., 2019).

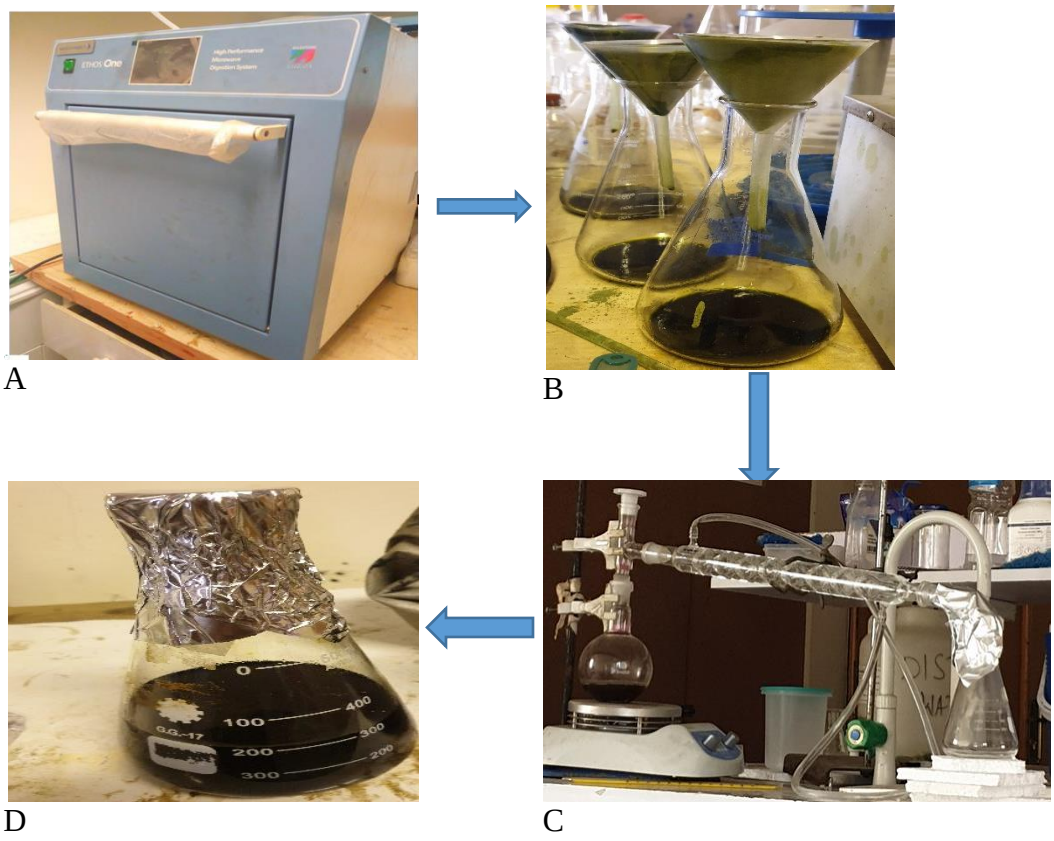


Figure 8. Microalgae oil extraction procedure: (A) microwave digester, (B) filtration, (C) distillation and evaporation and (D) purified microalgae oil

3.2.8. Physicochemical Properties of Extracted Oil

The oil was analyzed for physicochemical properties such as density, kinematic viscosity, acid value, FFA content, saponification value, ash content and moisture content. These parameters directly or indirectly may affect the quality of the biodiesel (Ejim & Kamen, 2013).

3.2.8.1. Density

The weight of a pycnometer was determined using an electronic weighing balance. A 25 ml sample that was heated to a temperature of 20 ^{o}C was filled into pycnometer and the weight of the bottle and oil was determined. The density was calculated using the equation 3.

$$\text{Density} = \frac{W2 - W1}{V} \ldots\ldots\ldots\ldots\ldots\ldots \text{Equation (3)}$$

Where; W1= weight of pycnometer, W2= weight of pycnometer with oil, V= volume of oil

3.2.8.2. Kinematic Viscosity

The viscosity of the oil was determined using a viscometer. The oil sample filled in the viscometer cup was kept at a constant temperature of 40 ℃ water bath for 30 minutes. The viscometer tip was inserted into the cup containing the sample and the reading was recorded for a fixed volume of liquid. The reading of the viscometer was dynamic viscosity therefore the value was used to calculate kinematic viscosity using equation 4.

$$N = \frac{\mu}{\rho} \ldots\ldots\ldots\ldots\ldots\ldots \text{Equation (4)}$$

Where; N = kinematic viscosity in mm^2/s, μ = dynamic viscosity, ρ = density in kg/m^3

3.2.8.3. Acid Value

To determine the acid value of the extracted microalgal oil, a titration solution of 0.1N of KOH in distilled water was prepared. Two grams of oil was added to a beaker and heated at 70^{o}C for 3 minutes. Then, 20ml of anhydrous ethanol (99.5% w/w), 20ml of diethyl ether, and 5 drops of phenolphthalein were added to the titration beaker that contains the sample oil. Finally, in the titration solution, 0.1N of KOH was added 1 drop at a time until the first color (until pinkish color appeared) change was observed. Once the color change was observed, the volume of the titrant KOH (ml) was recorded and the recorded volume was used to calculate the acid value using equation 5.

$$\text{Acid value (AV)} = \frac{\text{N} \times \text{V} \times 56.1}{\text{W}} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{Equation (5)}$$

Where; V = volume of KOH used in ml, N = normality of KOH, W = mass in gram of oil sample, 56.1 = molar mass of KOH

3.2.8.4. Free Fatty Acid Value

The free fatty acid value, FFA of the oil samples was calculated using the equation (6) from the acid values (AV) of the oil.

$$\%\text{Free faty acid value (FFA)} = \frac{\text{AV}}{2} \ldots\ldots\ldots\ldots \text{Equation (6)}$$

Where: %FFA = percentage of free fatty acid, AV = Acid Value of the oil

3.2.8.5. Saponification Value

Saponification value (SV) is expressed as the number of milligrams of KOH required to saponify 1g of fat (Gopinath et al., 2009). Two grams of the oil sample was weighed and added to a 250 ml conical flask then 25 ml of 0.5 mol/L ethanolic potassium hydroxide solution was also added and refluxed at 70 ℃ for 30 minutes. After that, the heated mixture was cooled immediately, and 5 drops of phenolphthalein were added as an indicator then the solution was titrated with 0.5 mol/L hydrochloric acid solution. As the color change was observed, the titration was stopped and the value V_a was recorded. The same procedure was also performed using a blank sample (without oil) and volume V_b was recorded. Then saponification value was calculated using equation 7.

$$\text{Saponification Value (SV)} = \frac{\text{MW} \times \text{N} (\text{V}_\text{b} - \text{V}_\text{s})}{\text{W}} \ldots\ldots\ldots\ldots \text{Equation (7)}$$

Where; SV = saponification, MW = molecular weight of KOH, N = normality of HCl solution, V_b = volume of HCl solution used in blank V_s =volume of HCl solution used in the sample and W= weight of oil used.

3.2.8.6. Ash Content

The ash content of the oil was determined using a muffle furnace. A 20g of sample was added to a burning cup then, the sample was placed in a muffle furnace. A furnace was adjusted at a temperature of 500 ℃ for 2 hours and then after burning, weighing the residue sample gave the ash content. Ash content was calculated using equation 8.

$$\text{Ash Content}(\%) = \frac{\text{Mass of oil after burning}}{\text{Intial mass of oil}} \times 100 \ldots\ldots\ldots\ldots\ldots \text{Equation (8)}$$

3.2.8.7. Moisture Content

A dish was weighed with and without oil. The dish with oil was dried in an oven at 105 ^{o}C for 7hr, weighing every 2 hrs till constant weight is obtained and finally the weight was taken and compared with the initially recorded weight. The percentage weight in the oil was calculated using Equation 9.

$$\text{Moisture Content } (\%) = \frac{W1-W2}{W1} * 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\text{Equation (9)}$$

Where; W1 = Original weight of the sample, W2 = Weight of the sample after drying

3.2.8.8. Molecular Weight Determination

The molecular weight of the microalgal oil was determined according to the equation 10 below:

$$MW = \frac{168300}{\text{SV}-\text{AV}} \ldots\ldots\ldots\ldots\ldots \text{Equation (10)}$$

Where; MW= molecular weight of oil, SV = saponification value of oil, AV = acid value of oil

3.2.9. Catalyst Preparation

3.2.9.1. Eggshell Sample Collection and Preparation

Calcium oxide (CaO) nanocatalyst was prepared from eggshells by collecting eggshells from local restaurants and then was soaked in boiled water for 10 minutes to solidify the gelatinous materials adhering to the inner wall of the eggshell for easy removal, washed several times with tap water and distilled water to remove impurities and all organic matter eventually adhered to them. After washing, the eggshells were dried in an oven at 105°C for 24 hours. Then, the eggshells were grinded and then sieved using a 63 µm sieve (Figure 9) and gotten a fine powder (Da Silva Castro et al., 2018).

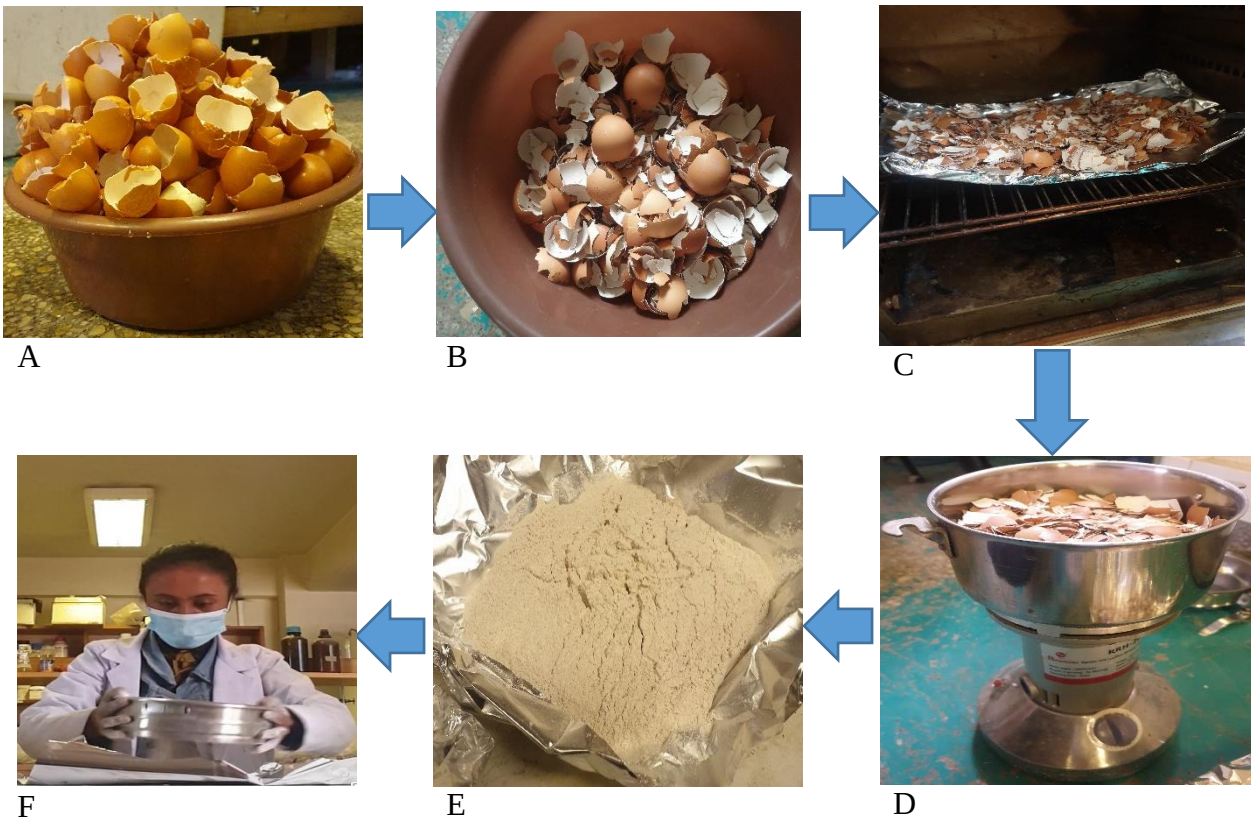


Figure 9. Preparation Process of eggshell-based CaO nanocatalyst: (A) Collected eggshell, (B) Rinsed with distilled water, (C) Oven dried eggshell, (D) Grinding using a grinder, (E) Grinded eggshell, (F) Sieving using 63 µm sieve

3.2.9.2. Calcination of Eggshell Powder

The eggshell fine powder was put in a crucible and calcinated in a muffle furnace at 900 ℃ for 3 hours (Figure 10) and kept in a desiccator.

A B

Figure 10. (A) Eggshell powder before calcination, (B) Calcinated eggshell powder at 900 ℃

3.2.9.3. Zn Substituted CaO Nanocatalyst

The Zn-doped CaO nanocatalyst was prepared by wet impregnation method (Figure 11), and in a typical preparation, CaO (10 g) was suspended in 40 mL of distilled water. To this, 10 mL of an aqueous solution of zinc sulfate of the desired concentration was added. The concentration of the zinc sulfate was 1.5 wt % Zn^{2+} concentration in CaO. The slurry thus obtained was stirred and refluxed at 60 ℃ for 6 hours and then, dried in an oven at 105 ℃ overnight (Borah et al., 2019; Kumar & Ali, 2013). The Zn/CaO characterization studies reveals that 1.5 wt % Zn^{2+}-doped CaO nanoparticles have the smallest size, possess higher basic strength and larger surface area, and hence, are expected to show higher activity toward the transesterification reaction (Kumar & Ali, 2013).

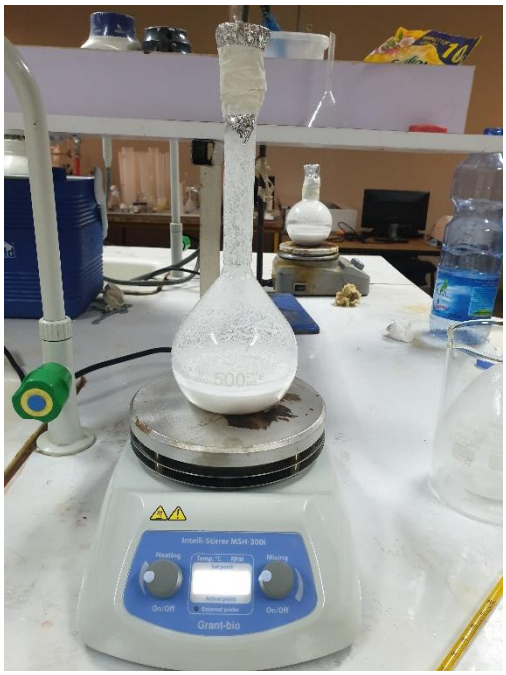


Figure 11. Impregnation of CaO by zinc doping

3.2.9.4. Calcination of the Zn Substituted CaO Nanocatalyst

The dried product was then calcined at 800 °C for 3 hours to activate it (Figure 12) and kept in a desiccator for future use (Borah et al., 2019; Kumar & Ali, 2013).

A B

Figure 12. Calcinated Zn/CaO nanocatalyst

3.2.10. Characterization of Nanocatalyst

The two different samples of CaO nanocatalyst prepared by the method of calcination of eggshell at a temperature of 900 ℃ and enhanced by zinc doped CaO (wet impregnation) method were characterized using XRD, SEM, and FTIR (Da Silva Castro et al., 2018). XRD and FTIR data interpretation was done using Origin 2018 software.

3.2.11. Optimization of Transesterification Parameters using Taguchi method

Taguchi's experimental design was used for the optimization of biodiesel production using MINITAB software (version 18). This method for the design of experiments (DOE) makes use of orthogonal arrays to optimize different process influencing parameters and the extent to which they can be varied. The very specialty of this method is that it only investigates a few pairs of combinations rather than all the possible parameter combinations. This method makes it possible to collate data for the determination of factors that have the most influence on product quality with a minimal number of experiments to conserve valuable time and resources (Sathish Kumar et al., 2015).

Among the different parameters influencing the production yield of biodiesel only the four most influencing parameters and three levels (L = 3, P = 4 as shown in Table 6) were considered in this study. By conducting only nine experiments as per L9 OA shown in Table 7, the effects of the four selected parameters such as A) catalyst loading (% wt.), B) methanol to oil ratio (molar ratio), C) reaction time (min), and D) reaction temperature (^oC) at three different levels were studied.

Table 6. Chosen parameters and their levels.

Parameters	**Levels**		
	1	**2**	**3**
A: Concentration of catalyst (wt%)	1	3	5
B: Methanol to oil (molar ratio)	9:1	11.53:1	16:1
C: Time of reaction (min)	60	120	180
D: Temperature of reaction (^oC)	50.0	57.5	65.0

Table 7. Experimental design with four parameters for optimization in transesterification reaction using the Taguchi method.

Run	Concentration of catalyst (wt%)	Methanol/oil (molar ratio)	Time for reaction (min)	Reaction temperature (°C)
1	1	1	1	1
2	1	2	2	2
3	1	3	3	3
4	2	1	2	3
5	2	2	3	1
6	2	3	1	2
7	3	1	3	2
8	3	2	1	3
9	3	3	2	1

3.2.12. Biodiesel Production Process and Experimental Design

For the reaction to be carried out, three-necked 250 and 500 ml round bottom flasks were used as a reactor. The three-necked round bottom flask was immersed in a dish used as a water bath to control the temperature in the hot plate with a magnetic stirrer. One of the side necks was used for placing the thermometer in order to observe the reaction temperature; the other side neck was connected with a water-cooled condenser owing to reduce the loss of methanol and reagents were added through the middle neck (Chowdhury et al., 2019). During the reaction the microalgal oil was measured and poured into three-necked flask and it was preheated at 50℃ and the catalyst was weighed and dissolved in the required amount of preheated alcohol (methanol) then, the calcium methoxide solution was added to preheated oil, the transesterification process was performed to obtain the maximum biodiesel yield (Figure 13) by differing the operating variables like the catalyst loading, alcohol /oil molar ratio, reaction time, and temperature. All experiments were conducted with a constant volume of microalgae oil of 20ml and an agitation speed of 600 rpm.

Figure 13. Experimental setup for transesterification reaction

After the reaction was completed, the solution was poured into a separating funnel. The separate layers of glycerol, catalyst, and methyl ester were distinguished; for proper separation the product was standing for overnight. After a one-night stand, the layers of glycerol, catalyst, and crude biodiesel become very prominent. Glycerol being dense was at the bottom and biodiesel on the top.

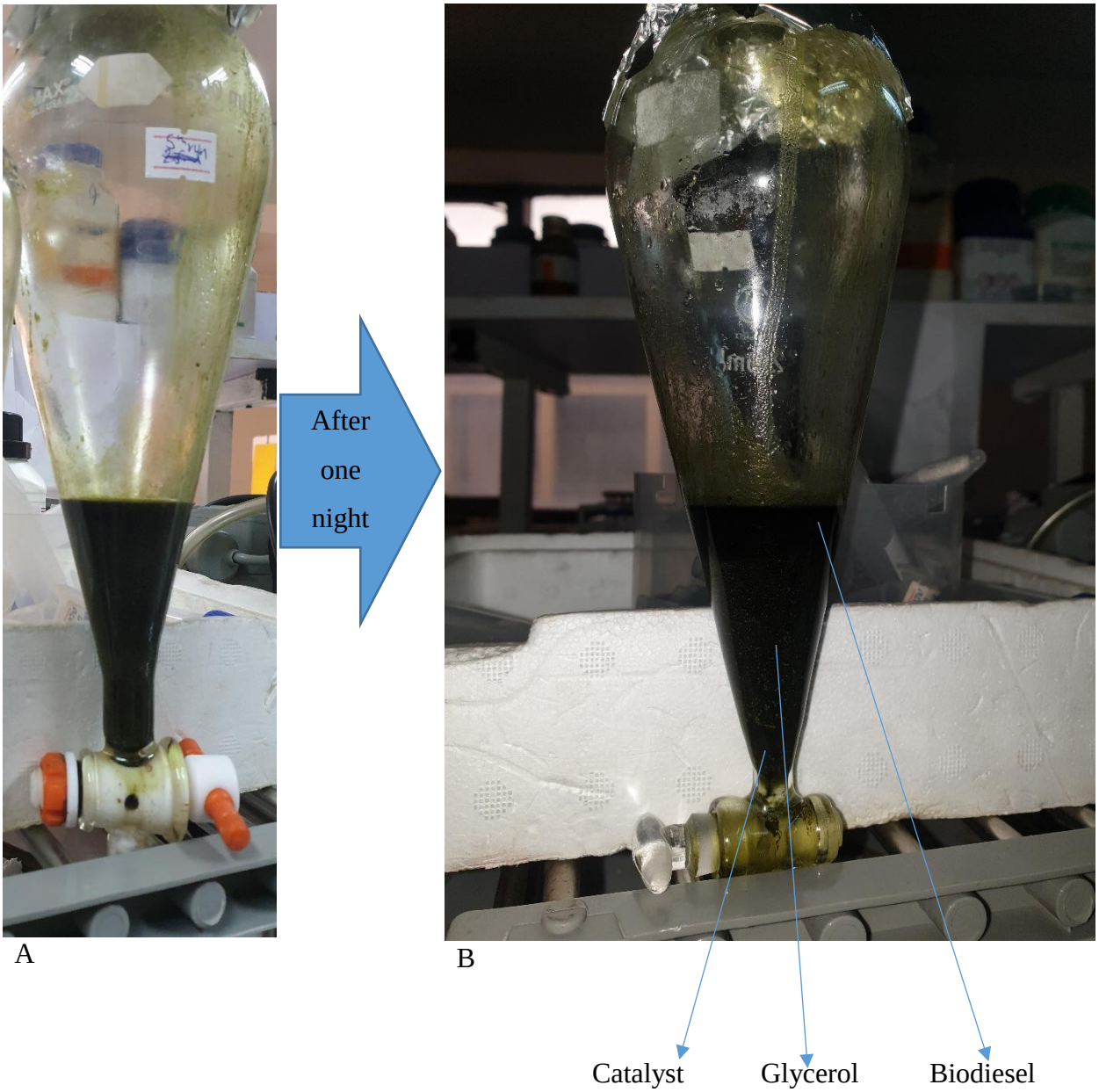


Figure 14. Biodiesel separation (A) after reaction completed and before the separation of biodiesel, glycerol and catalyst phase (B) After one-night stand biodiesel, glycerol, and catalyst phase

As shown in figure 14, after the reaction was completed, the mixture was transferred into a separating funnel and allowed to stand overnight and three phases were formed. The upper phase was biodiesel, the middle phase was glycerol, and the lower phase was the solid catalyst. The

separated biodiesel product obtained was heated to remove excess methanol and finally the purified biodiesel was obtained.

Figure 15. The purified produced biodiesel

The produced biodiesel yield was determined using equation 11.

$$\text{Biodiesel Yield (\%)} = \frac{\text{volume of biodiesel}}{\text{volume of microalgal oil}} \times 100 \ldots\ldots\ldots\ldots \text{Equation (11)}$$

3.2.13. Signal-to-Noise Ratio (SNR) and Statistical Analysis of Variance (ANOVA)

Statistical data analysis of the optimization of biodiesel production data was analyzed using Taguchi methodology and MINITAB software (version 18) was used. To determine the deviation between the experimental value and desired value of performance characteristics, Taguchi recommended to use the loss function and then a signal-to-noise ratio (SNR) has been created from the value of the loss function. To identify the combined effect of catalyst concentration, molar ratio of alcohol to oil, reaction temperature, and reaction time on yield, the S/N ratio of the Taguchi method was used for data analysis and prediction of optimum parameters. The expected outcome's log functions, or SNRs, serve as the objective of the optimization problem. Then, SNR was employed to calculate how far the quality function deviates from the expected value. Depending upon the objective of the problem, there are three types of SNRs used in the Taguchi method. For maximization problems (Larger-the-Better (LTB)), for minimization problem (Smaller-the-Better (STB)) and for normalization problems (Nominal-the-Better (NTB)) can be adopted. For NTB,

STB and LTB models, the SNR can be calculated as shown below (Saravanakumar et al., 2016; Sathish Kumar et al., 2015).

$$\text{Nominal the best} - \text{SNR}_i = 10 \log \left(\frac{\overline{y}_i^2}{s_i^2} \right) \quad \text{.....Equation(12)}$$

$$\text{Smaller the better} - \text{SNR}_i = -10 \log \left(\sum_{j=1}^{n} \frac{y_j^2}{n} \right) \quad \text{.....Equation(13)}$$

$$\text{Larger the better} - \text{SNR}_i = -10 \log \frac{1}{n} \left(\sum_{j=1}^{n} \frac{1}{y_j^2} \right) \quad \text{.....Equation(14)}$$

where

$$y_i = \frac{1}{n} \left(\sum_{j=1}^{n} y_{i,j} \right) \text{ (mean value of response)} \quad \text{.....Equation(15)}$$

$$s_i^2 = \frac{1}{n-1} \left(\sum_{j=1}^{n} y_{i,j} - \overline{y}_i \right) \text{ (variance)} \quad \text{.....Equation(16)}$$

Where: i-the experiment number, j- the trial number and n-the number of trials.

The evaluation of experimental data based on SNR has been done in order to identify the optimal parameter combinations. Out of the three different SNR quality parameters that are available based on the nature of variables, Larger-the-Better (LTB) has been used in the present study since the objective is to get maximum biodiesel yield. Therefore, the optimal design parameter or level of control was the level with the maximum SNR. SNR analysis can be used to obtain the optimum level of each parameter and the optimum set of parameters that would provide the highest yield of biodiesel, but it cannot identify which factor has influenced the output significantly and how much each factor contributed to the output. By doing a statistical analysis of variance (ANOVA) on the response data, this might be accomplished (Sathish Kumar et al., 2015). Therefore, a better feel for the relative effect of the different process parameters on the methyl ester conversion ratio was obtained by decomposition of variance, which is called analysis of variance. The ANOVA was studied to find out the effects of process parameters on FAME production (Saravanakumar et al., 2016).

For carrying out ANOVA of response data also, calculating the sum of squares is crucial. The following equation was used to calculate the percentage of contribution.

$$\%\ \text{contribution of factor} = \frac{\text{SSf}}{\text{SST}} \text{x } 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{Equation (17)}$$

Where SSf refers to the sum of the squares for the parameter and SST refers to the total sum of the squares of all parameters.

CHAPTER FOUR

4. RESULTS AND DISCUSSION

4.1. Physicochemical Parameters of Lake Hawassa

Four physicochemical parameters were determined during this study and the physicochemical parameters at the sampling sites were shown in Table 8.

Table 8. Selected physicochemical parameters of Lake Hawassa

Sample code	pH	Temperature	Conductivity	Salinity
H1	6.75	20.8 ^{o}C	8.92 mS/cm	5.5 ppt
H2	6.75	21.2 ^{o}C	8.78 mS/cm	5.3 ppt
H3	7.38	20.3 ^{o}C	8.66 mS/cm	5.4 ppt
Average	6.86	20.8 ^{o}C	8.78 mS/cm	5.4 ppt

The pH is an important factor for water analysis since it measures the free acidity or alkalinity of the water solution. One of the most important factors that influence aquatic production is the pH. Since most aquatic organisms are adapted to an average pH and do not withstand abrupt changes, pH variation is an essential parameter in water bodies (Melaku & Endale, 2019). In this study, pH values ranged from a minimum of 6.75 to a maximum of 7.38 with an average value of 6.86. Therefore, the highest values of pH were recorded in H3 and the lowest value in H1 and H2.

The algal cell factory's biochemical processes including photosynthesis are directly influenced by temperature, which is another crucial factor in the growth of microalgae. Water temperature obtained during the sampling period for the three sites varies from 20.3 to 21.2°C, with an overall mean value of 20.8°C. Therefore, relatively high-temperature values were recorded at H2 and the lowest value in H3. The pH and temperature of the *B. braunii* culture growth conditions used in this study were comparable to those of Lake Hawassa.

The ability of water to conduct an electric current is measured by its conductivity. It is sensitive to changes in dissolved solids, primarily mineral salts. High conductivity values are a sign of an increase in the amount of polluting particles, while a decrease in conductivity may be caused by dilution by rainwater (Melaku & Endale, 2019). The specific conductivity (SC) of Lake Hawassa

ranged from 8.66 mS/cm to 8.92 mS/cm and the overall mean value was 8.78 mS/cm. The lowest SC value was recorded at H3 and the highest was recorded at H1. Salinity was calculated from conductivity measurements and ranged from 5.3 ppt to 5.5 ppt with an average of 5.4 ppt. Therefore, the lowest salinity value was recorded at H2 and the highest was recorded at H1.

4.2. Isolation and Identification of *Botryococcus braunii* Microalgae

Initially, observation and identification of species were conducted using Lugol's iodine solution under an inverted microscope. Lugol's iodine solution is generally recognized as being the most suitable preservative to use when studying phytoplankton taxa. It is better for accurately quantifying cells than many other fixatives as it stains cells a dark brown color. This facilitates enumeration and aids with identification; it also adds weight to cells and helps phytoplankton cells sink to bottom of settling chambers (Williams et al., 2016). Therefore, identification, availability and abundance of *B. braunii* species were checked.

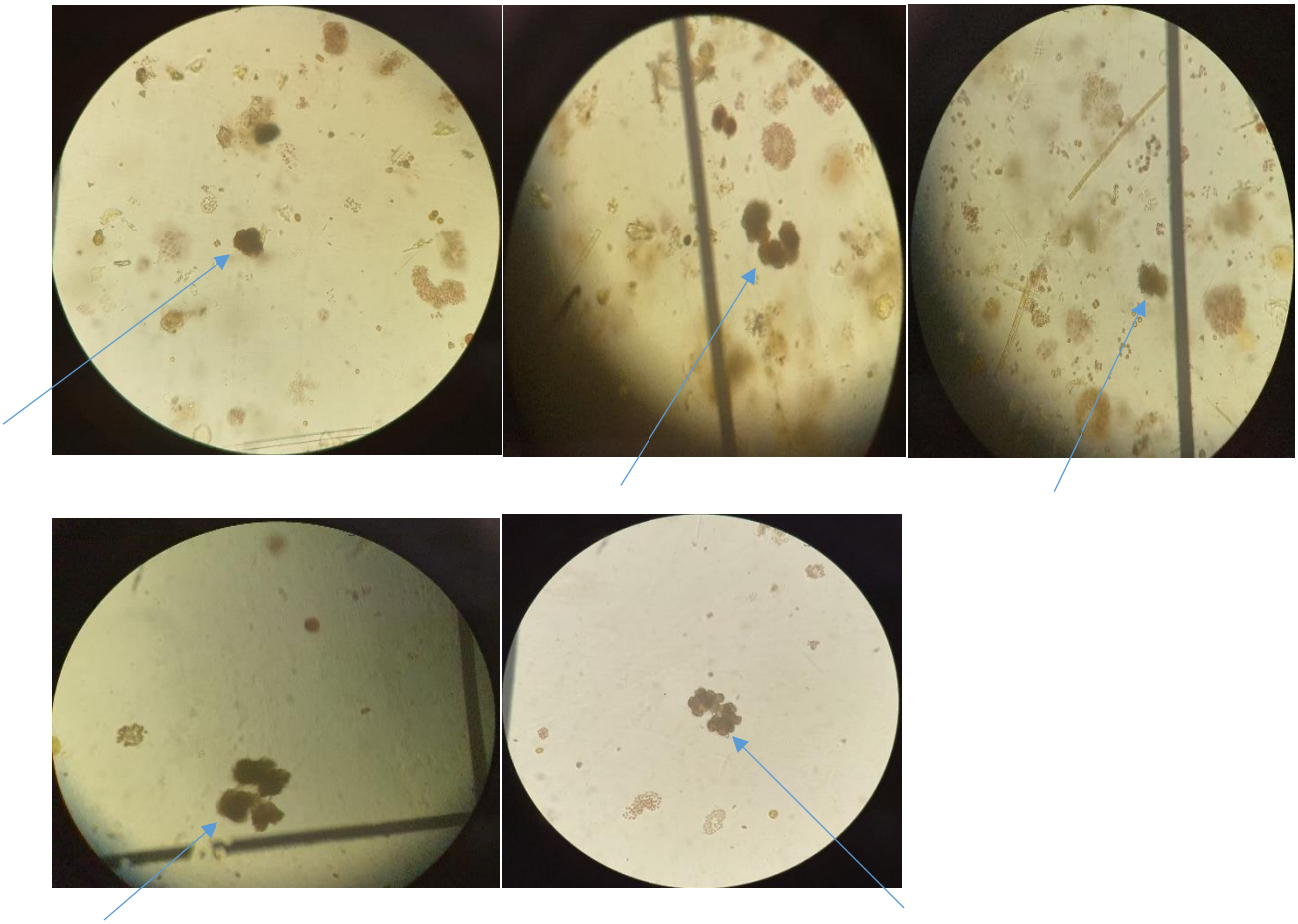
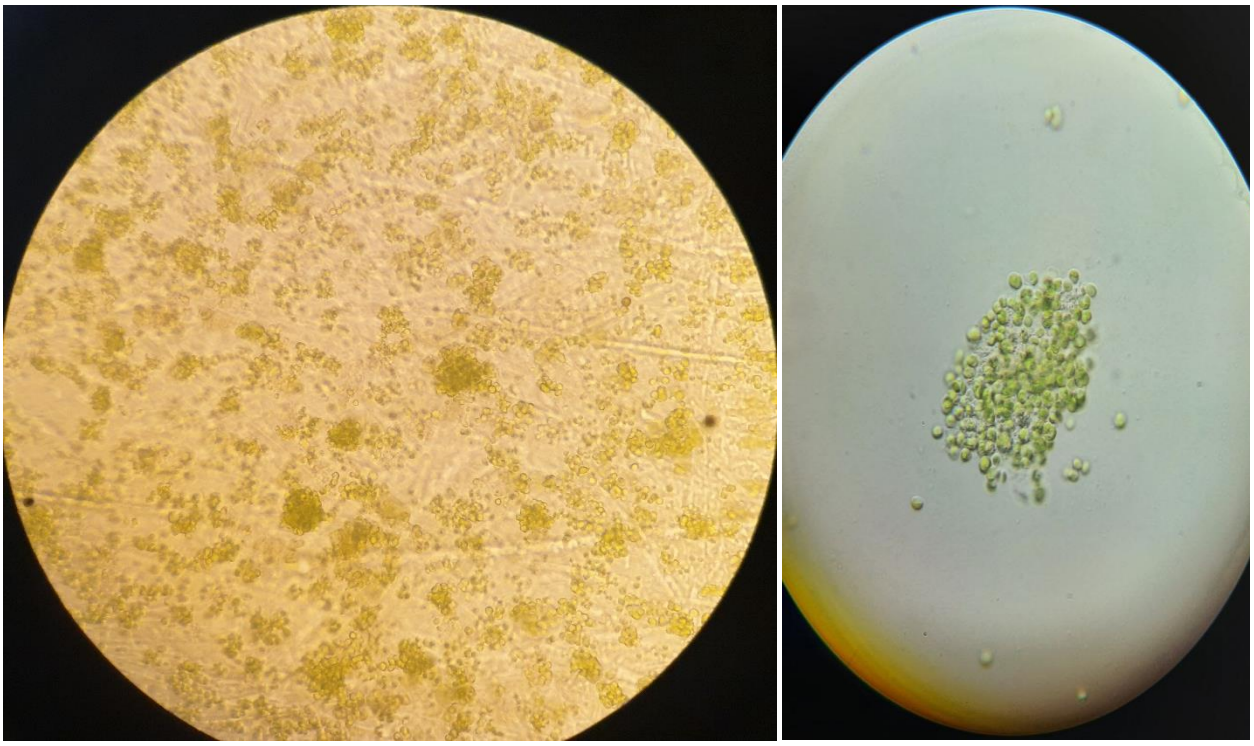


Figure 16. *B. braunii* identified samples collected from Lake Hawassa

Cells were spherical in shape and the colonies remain attached to one another. Cells are generally green to yellowish green in color. Similar observations were made by (Asma et al., 2015; Kleinert & Griehl, 2021). *B. braunii* is a colonial green microalga and was mainly distinguished based on colony size and details of cell shape, therefore the colony characteristics and morphological features of the Lake Hawassa isolate have demonstrated its similarity with *B. braunii*. Determination of inoculum size was followed for the given density of algae culture and after consecutive trial 10 % (v/v) of inoculum size was taken for all cultures during the whole culturing time.



A B

Figure 17. Isolates of *B. braunii* Species (A) and (B) at 40x and 100x magnification respectively.

4.3. Optical Density Measurement

The optical density of the *B. braunii* microalgal culture was estimated using the spectrophotometer for 15 days to observe its growth profile. As growth indicators, the growth curve of the optical density (absorbance) was plotted against the number of days (Figures 19 and 20). The growth of microalgae in the modified Chu-13 medium with an inoculum showed a typical pattern with 3 phases consisting of adaptation, logarithmic, and stationary phases. The adaptation phase was occurred at days 0-1 characterized by no significant growth, because the microalgae need initial

adaptation to a new environment. The logarithmic phase occurred on days 1–14 indicated by a significant increase in the growth of *B. braunii* in this phase, the microalgal cells uptake the excessive nutrients in the medium to support their growth to obtain energy, so the number of cells was increased logarithmically. On day 14, the growth of *B. braunii* reached the stationary phase, characterized by stagnant growth. In this phase, the cell number of growth and death were balanced (Purkan et al., 2019).

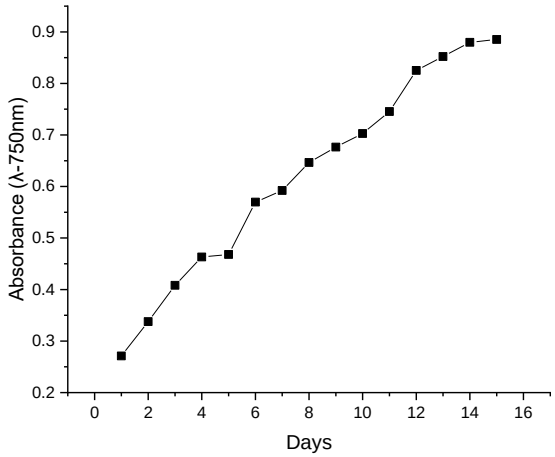


Figure 18. Absorbance of *B.braunii* microalgae culture at 750 wave number

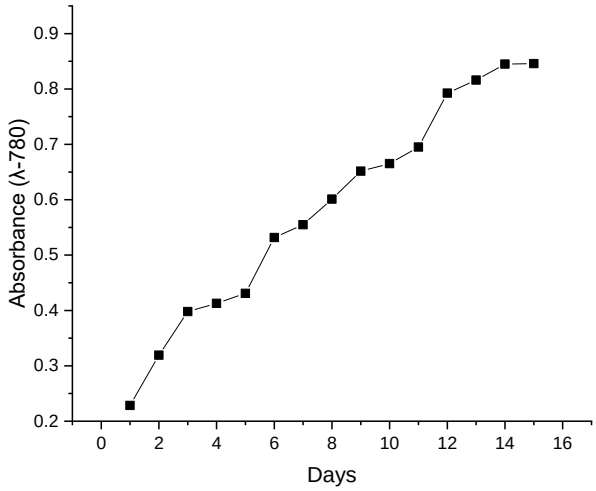


Figure 19. Absorbance of *B. braunii* microalgae culture at 780 wave number

The growth of the culture was calculated using Equation 2 and the result gave 0.085 g d^{-1} at 750nm and 0.094 g d^{-1} at 780nm. The results obtained from this experiment are comparable with other previous works which ranged from 0.078 g d^{-1} to 0.090 g d^{-1} and from harvesting on average 133 g of microalgae powder was obtained and also dry biomass of 4.661 g L^{-1} was obtained. The dry biomass result is comparable with other works which ranged from 0.430 g L^{-1} to 0.543 g L^{-1} (Lee et al., 2015).

4.4. Oil Extraction and Characterization

4.4.1. Microwave-assisted Oil Extraction

The use of a modified solvent mixture (n-hexane/methanol) using microwave in the extraction processes resulted in a higher yield than the other extraction methods. Since n-hexane is a non-polar solvent with high oil solubility; thus, it is able to solve and attract oil content from the microalgae matrix cells. Additionally, because methanol is the polar solvent, it allows microalgae to absorb more microwave energy; as a result the rate of mass transfer and disruption of cells rises during microwave extraction. Utilizing the mixing solvent noticeably higher extractable yield from microalgae was obtained than the yield with hexane (Kalsum et al., 2019).

Oil extractions were done by following the procedure put in section 3.2.7. and the result was summarized in Table 9 below:

Table 9: Oil extraction yield

Algae Powder /g/	**Solvents /ml/**	**Extraction time /min/**	**Oil yield /g/**	**Oil content /%/**
10	100(n-hexane) 50(methanol)	40	3.77	37.7

The total lipid content of *B. braunii* biomass in this study was found to be 37.7% which is greater than what was found in the other works. Previous study on *B. braunii* shows the biomass yield of 2.46 g L^{-1} with the highest lipid content of 37.5 % (Yeesang & Cheirsilp, 2014). Similar results have been observed with a 26.3% wt of lipid content (of dry microalgae) extracted from *B. braunii* by methanol/chloroform mixture using a soxhlet extractor (Hidalgo et al., 2015). Other results about total lipids for *B. braunii* grown in BBM are 20.3% and 25.2% (Hidalgo et al., 2014).

Therefore, due to their high lipid content *B. braunii* microalgae investigated in this study, represents a good raw material for the production of biodiesel.

4.4.2 Characterization of Microalgal Oil

Physicochemical properties of microalgal oil were determined after distillation and evaporation and the obtained results are presented in Table 10.

Table 10: Summary of physicochemical properties of the oil obtained from *B. braunii*

No.	physicochemical Properties	Unit	Measured Value
1	Color	-	Dark yellow
2	PH	-	5.88
3	Density@20 ℃	g/ml	0.84
4	Kinematic viscosity @40 ℃	mm^2/s	35.47
5	Acid value	mg KOH/g oil	1.59
6	Free fatty acid value	%	0.795
7	Saponification value	mg KOH/g oil	212.78
8	Molecular weight	g/mol	791
9	Moisture content	%	0.98
10	Ash Content	%	1.86

In order to design an advanced technological process determining the physicochemical properties of the extracted microalgae oils are very important parameters. The results tabulated in Table 10 show that at a temperature of 20°C the values of the density was 0.84g/mL. Similar results was observed which is 0.9694 g/mL and 0.9223 g/mL for Mustard and Corn oils respectively. Table 10 also shows that at temperature of 40°C the viscosity is low (35.47) when compared with Mustard and Corn oil which is 117.27 and 112.00 respectively (Zahir et al., 2017). It explains that the viscosity and density decreases with an increase in unsaturation and increases with high saturation and polymerization (Kim et al., 2010). Viscosity also depends on temperature.

As the temperature rises the kinetic energy also rises, enhancing molecules movement and lowering intermolecular forces. The liquid's layers easily passing over one another and then contributes to the viscosity reduction. This phenomenon is also verified by other researchers since

oil viscosity depends on molecular structure and decreases with the unsaturation of fatty acids (Kim et al., 2010; Zahir et al., 2017).

The acid value of microalgae oil in this study were 1.59 mg KOH/g of oil. This result indicates low oil acidity, enabling the production of biodiesel through the use of a basic catalyst, since the problems related to soap formation during the transesterification reaction will be minimized, making the biodiesel purification steps easier (Batista et al., 2018). The saponification value obtained for the microalgae oil in Table 10 showed 212.78 mg KOH/g. The lower value of saponification values suggests that the mean molecular weight of fatty acids is lower (saturated and unsaturated) (Zahir et al., 2017). The SV result obtained compared favorably with that of soybean oil (193 mg KOH/g). The free fatty acid of microalgae oil in Table 10 also showed 0.795% (Ejim & Kamen, 2013).

4.5. Characterization of CaO Nanocatalyst Prepared from Eggshell

4.5.1. X-Ray Diffraction (XRD) Analysis

The crystal structure of the prepared samples was determined by X-ray diffractometer with Ni filtered CuKα radiation at λ = 0.154 nm over a 2Θ range of 10–60° at a scanning rate of 2°min^{-1}. The sharp diffraction peaks for undoped CaO synthesized by calcination of eggshells at 900 ℃ at 2Θ were 32.45°, 33.87°, 37.61°, 48.56°, 54.14° as shown in Figure 20. For Zn doped CaO synthesized by wet impregnation method the sharp peaks of the sample at 2θ were 32.52°, 33.94°, 37.68°, 48.62°, 54.20° as shown in Figure 21. The results are comparable with the main peak values characteristics of undoped CaO produced from eggshell and Zn doped CaO reported by (Borah et al., 2019). All of the samples showed high crystalline diffraction peaks. The formation of a sharp peak indicates the formation of highly crystalline materials and the XRD pattern of undoped and Zn doped CaO is shown in Figure 22. No impurities were detected in the XRD patterns, confirming the high purity of the synthesized products.

The crystallite size diameter (D) in the nanometer of the undoped CaO and Zn doped CaO nanoparticle has been calculated using the Debye Scherrer equation 18 shown below for each 2θ value and FWHM Bsize (°) as shown in Tables 11 and 12.

$$\text{Scherrer equation: } D = \frac{K\lambda}{\beta \cos\theta} \dots\dots\dots\dots\dots\dots \text{Equation (18)}$$

where, D: average crystallite size diameter, λ: is the X-ray wavelength which is 1.5406 Å for Cu Kα, β: is the peak width of the diffraction peak profile at half maximum height resulting from small crystallite size in radians, full-width at half-maximum or half-width (FWHM) is in radian, K: is a constant related to crystallite shape normally taken as 0.94, θ: Bragg angle can be in degrees or radians.

Table 11: Result of X-Ray Diffraction pattern of CaO nanocatalyst prepared by calcination at 900 °C

2theta Value	**β-beta FWHM (radians)**	**K-constant**	**λ (Å)**	**Intensity**	**Dp (nm)**	**Dp Average (nm)**
32.45	0.25306	0.94	1.5406	846.48	34.15	
33.87	0.22672	0.94	1.5406	548.69	38.26	
37.62	0.24838	0.94	1.5406	2287.63	35.29	39.12
48.56	0.17568	0.94	1.5406	341.58	51.81	
54.14	0.25816	0.94	1.5406	1239.60	36.09	

Table 12: Results of X-Ray Diffraction pattern of the Zn/CaO nanocatalyst

2theta Value	**β-beta FWHM (radians)**	**K-constant**	**λ (Å)**	**Intensity**	**Dp (nm)**	**Dp Average (nm)**
32.52	0.20855	0.94	1.5406	1232.43	41.44	
33.94	0.19067	0.94	1.5406	772.86	45.49	
37.68	0.21613	0.94	1.5406	3253.70	40.56	44.74
48.62	0.15876	0.94	1.5406	462.77	57.35	
54.20	0.23995	0.94	1.5406	1576.23	38.84	

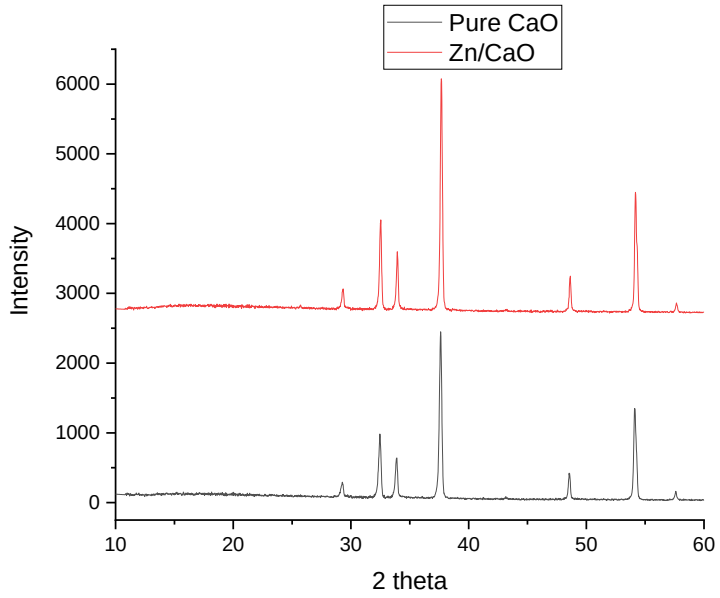


Figure 20. X-Ray Diffraction pattern of undoped CaO and Zn doped CaO nanocatalyst

The average crystallite size of undoped CaO and Zn doped CaO nanoparticles is calculated as 39.12 nm and 44.74 nm respectively; and a similar trend has also been observed by (Abdala et al., 2020).

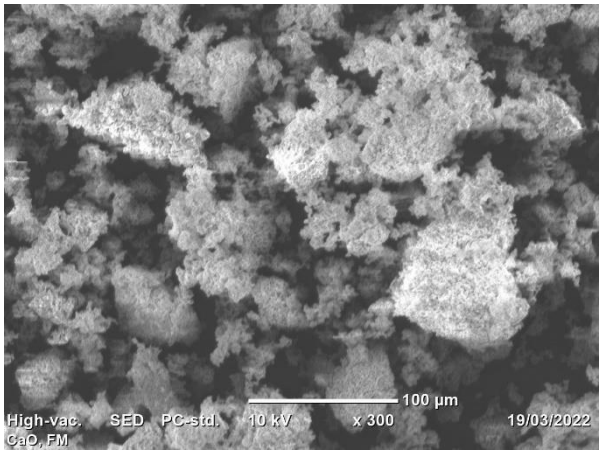
4.5.2. Scanning Electron Magnification (SEM) Analysis

The surface structure and morphology of the prepared nanocatalysts (undoped CaO and Zn doped CaO) were characterized under a scanning electron microscope (SEM). Micrographs were recorded at 100, 20, and 10 μm magnification. Although irregular shaped particles were mainly present in the natural eggshells, the calcination and wet impregnation treatment resulted in more regularity in the particle shape, forming a termite nest-like structure (Fayyazi et al., 2018). After calcination of the eggshell as shown in Figure 23, the obtained CaO material displayed high porosity and high surface area. This phenomenon can be described that the $CaCO_3$ compound of eggshell was transformed to CaO by losing CO_2 gas which produced high porous on the surface. Consequently, this physical property was expected not only to lead a highly active site but also directly to result in high biodiesel yield.

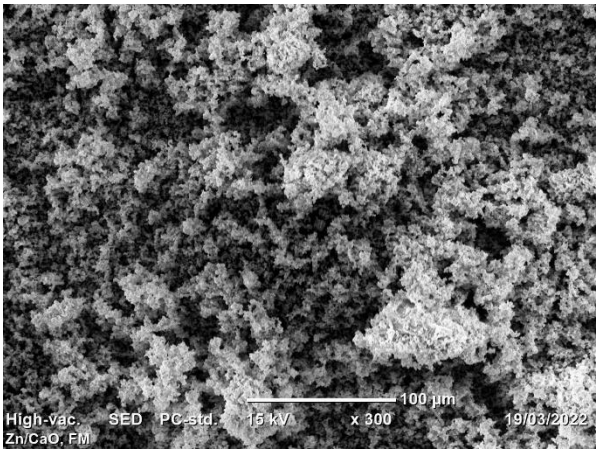
The Zn doped CaO particles are densely agglomerated with each other with a regular shape. The image shows more high porosity and high surface area, and the material is crystalline in structure.

A regular and ordered shape offers the advantage of having a better interconnected regular pore distribution system (Bharti et al., 2019). The Zn/CaO characterization studies reveal that 1.5 wt % Zn doped CaO nanoparticles possess higher basic strength and larger surface area than the rest of the samples, and hence, are expected to show higher activity toward the transesterification reaction (Kumar & Ali, 2013).

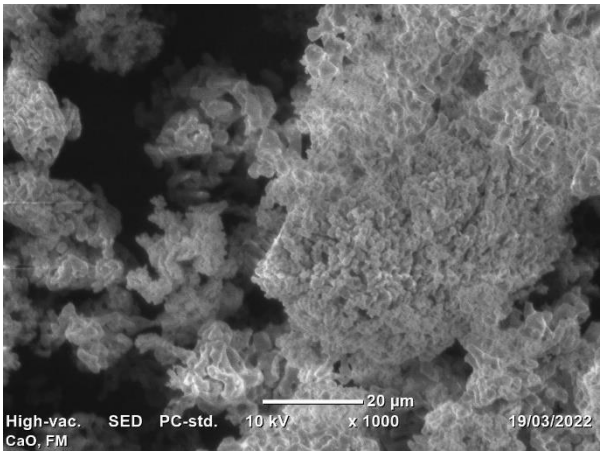
CaO **Zn/CaO**



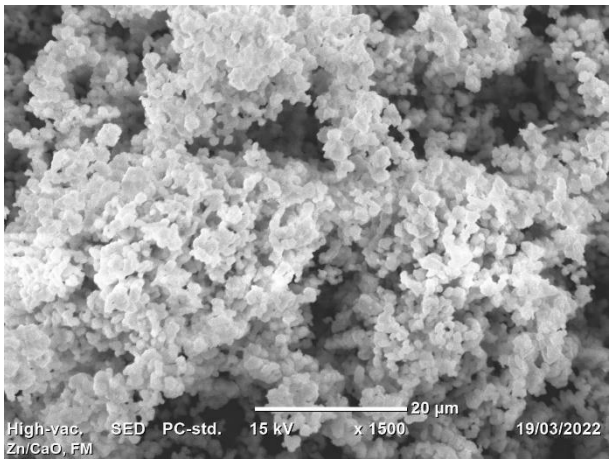
A



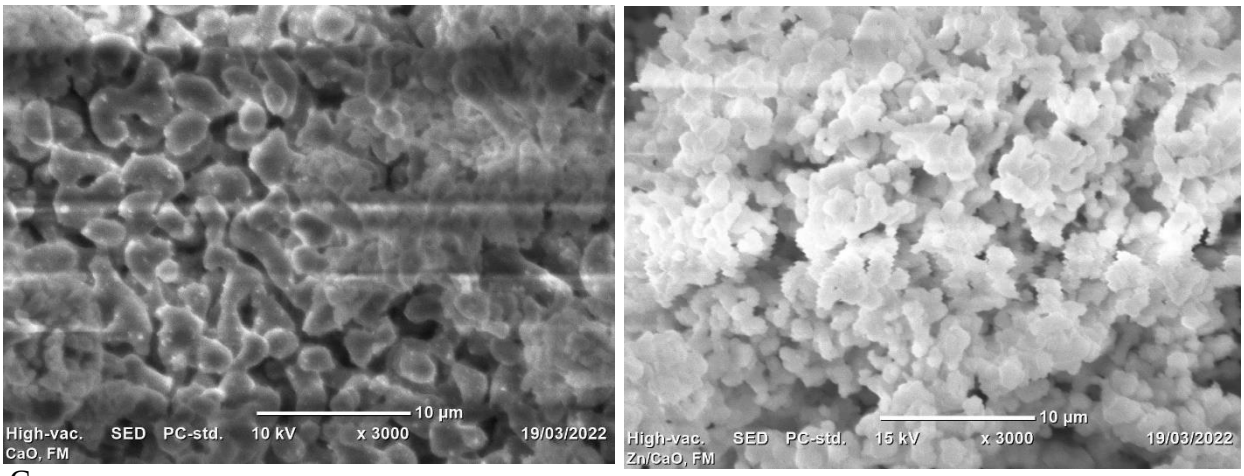
D



B



E



C F

Figure 21. SEM images of CaO nanocatalyst produced by calcination of eggshell at 900 ℃ (A, B and C) and SEM images of 1.5% Zn doped CaO nanocatalyst produced by calcination and wet impregnation method (D, E, and F) at 100, 20, and 10 µm magnification, respectively.

4.5.3. Fourier Transform Infrared Spectroscopy (FTIR) Analysis

The functional groups present on the nanocatalyst surface were identified with the help of FTIR; the method used was the KBr method at room temperature in the IR range of 500–4000 cm^{-1} (Bharti et al., 2019). The FTIR spectra of undoped CaO and Zn doped CaO are shown in Figure 24. The Ca–O vibrations appeared at around 500 cm^{-1} and the vibrations of hydroxide appear at 3643 cm^{-1}. Because the hygroscopic nature of Ca–O is well recognized, the appearance of $Ca(OH)_2$ spectra were justified. The characteristic signals at 500 cm^{-1} suggested the synthesis of Ca–O. The results obtained by this experiment are comparable to values reported by (Bharti et al., 2019).

The FT-IR spectra for the nanocatalysts with Zn loading are also presented in Figure 24. The peak which appeared around 3643 cm^{-1} corresponds to the vibrations of the OH group bonded to the metal ions and becomes more intense with Zn addition. The most obvious band assigned to ZnO is the one observed at around 527–876 cm^{-1}. The band around 1378–1536 cm^{-1} may have arisen due to the asymmetric stretching vibrations of the Zn–O bond. The absorption peak at around 3643 cm^{-1} is attributed to the O–H stretching vibration of H_2O. The peak observed at around 502–553 cm^{-1} is described as a stretching vibration of the Zn–O bond. The results obtained by this experiment are comparable to (Borah et al., 2019).

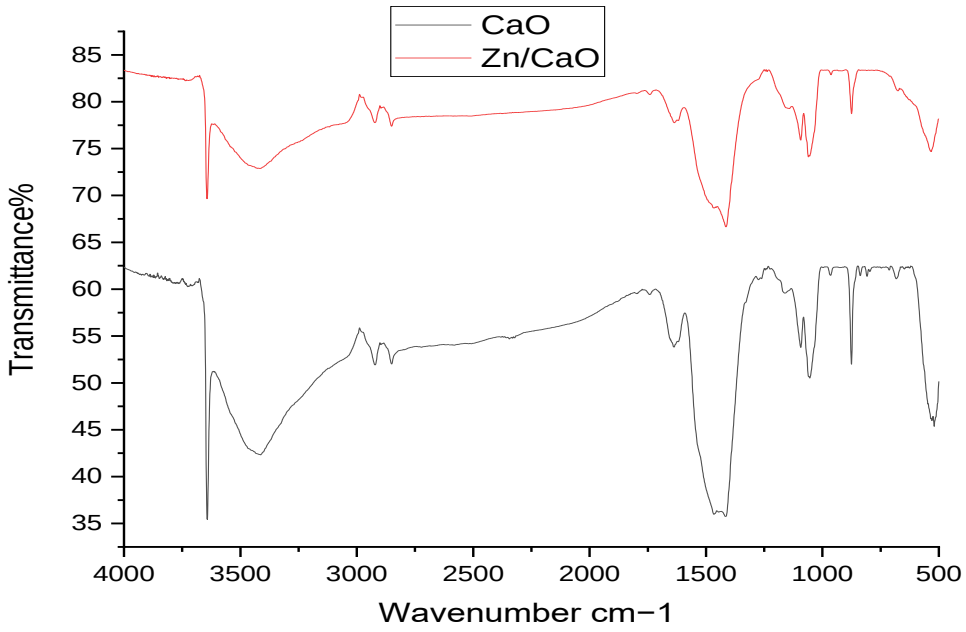


Figure 22. FT-IR spectra of undoped CaO and Zn-doped CaO nanocatalyst

4.6. Optimization of Biodiesel Production

4.6.1. Determination of Optimal Experimental Condition by Taguchi Methodology

Table 13: Experimental design for optimization of various reaction parameters in transesterification reaction using Taguchi methodology.

Run	A Catalyst (wt%)	B Methanol/oil (Molar ratio)	C Time (Min)	D Temperature (ºC)	Experimental FAME (%)	SNRA1	Predicted FAME (%)
1	1	7.73	60	50.0	55.72	34.92	56.10
2	1	9.91	120	57.5	66.28	36.43	63.37
3	1	13.75	180	65.0	76.54	37.68	77.48
4	3	7.73	120	65.0	71.92	37.14	69.08
5	3	9.91	180	50.0	55.10	34.82	60.34
6	3	13.75	60	57.5	94.58	39.52	95.36
7	5	7.73	180	57.5	67.41	36.57	66.05

Table 13. Continued

8	5	9.91	60	65.0	90.58	39.14	94.23
9	5	13.75	120	50.0	96.21	39.66	92.33

4.6.2. Static Design Analysis of Signal-to-Noise Ratio (SNR)

The percentage yield of methyl ester from microalgal oil under the designed nine sets of experiments (runs), their SNRs, and overall mean SNR are tabulated in Table 13. In this study, the maximization of the yield of biodiesel was set as objective, as a result the larger the better (LTB) SNR model has been employed. The results show that experiment number 9 had been the highest yield of 96.21% and experiment 5 had the lowest yield of 55.10%. Though the set of parameters corresponding to experiment 9 had the highest yield, this would not be the optimum set of parameters. It has been determined that the level mean signal to noise ratio (SNRL), which is the algebraic mean of all the SNRs of a certain control parameter at a given level, exists. The SNR, DSNR (difference in maximum SNRL to minimum SNRL of particular parameters), and rank for all four parameters have been calculated. The rank was determined using the DSNR value. A higher DSNR value was assigned rank 1. Based on the rank as shown in tables 15, the molar ratio of methanol to oil has been identified as the most influencing parameter on the yield of FAME. The concentration of catalyst and temperature of reaction are the second and third influencing factors followed by the time of reaction. The effects of each parameter at three different levels on FAME yield in terms of mean and SNR are shown in Figures 25 and 26. A higher value of SNR infers a greater influence of the particular parameter at that level. The maximum value in each graph specifies the optimum level of that particular parameter on the yield of FAME. Therefore, the optimum level of each parameter for the maximum yield of FAME were A: (concentration of catalyst) at level 3 (5%), B (Molar ratio of methanol to oil) at level 3 (16:1), C (time of reaction) at level 1 (60 min) and D (temperature of reaction) at level 3 (65 ^{o}C) where the agitation speed of the stirrer during the reaction was kept constant at 600 rpm.

Table 14: Response table for means

Level	Catalyst (wt%)	Methanol/oil (Molar ratio)	Time (min)	Temperature (ºC)
1	66.18	65.02	80.29	69.01
2	73.87	70.65	78.14	76.09
3	84.73	89.11	66.35	79.68
Delta	18.55	24.09	13.94	10.67
Rank	2	1	3	4

Table 15: Response table for Signal-to-Noise Ratios (Larger is better)

Level	Catalyst (wt%)	Methanol/oil (Molar ratio)	Time (min)	Temperature (ºC)
1	36.34	36.21	37.86	36.47
2	37.16	36.80	37.74	37.51
3	38.46	38.95	36.36	37.99
Delta	2.12	2.74	1.50	1.52
Rank	2	1	4	3

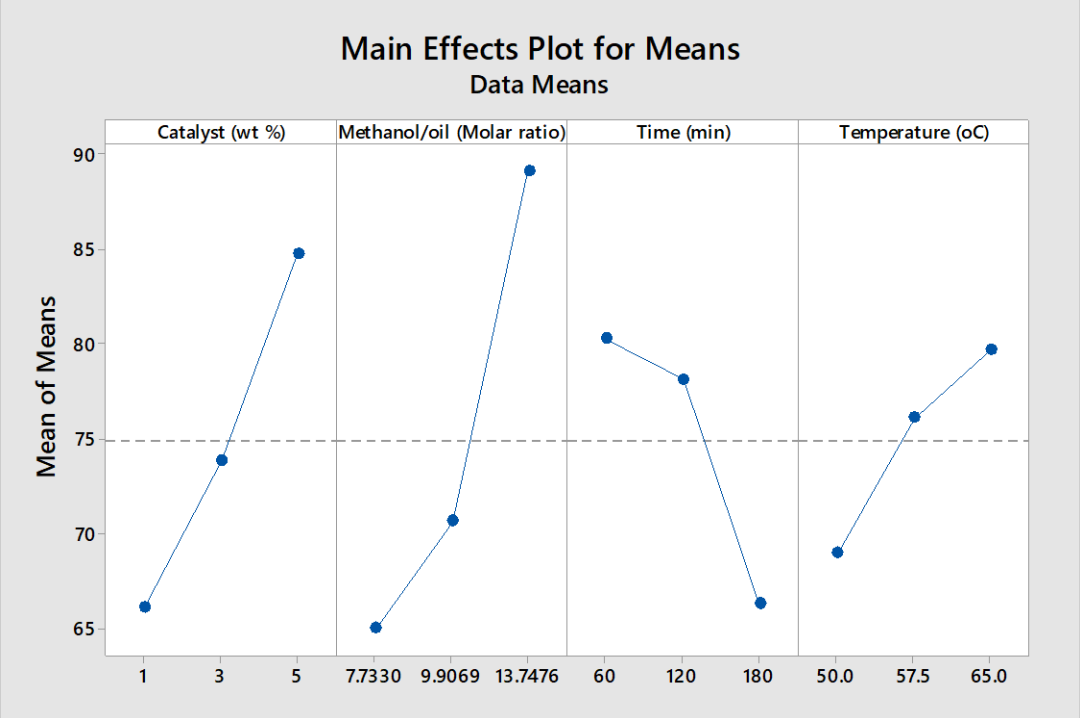


Figure 23. The main effects plot for means of each parameter at different levels on the conversion rate of microalgal oil methyl ester.

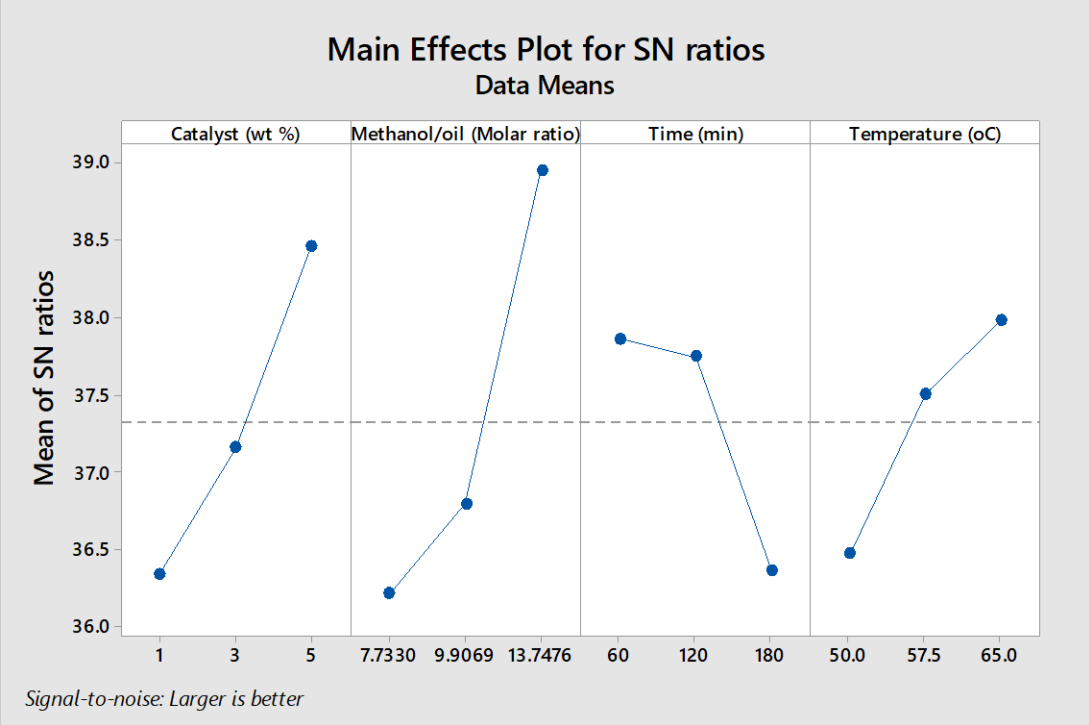


Figure 24. The main effects plot for the SN ratio of each parameter at different levels on the conversion rate of microalgal oil methyl ester.

4.6.2. Analysis of Variance (ANOVA)

ANOVA was employed in this study, to identify and quantify the most important factor that contributes to the highest yield (Saravanakumar et al., 2016; Sathish Kumar et al., 2015). ANOVA computes the relation with each parameter of biodiesel production as given in Equation 19 and Table 16. The most significant process parameter was also identified by calculating the percentage contribution of each parameter to the biodiesel yield. Table 17 shows the calculated SSf and % contribution. From the contribution table it was observed that the molar ratio of methanol to oil was the most significant parameter with 46.1% contribution to the biodiesel yield from *B. braunii* microalgal oil followed by the concentration of catalyst with 25.22% contribution. The time of reaction and temperature of reaction with 15.41% and 13.27% contribution to biodiesel yield respectively.

Experimental FAME (%) = -8.8 + 4.638 Catalyst (wt %) + 4.098 Methanol/oil (Molar ratio) - 0.1162 Time (min) + 0.711 Temperature (^oC) ……Equation 19

Equation 19 shows the regression equation, it is a statistical model that determines the specific relationship between the input parameters and output parameters. It gives the outcome with a relatively small amount of error.

Table 16: Analysis of Variance (ANOVA) for FAME conversion (%)

Source	DF	Adj SS	Adj MS	F-Value	P-Value	Remark
Regression	4	1913.25	478.31	25.20	0.004	Significant
Catalyst (wt %)	1	516.34	516.34	27.21	0.006	
Methanol/oil (molar ratio)	1	934.51	934.51	49.24	0.002	
Time (min)	1	291.62	291.62	15.37	0.017	
Temperature (^oC)	1	170.77	170.77	9.00	0.040	
Error	4	75.91	18.98			
Total	8	1989.16				

The ANOVA given in Table 16 revealed a significant ($p<0.05$) interactive effect of the four factors that have been investigated (Priyadarshi & Paul, 2019).

Table 17: Percentage contribution of process parameters

Source	DF	Seq SS (sum of squares)	% Contribution
Catalyst (wt %)	2	6.8460	25.22
Methanol/oil (molar ratio)	2	12.5101	46.1
Time (min)	2	4.1821	15.41
Temperature (^oC)	2	3.6025	13.27
Total	8	27.1407	100

Table 18: Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
4.35639	96.18%	92.37%	81.17%

The significance of the Taguchi model was calculated by determining the R square and adjusted R square. Coefficient of determination (R square): 96.18% and R square (adjusted): 92.37% were obtained for transesterification reactions (Table 18). Therefore: In the model summary, the R square value provides a measure of how well the model is fitting the actual data (Priyadarshi & Paul, 2019). Here R square value was 96.18%, this shows that the model obtained is fitted to actual data.

4.6.3. Normal Probability Plot of Residuals for %Yield

A graphical tool for comparing a data set with the normal distribution is the normal probability plot. The data fit a normal probability distribution as shown by a straight line in this plot. There were very low residual values and all residuals obtained were almost along the line.

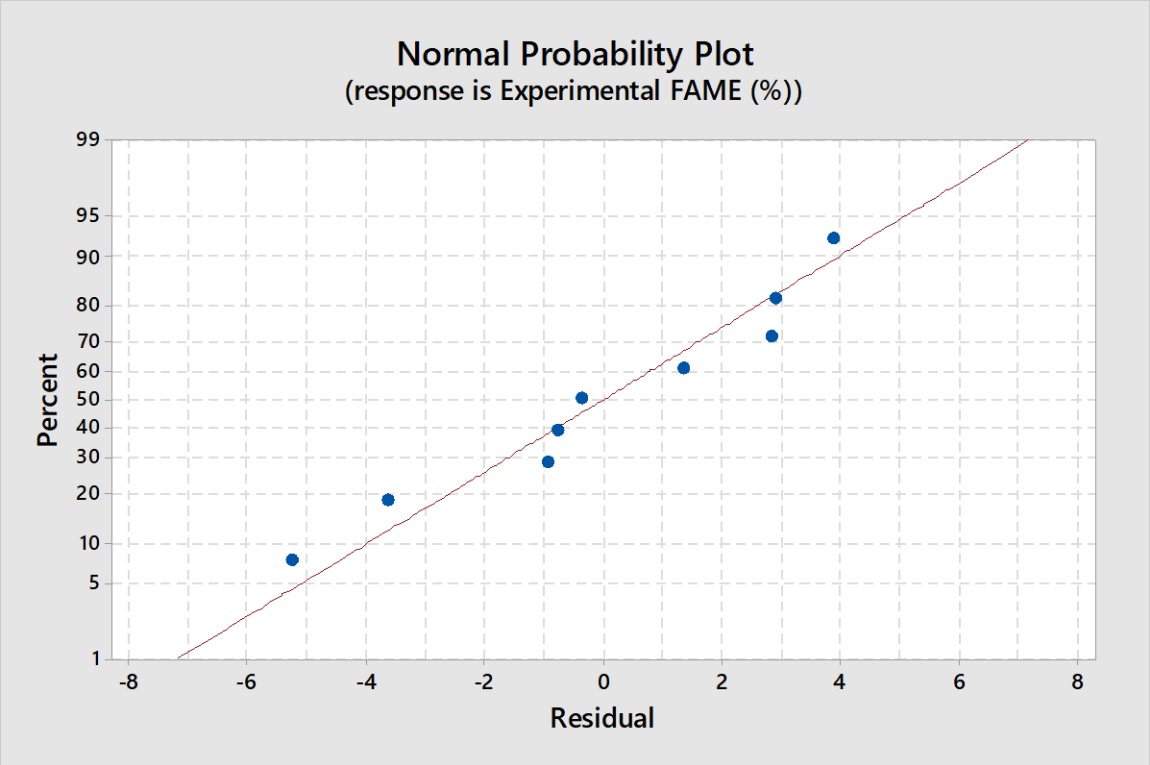


Figure 25. Normal probability plot

4.7. Effect of Operational Parameters on the Biodiesel Production Process

Contour plots were used to study the concurrent effect of the independent variables on biodiesel conversion (%). They are topographical maps drawn from three-dimensional data. On the horizontal axis one variable was represented, and on the vertical axis a second variable was represented. A color gradient and isolines (lines of constant value) were used to represent the third variable. These plots are often useful in data analysis, especially when searching for minimums and maximums in a set of trivariate data. Figures 26-31 describe the contour plot linked to the effect of two variables on the yield of biodiesel (response). The curvatures nature indicate the mutual interaction of the methanol/oil molar ratio with reaction time, methanol/oil molar ratio with reaction temperature, methanol/oil molar ratio with catalyst amount, reaction time with reaction temperature, reaction time with catalyst loading, and, reaction temperature with catalyst loading, respectively.

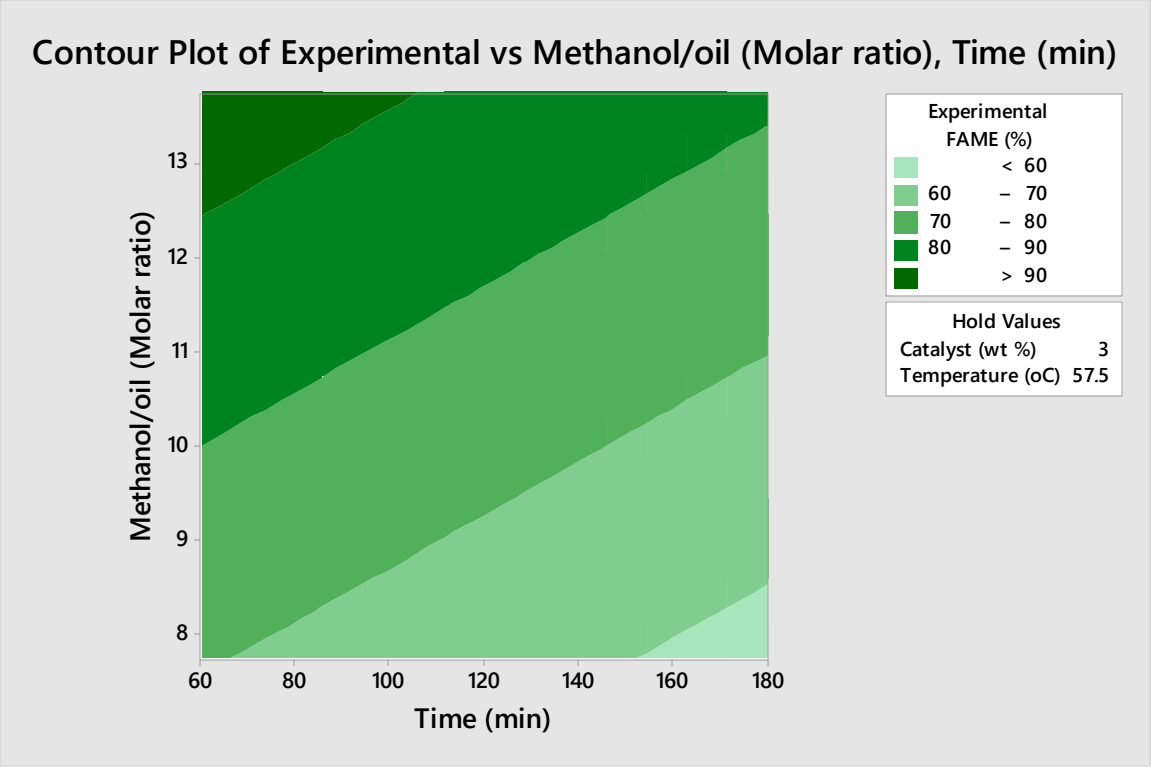


Figure 26. Contour plot of experimental fame vs Methanol to oil ratio, Time

Figure 26 shows the contour plot of the interaction between methanol to oil ratio and reaction time. The conversion of biodiesel rises with the increase in methanol to oil ratio and in less increase in reaction time. In this study, conversion raised with the increase in methanol to oil ratio from 55.72% (run 1) to 96.21 % (run 9) of yield in the range of 9:1 methanol ratio with respect to 60 min reaction time to 16:1 methanol to oil ratio with respect to 120 min reaction time, respectively. One of the most important variables that affect FAME yield efficiency as well as the production of biodiesel is the molar ratio of methanol to oil. Therefore, in order to enhance the equilibrium conversion of reaction based on the reversible reaction mechanism (i.e. transesterification reaction), an excess amount of methanol is used to drive the reversible reaction (transesterification) to the right side to produce more biodiesel (Çakırca et al., 2019). The maximum biodiesel conversion of 96.21% was achieved under optimized reaction conditions of 16:1 methanol to oil molar ratio, 5 wt % catalyst loading, 50 °C reaction temperature and 2 hr reaction time using Zn doped CaO nanocatalyst.

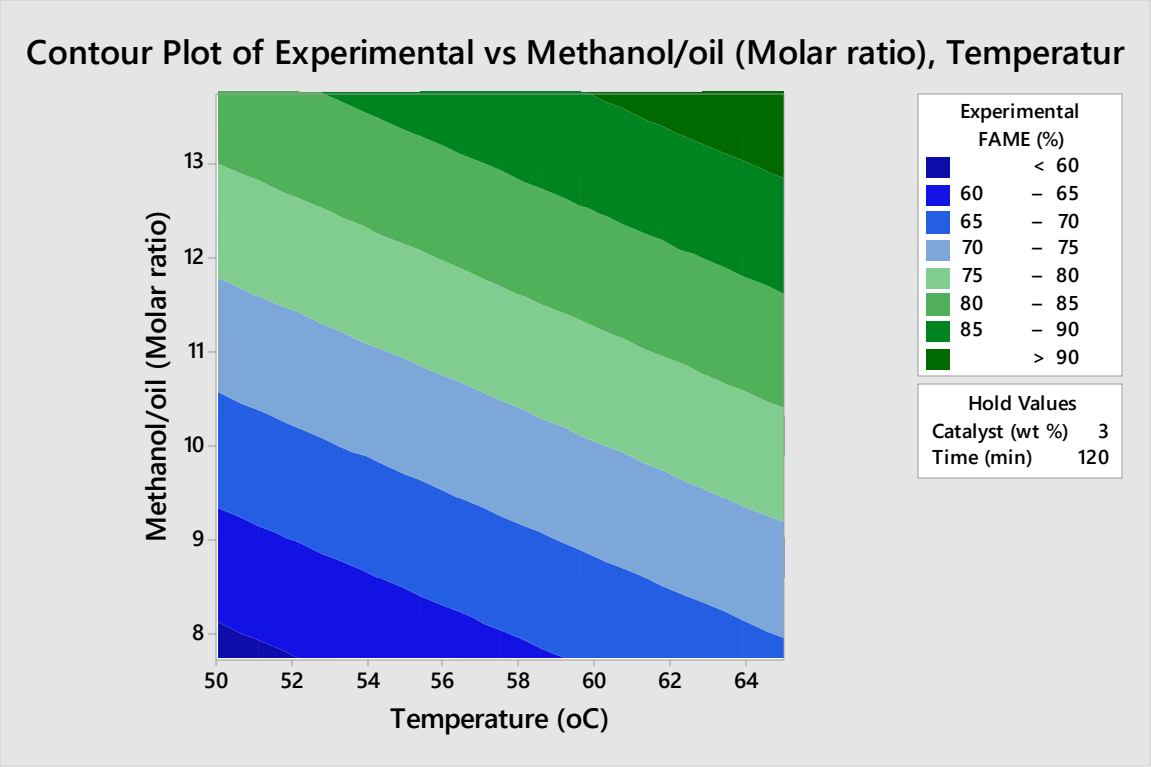


Figure 27. Contour plot experimental fame vs Methanol/oil, Temperature

The contour plot of the combined effect of temperature and methanol to oil ratio is depicted in Figure 27. In order to improve the yield, reaction temperature also plays an important role. An increase in temperature increases the reaction rate and kinetics (Gashaw et al., 2015). Depending on the type of oil used, the trans-esterification process is often carried out below the boiling temperature of alcohol within an optimum temperature range of 50–60 °C. Due to the subcritical state of methanol, relatively low conversion to methyl ester is evident at low temperatures. High temperatures mostly cause the utilized alcohol to evaporate (Bano et al., 2020). There is an increase in conversion with the increase of the methanol to oil ratio. The reason can be explained by the fact that increasing methanol concentration will cause the equilibrium to shift to the product side, hence favoring the biodiesel production (Borah et al., 2019). Therefore, with an increasing temperature of the reaction, the yield of biodiesel increased quickly from 55.72 % (run1) to 94.58% (run 6) yield in the range of methanol to oil ratio from 9:1 with respect to 50 ^{o}C temperature up to 16:1 methanol to oil ratio with respect of 57.5 ^{o}C temperature respectively.

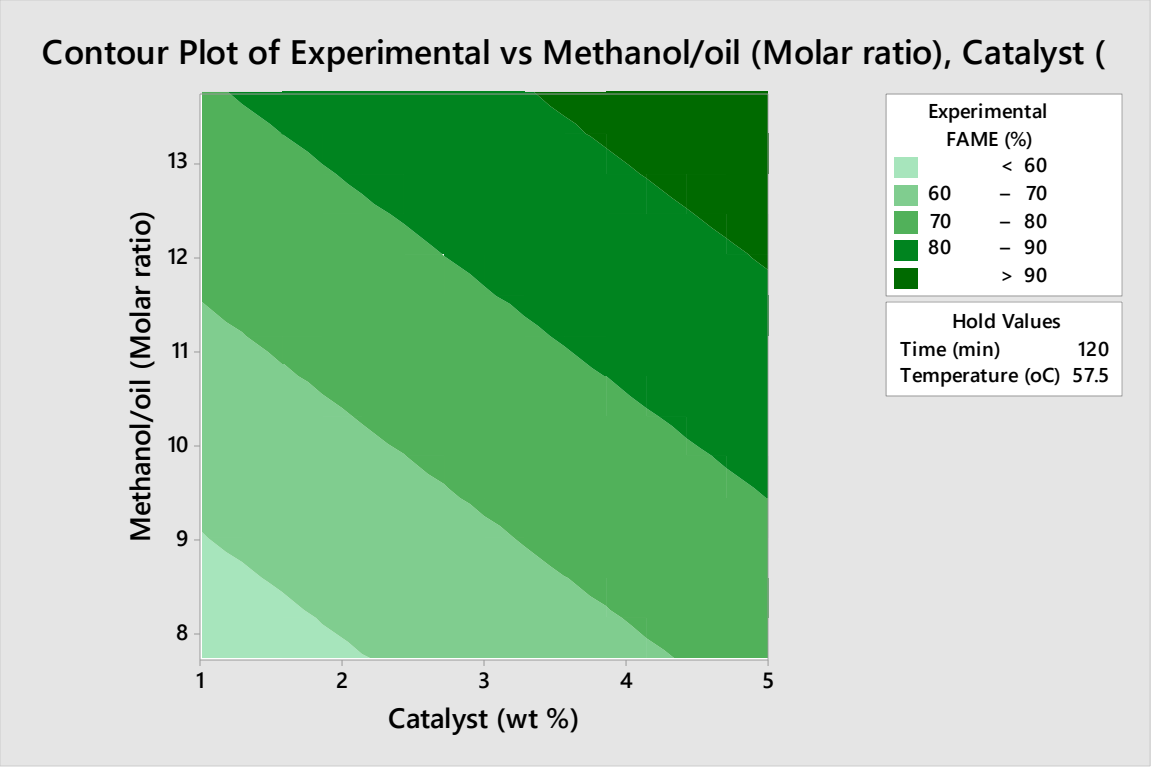


Figure 28. Contour plot of experimental FAME vs Methanol/oil, Catalyst

The contour plot of the combined effect of catalyst loading and methanol to oil ratio is depicted in Figure 28. The figure illustrates that the yield of methyl esters increases significantly with the increase of catalyst concentrations and methanol-to-oil ratio. In the beginning, the yield of methyl ester on catalyst loading 1 wt% was 55.72 % (run 1) in the range of methanol to oil ratio 9:1; While on catalyst loading 5 wt% in the range of methanol to oil ratio 16:1 the percentage of the yield was increased to 96.21% (run 9). The conversion of biodiesel increases with the increase in the amount of catalyst loading which may be due to the increased surface active sites of the catalyst and also there is an increase in conversion when the methanol to oil ratio increases. The reason can be explained by the fact that increasing methanol concentration will cause the equilibrium to shift to the product side, hence favoring biodiesel production. Furthermore, using excess methanol not only accelerates the rate of transesterification but also removes product molecules from the surface of the catalyst to regenerate the active sites (Borah et al., 2019). Studies show that a higher catalyst loading (≥5 wt %) was not found to change the FAME yield significantly. This may be a result of the reaction mixture being more viscous with higher catalyst loadings, which might resist the mass transfer in the liquid–liquid–solid system (Kumar & Ali, 2013).

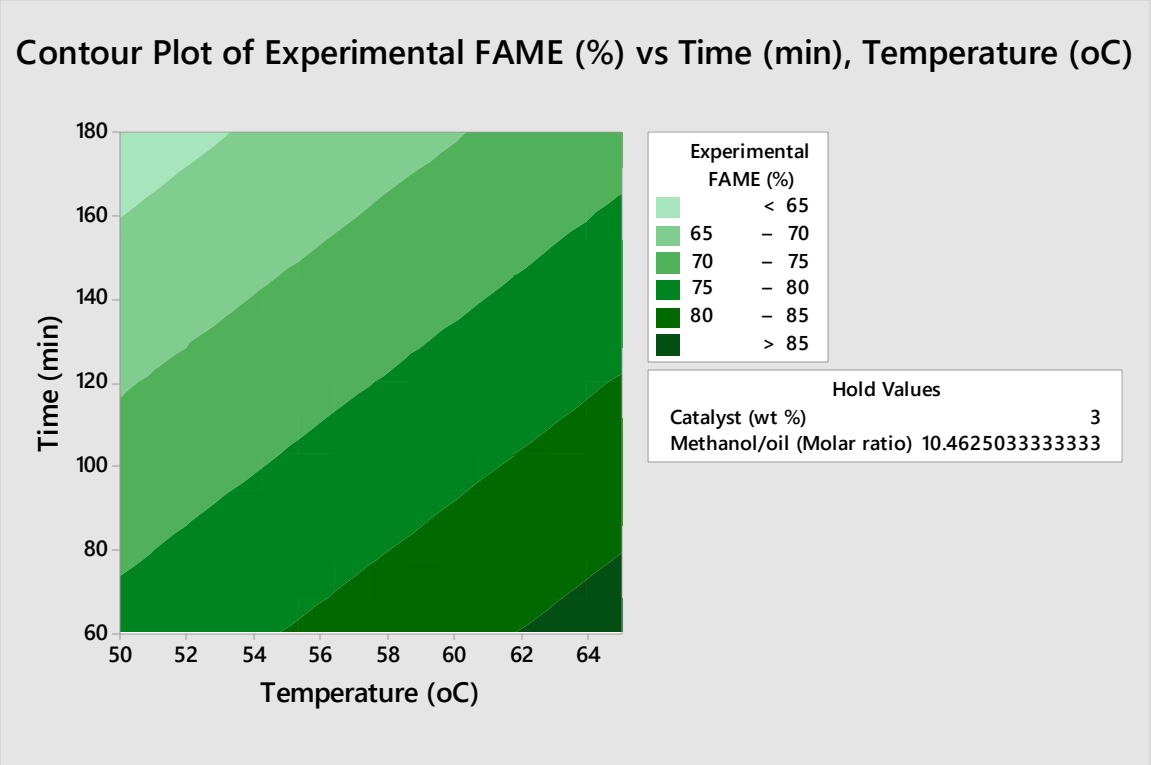


Figure 29. Contour plot experimental fame vs Time, Temperature

The contour plot of the combined effect of temperature and time is depicted in Figure 29. In order to increase the yield, reaction temperature and time play a significant role. A rise in temperature increases the rate of reaction and kinetics ultimately reducing the reaction time (Gashaw et al., 2015). Depending on the type of oil used, the trans-esterification process is often carried out below the boiling temperature of alcohol within an optimum temperature range of 50–60° C. High temperatures mostly cause the utilized alcohol to evaporate. Therefore, the figure shows that with an increasing temperature and time of the reaction, the yield of biodiesel increased quickly from 55.72 % (run 1) to 94.58% (run 6) of yield in the range of time 60min with respect to 50^{o}C temperature up to 57.5 ^{o}C of temperature respectively. As reported by many groups, at normal temperature, a higher conversion is easily achieved within a 60–90 min time period (Hossain and Boyce, 2009) (Bano et al., 2020).

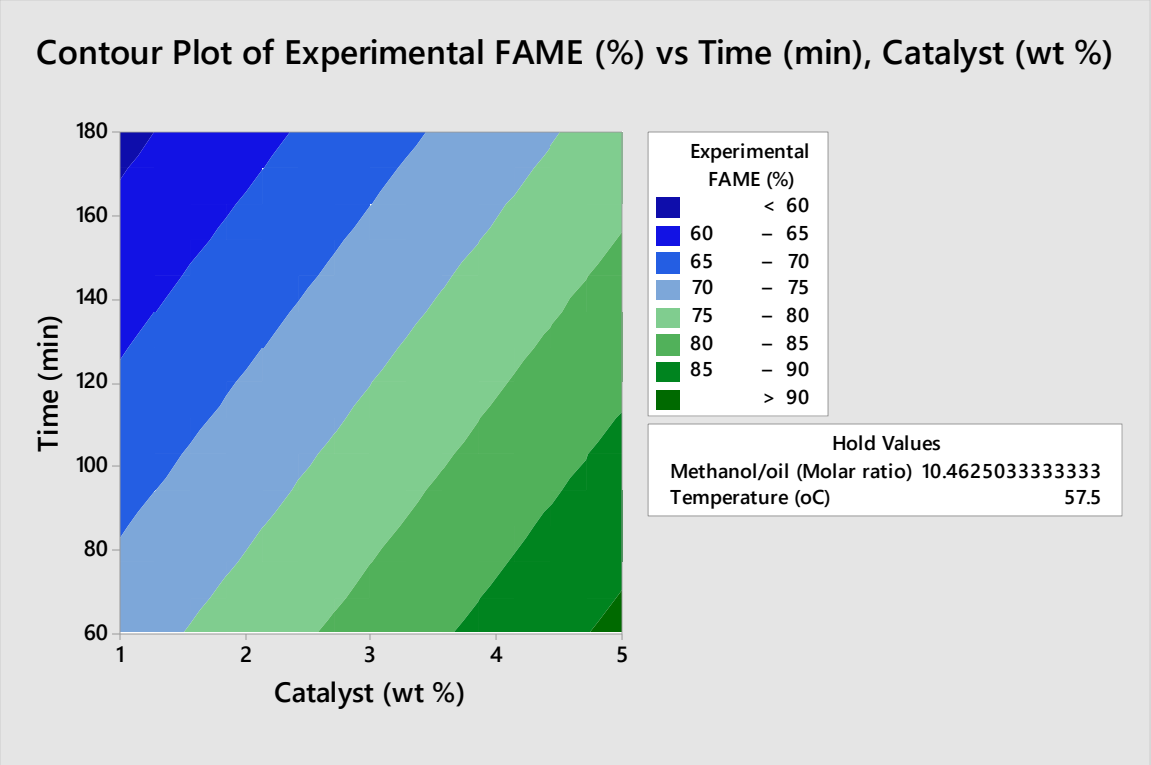


Figure 30. Contour plot of experimental FAME vs Time, Catalyst

The effect of interaction between catalyst loading and reaction time on biodiesel conversion is demonstrated in Figure 30. The conversion of biodiesel rises with rising catalyst concentrations, and it also increases with an increase in reaction times, which is due to the fact that rising reaction time the interaction between reactants rises which aids to boost the conversion (Borah et al., 2019). In this study, with an increasing catalyst loading of the reaction, the yield of biodiesel increased quickly from 66.28 % (run 2) to 96.21% (run 9) yield in the range catalyst loading from 1 wt% with respect to 120 min up to 5 wt% catalyst loading with respect to 120min time respectively. As can be seen from the slope of the catalyst loading which is very sharp, conversion has been shown to be more reliant on catalyst loading than reaction time, but the increase in conversion for reaction time is low. The increase in conversion with catalyst loading may be due to the presence of more catalysts active sites with an increase in catalyst loading.

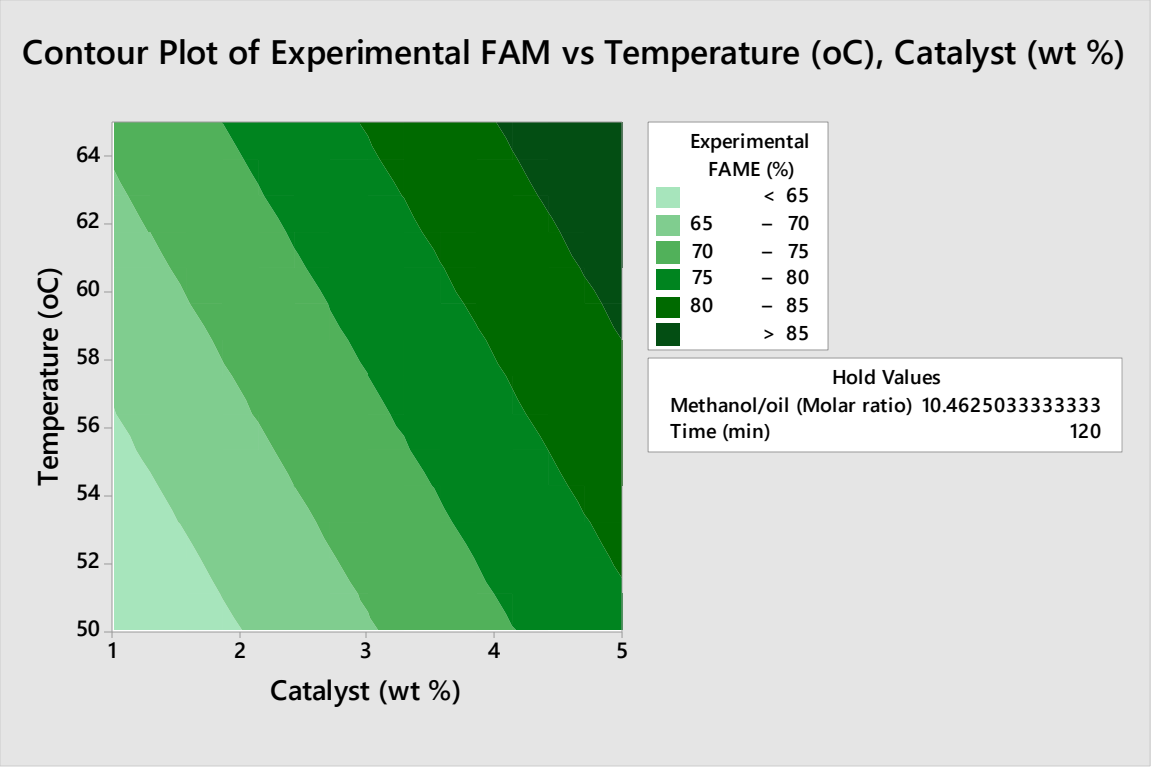


Figure 31. Contour plot of experimental FAME vs Temperature, Catalyst

The effect of catalyst loading and reaction temperature interaction on biodiesel conversion is illustrated in Figure 31. The conversion of biodiesel rises when catalyst loading increases which may be due to the increased surface-active sites of the catalyst. There is also an increase in conversion with the increase in temperature (Borah et al., 2019). In the present study, the FAME yield was found to increase as the reaction temperature and catalyst loading increased from 55.72% (run 1) to 90.58% (run 8) of yield at the range catalyst loading of 1 wt% with respect to 50^{o}C and 5 wt% catalyst loading with respect to 65^{o}C respectively. The Figure shows that the biodiesel conversion increases with both catalyst loading and temperature. But herein, catalyst loading is the most dominant variable as can be seen clearly from the very sharp rise in conversion with increasing catalyst loading.

4.7. Characteristics of Produced Biodiesel

The fuel properties of the biodiesel product are very important for application in the diesel engine. The major properties of biodiesel fuels including density, dynamic viscosity, kinematic viscosity, acid vale, free fatty acid value, saponification value, cloud point, pour point, and flash point were measured following the ASTM standard methods as summarized in Table 19. The kinematic

viscosity (3.2 mm^2/s) of the microalgae biodiesel in this study was in the range of ASTM (1.9-6.0 mm^2 /s) standard value. Important factors that determine viscosity include the nature of dominant fatty acids, carbon chain length, and degree of saturation. For instance, viscosity increases with the number of CH_2 moieties in the fatty ester chain and decreases with an increasing unsaturation. This implies that transesterification favors reduction of the viscosity of the microalgae oil which, is measured as 35.47 mm^2/s before transesterification. Compared with the literature value, the viscosity of the algae biodiesel in the present work (3.2 mm^2/s) is much lower than microalgae and castor oil biodiesel which is 6.71 mm^2/s and 10.43 mm^2/s respectively (Dawit Firemichael et al., 2020).

The degree of fuel ageing during storage is indicated by the acid value, as it gradually increases as a result of hydrolytic cleavage of ester bonds. High fuel acidity has been considered in relation to corrosion and the formation of deposits within the engine. The EN and ASTM Standards allow a maximum acid value of 0.5 mg of KOH/g to mitigate these negative effects. The acid value of the microalgae biodiesel gave 0.47 mg KOH/g which is in agreement with the specified limit, implying that the algae biodiesel will not pose a problem for the long-term performance of the engine. Cold flow properties are significant parameters for biodiesel and could be measured by cloud and pour points. The reduction of temperature may cause the biodiesel to form visible crystals ($d \geq 0.5$ μm) at a limit known as the cloud point. The temperature at which biodiesel ceases to flow is known as the pour point (Prathima & Karthikeyan, 2017). The cloud and Pour point of microalgae biodiesel were 6 and -9.8 °C, respectively. The CP and PP values of the algae biodiesel in the present study are comparable to the CP and PP values of biodiesels derived from other feedstocks (microalgae oil: -3 and -7 °C; olive oil: -2 and -3 °C; rapeseed oil: -2 and-9 °C; soybean oil: 0 and -2 °C; sunflower oil: 2 and -1 °C; tallow: 17 and 15 °C). The flashpoint of the biodiesel indicates that the fuel is safe for storage and not flammable at low temperatures (Borah et al., 2019 ; Firemichael et al., 2020). Consequently, biodiesel oil produced in this work shows the high–quality properties according to the ASTM standard specifications for biomass automotive fuel.

Table 19: Physicochemical properties of produced biodiesel

No.	Physicochemical property	Units	Test method ASTM	ASTM limit for B100	Produced biodiesel values
1	Color		Observed		Dark yellow
2	pH				8.12
3	Density @ 20^{o}C	Kg/m^{3}	D4052	880	784
4	Density @ 40^{o}C	Kg/m^{3}	D4052	880	782
5	Dynamic viscosity @ 40^{o}C	mpa.sec			2.5
6	Kinematic viscosity @ 40^{o}C	mm^{2}/s	D445	1.9-6	3.2
7	Acid value	mg KOH/g	D974	0.50	0.47
8	Free fatty acid value	%			0.235
9	Saponification value				205.63
10	Cloud point	^{o}C	D2500	-3 to -15	6
11	Pour point	^{o}C	D97	-5 to -10	-9.8
12	Flash point	^{o}C	D93	100-170	120

CHAPTER FIVE

5. CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

B. braunii, a green colonial microalga, has already been proposed as a renewable energy source due to its well-known high lipid content. In this study, biodiesel was prepared from the *B. braunii* oil with 37.7% lipid content through transesterification with methanol and Zn doped eggshell-derived CaO nanocatalysts. This oil yield is much better than most of the microalgae strain reported from previous studies. The nanocatalyst was synthesized from eggshell and enhanced by the Zn doped CaO wet impregnation method. From the XRD analysis of the nanocatalysts prepared from eggshell and calcinated at 900℃, the crystalline size obtained were 39.12 and 44.74 nm for CaO and Zn/CaO respectively. The SEM result showed calcination and wet impregnation treatment resulted in more regularity in the particle shape, forming a termite nest-like structure. Calcinated eggshell CaO material displayed high porosity and high surface area. The Zn doped CaO particles are densely agglomerated with each other with a regular shape and have more high porosity and material is crystalline in structure. Thus, waste eggshell-derived CaO catalysts with slight modification can be employed for producing biodiesel from microalgae oil at low cost as the catalyst is derived from a renewable precursor. The transesterifcation of *B. braunii* oil using Zn doped CaO nanocatalyst was optimized through the Taguchi methodology. The results of this study showed that the optimal conditions for maximum FAME yield (96.21 %) were found to include methanol to oil molar ratio 16:1, the catalyst loading of 5 wt%, the reaction time of 120 min, and reaction temperature of 50°C and this was well close to the predicted value, verifying the appropriateness of the model. Furthermore, Taguchi's method of optimization results showed that the developed models are significant and their predictions are in line with the experimental results. The statistical analysis also showed that methanol to oil molar ratio and catalyst loading give a significant effect on the percentage conversion of oil to biodiesel. The fuel properties of the produced biodiesel were analyzed according to ASTM D6751 standards and were also better in comparison to conventional diesel. Therefore, *B. braunii* microalgae investigated in this study is proven to be suitable as raw materials for biodiesel production and can be considered as a potential feedstock for biodiesel production to fight the future energy crisis.

5.2. Recommendations

This research shows microalgae are the potential resources for the third-generation biofuel and an excellent candidate for biodiesel production to replace the petroleum fossil energy and to mitigate environmental pollution caused by fossil fuel consumption and nanocatalyst production from eggshell waste. Further research is required to evaluate the strain type such as 18S rRNA sequence analysis is needed to confirm the taxonomical relationship and to show similarities with the reported species of the genus *Botryococcus* and in particular to *B. braunii* and also an investigation on hydrocarbon composition of this particular *B. braunii* isolate is needed.

Further work on the productivity of isolated strains, determination of conductive environmental conditions (i.e. growth media or nutrients requirement, optimal temperature, light intensity) for optimal growth of strains with higher fatty acid yield per unit area of land should be carried out to grow microalgae on outdoor large scale open pond system. In the case of nanocatalysts more studies should be implemented and strengthened on the reusability of eggshell-based Cao and Zn doped CaO nanocatalysts for transesterification reactions. Glycerol has relevance in soap industries but in this study, the property of glycerol was not determined. Therefore, in addition to the production of biodiesel the property of the byproduct glycerol should be analyzed and purified for further applications. Besides, further studies on fuel properties such as blending conditions, engine performance, and emission tests should be carried out in future studies.

Research Fund Acknowledgment

This research project is funded by Adama Science and Technology University under the grant number ASTU/SM-R/303/21 Adama, Ethiopia.

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APPENDICES

Appendix 1. Components of modified Chu-13 culture media

No.	Component	mg/l
1	KNO_3	400
2	K_2HPO_4	80
3	$MgSO_4.7H_2O$	200
4	$CaCl_2.6H_2O$	107
5	Ferric citrate	20
6	Citric acid	100
7	H_3BO_3	5.72
8	$MnCl_2.4H_2O$	3.62
9	$CUSO_4.5H_2O$	0.16
10	$Co(NO_3)_2.6H_2O$	0.02
11	$Na_2MoO_4.2H_2O$	0.084
12	$CoCl_2$	0.02
13	0.072N H2SO4	I drop
14	$ZnSO_4$	0.04

- The volume was then brought to 1L by adding more distilled water. Because it was difficult to weigh out some of the trace minerals, all the mixture were made in large concentration and the appropriate amount were mixed, and the pH was corrected to 7.2 using pH meter. The modified Chu-13 medium was autoclaved at 125 °C for 15 minutes.

Appendix 2. Lugol's iodine solution

No.	Component
1	Potassium iodide
2	Iodine crystals
3	Glacial acetic acid

- First dissolve 10g potassium iodide in 100ml of distilled water, slowly add 5g iodine crystals in 10ml glacial acetic acid while shaking. Then mix these two solutions and finally store in a tightly stoppered brown bottle.

Appendix 3. Absorbance of microalgae culture at 750 wave number

Day	**Absorbance Measurements**			**Average**
	1	**2**	**3**	**Absorbance**
1	0.230	0.341	0.243	0.271
2	0.312	0.366	0.336	0.338
3	0.293	0.504	0.428	0.408
4	0.408	0.522	0.460	0.463
5	0.404	0.522	0.478	0.468
6	0.501	0.610	0.599	0.570
7	0.535	0.633	0.610	0.592
8	0.599	0.665	0.676	0.647
9	0.599	0.710	0.721	0.677
10	0.612	0.726	0.771	0.703
11	0.664	0.745	0.828	0.746
12	0.755	0.815	0.906	0.825
13	0.799	0.839	0.919	0.852
14	0.825	0.872	0.943	0.880
15	0.821	0.874	0.962	0.885

Appendix 4. Absorbance of microalgae culture at 780 wave number

Day	**Absorbance Measurements**			**Average**
	1	**2**	**3**	**Absorbance**
1	0.128	0.332	0.226	0.228
2	0.281	0.380	0.297	0.319
3	0.334	0.448	0.412	0.398

Appendix 4 Continued

4	0.342	0.488	0.409	0.413
5	0.367	0.497	0.429	0.431
6	0.469	0.576	0.551	0.532
7	0.497	0.606	0.562	0.555
8	0.550	0.632	0.621	0.601
9	0.588	0.673	0.695	0.652
10	0.576	0.679	0.741	0.665
11	0.639	0.697	0.750	0.695
12	0.724	0.803	0.851	0.7925
13	0.761	0.799	0.889	0.816
14	0.778	0.829	0.928	0.845
15	0.781	0.825	0.932	0.846

Appendix 5. Materials used for transesterification reaction

I. The amount of Zn/CaO nanocatalyst required

The amount of Zn/CaO required when catalyst weight is 1%

$$\frac{\text{mass of catalyst}}{\text{mass of oil}} = \frac{1}{100}; \frac{\text{mass of catalyst}}{\rho \text{ oil} \times \text{V oil}} = \frac{1}{100}$$

$$\frac{\text{mass of catalyst}}{\frac{0.84\text{g}}{\text{ml}} \times 20\text{ml}} = \frac{1}{100}$$

$$\frac{\text{mass of catalyst}}{16.8} = \frac{1}{100}$$

Mass of catalyst = 0.168g

Table 5A: The amount of catalyst required with various percentage

No.	Catalyst Loading (%)	Catalyst loading required in (g) for 20 ml of microalgae oil
1	1	0.168
2	3	0.504
3	5	0.840

II. The amount of methanol required

The amount of methanol required when the molar ratio oil to methanol 1:9

$$\frac{\text{mole of methanol}}{\text{mole of oil}} = \frac{9}{1}$$

$$\frac{\text{mole of methanol}}{\text{mole of oil}} = \frac{\text{given mass of methanol} / \text{Mwt. methanol}}{\text{given mass of oil} / \text{Mwt. oil}} = \frac{9}{1}$$

$$\frac{\text{mole of methanol}}{\text{mole of oil}} = \frac{\rho\ \text{methanol} \times \text{V methanol} / \text{Mwt.methanol}}{\rho\ \text{oil} \times \text{Voil} / \text{Mwt.oil}} = \frac{9}{1}$$

$$\frac{0.791\text{g/ml} \times \text{Vmethanol} / 32\text{g/mol}}{0.84\text{g/ml} \times 20\text{ml} / 791\text{g/mol}} = \frac{9}{1}$$

Volume of methanol with methanol to oil ratio 9:1 = 7.733 ml

Table 5B: Volume of methanol used with different ratio

No.	Methanol to oil molar ratio	Volume of methanol required (ml)
1	9:1	7.733
2	11.53:1	9.907
3	16:1	13.750

Appendix 6. Transesterification results using Taguchi methodology

Table 6A: Experimental FAME yield using Taguchi methodology

Runs	A Catalyst (wt%)	B Methanol/oil (Molar ratio)	C Time (Min)	D Temperature (ºC)	Experimental FAME (%)
1	1	7.73	60	50.0	55.72
2	1	9.91	120	57.5	66.28
3	1	13.75	180	65.0	76.54
4	3	7.73	120	65.0	71.92
5	3	9.91	180	50.0	55.10
6	3	13.75	60	57.5	94.58
7	5	7.73	180	57.5	67.41
8	5	9.91	60	65.0	90.58
9	5	13.75	120	50.0	96.21

Appendix 7. Experimental designing using Taguchi methodology

1. Open the Minitab software, select Stat, DOE, Taguchi, and then click Create Taguchi Design.
2. When creating a Taguchi design, choose the design type which is 3-Level design with 9 runs for 4 continuous factors.
3. Assign the factors, such as name and level values, and then click ok.
4. The worksheet shows the design that can be run, and it produced 9 runs.