PRODUCTION AND OPTIMIZATION OF BIODIESEL FROM CHARA VULGARIS

A Thesis

SUBMITTED BY

Shaila Siddiqua
Examination Roll Number: MS 120701
Session: 2012-2013

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Biotechnology and Genetic Engineering Discipline
Life Science School, Khulna University
Khulna-9208, Bangladesh
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DECLARATION

This thesis report is the original research work of the author, except as otherwise stated. The material presented has not submitted either in whole or in part for a degree for this or any other university. The findings of the research has not been / will not be published anywhere without the concurrence of the concerned supervisor.

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ABSTRACT

Renewable biodiesels are needed as an alternative of petroleum-derived transport fuels, which contribute to global warming and are of limited availability. Algae biomass, are a potential source of renewable energy, and they can be converted into energy such as biofuel oil and gas. The present study introduced an integrated method for the production of biodiesel from *Chara vulgaris* algae collected from local area. The Box–Behnken design based on response surface methods (RSM) used as the statistical tool to optimize three variables for predicting the best performing conditions (calorific value and yield) of algae biodiesel. The three parameters for production condition were chloroform (X_1), sodium chloride concentration(X_2) and temperature (X_3). Optimal conditions were estimated by the aid of statistical regression analysis and surface plot chart. The Optimal condition of biodiesel production parameter was observed to be 198 ml Chloroform with 0.75% sodium chloride at 65°C temperature, where the calorific value of biodiesel is 9255.106 kcal/kg and yield 3.6 ml.

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Chapter one

Introduction

1.1 Background

Every nation of the world gives their major effect on the energy security. Because energy is must for every sector from transportation to communication, to security and health delivery systems. This energy mainly produced from oil, coal and natural gas. In 2007, fuel for transport accounted for 27 per cent of total world delivered energy consumption [1]. In the same year, 53 per cent of liquid fuel supplied was consumed by the transport sector, and this proportion is predicted to rise to 61 per cent by 2035[2]. We have stressed before that it is vital to reduce consumption significantly over the next few decades. However, it is unrealistic to expect that the demand for liquid transport fuels will reduce significantly.

The idea of biofuels are hundred year old, Rudolf Diesel ran the peanut oil on the engine bearing his name when he first demonstrated it during the world's fair in Paris 1900 [3]. The idea of biofuels reinforced in the later decades of 20^{th} century. First Generation biofuels are produced directly from food crops by abstracting the oils for use in biodiesel or producing bioethanol through fermentation [4]. Crops such as wheat and sugar are the most widely used feedstock for bioethanol while oil seed has proved a very effective crop for use in biodiesel. However, first generation biofuels have a number of associated problems. There is much debate over their actually benefit in reducing green house gas and CO_2 emissions due to the fact that some biofuels can produce negative net energy gains, releasing more carbon in their production than their feedstock's capture in their growth. However, the most contentious issue with first generation biofuels is 'fuel vs. food'. As the majority of biofuels are produced directly from food crops the rise in demand for biofuels has lead to an increase in the volumes of crops being diverted away from the global food market. This has been blamed for the global increase in food prices over the last couple of years [4].

Second Generation biofuels have been developed to overcome the limitations of first generation biofuels. They are produced from non-food crops such as wood, organic

waste, food crop waste and specific biomass crops, therefore eliminating the main problem with first generation biofuels [5]. Second Generation biofuels are also aimed at being more cost competitive in relation to existing fossil fuels [6]. Life cycle assessments of second-generation biofuels have also indicated that they will increase 'net energy gains' over coming another of the main limitations of first generation biofuels.

The Third Generation of biofuels is based on improvements in the production of biomass. It takes advantage of specially engineered energy crops such as algae as its energy source [6]. The algae are cultured to act as a low-cost, high-energy and entirely renewable feedstock. It is predicted that algae will have the potential to produce more energy per acre than conventional crops. Algae can also be grown using land and water unsuitable for food production, therefore reducing the strain on already depleted water sources. A further benefit of algae based biofuels is that the fuel can be manufactured into a wide range of fuels such as diesel, petrol and jet fuel.

Several researches have been conducted worldwide on algae biodiesel. Biodiesel from microalgae; Algae-based biofuels: applications and co-products; Opportunities and challenges in algae biofuels production; A realistic technology and engineering assessment of algae biofuel production; combustion analysis of algal oil methyl ester in a direct injection compression ignition engine [8-12]. In current years some research was conducted In Bangladesh on biodiesel. Prospect of Algal Biodiesel Production in Bangladesh: Overview from Developed Countries; Biodiesel from Microalgae as a solution of third world energy crisis [13-14]. Still there is little research work has done on macro algae. Our research work was focused on biodiesel production from locally grown algae. Algae were collected from southern part of Khulna, Bangladesh. These algae grown in the brackish water as weed and have no useful function. Unlike the microalgae which required extensive cultivation process to produce sufficient amount of biomass, these macro algae grow naturally in the salty waste water system in a high quantity.

Chloroform, methanol, sodium chloride and temperature are important factor for chemical method of algal biodiesel production. For optimization of chemical method of algal biodiesel production Box–Behnken designs [15] were used. The Box–Behnken

design, which is the response surface methods (RSM), is a very useful statistical tool to optimize multiple variables for predicting the best performing conditions by using a minimum number of experiments [16].

1.2 Objective

The overall goal of this research were

1. Assessment of algae for biodiesel production
2. Modification of Bligh and Dyer method of oil extraction from algae and production of algae biodiesel
3. Optimization of production parameters using Box Behnken design.

Chapter Two
Literature Review

2.1 History:

Biofuels are fuels derived from biomass. Biomass is organic matter taken from or produced by plants and animals. It comprises mainly wood, agricultural crops and products, aquatic plants, forestry products, wastes and residues, and animal wastes. In its most general meaning, biofuels are all types of solid, gaseous and liquid fuels that can be derived from biomass. Examples of solid biofuels include wood, charcoal and bagasse. Wood and charcoal are widely used as fuel for domestic purposes such as cooking in the rural areas of most developing countries. Waste bagasse, the fibrous material produced from sugar cane processing, is extensively used for steam and electrical power generation in raw sugar mills. Examples of gaseous biofuels include methane gas and producer gas. Methane gas is produced from the anaerobic fermentation of animal wastes, wastewater treatment sludge and municipal wastes in landfills. On the other hand, producer gas can be made from the pyrolysis or gasification of wood and agricultural wastes. Examples of liquid biofuels include methanol, ethanol, plant oils and the methyl esters produced from these oils commonly referred to as biodiesel [17].

The concept of alternative, renewable energy has been in existence for over a century. Rudolf Diesel is credited as the inventor of the first diesel engine which was originally designed to run on fuel derived from peanut oil. With heavy anticipation, Rudolf's' first exhibition of the diesel engine was put on display at the 1911 World's Fair in Paris receiving much acclaim [3]. Rudolf Diesel was quoted as saying; "The diesel engine would help considerably in the development of agriculture of the countries which use it." Unfortunately, due to the low cost of mineral oils at the time, the diesel engine was modified to run on petroleum oil. Biodiesel technology was overlooked while the demand for crude oil increased significantly as the automotive and industrial age ensued. Rudolf Diesel was well aware that renewable fuel would not be of major relevance during his lifetime when he said, "The use of vegetable oils for engine fuel may seem insignificant

today. But such oils may become in course of time as important as petroleum and the coal tar products of the present time" [18, 19].

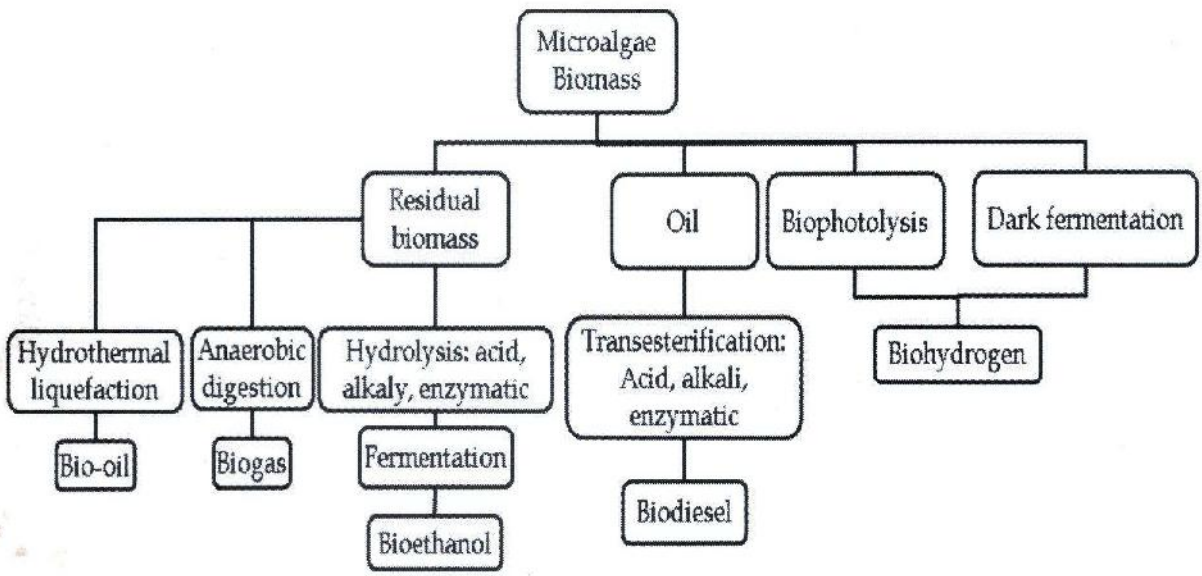


Figure 2.1: Different types of Biofuels derived from algae biomass.

Despite the extensive use of petroleum-derived diesel fuels, several countries during the 1930s and during World War II started showing curiosity in vegetable oils as fuels for internal combustion engines. The history of biodiesel fuel reflects that countries like Belgium, Portugal, France, Italy, Brazil, United Kingdom, Germany, Japan, Argentina, and China were reported to have tested and used vegetable oils as diesel fuels during this time. Although there were initial operational problems due to the high viscosity of vegetable oils as compared to petroleum diesel fuel. In 1937, G. Chavanne in Belgium was granted a patent for a "Procedure for the transformation of vegetable oils for their uses as fuels" Belgian Patent 422,877. This patent described the alcoholysis of vegetable oils using ethanol. This probably is the first account of the production of what is known as "biodiesel" today in the biodiesel fuel history [20].

The idea of using algae as a source of food, feed and energy goes back more than half a century. Production of methane gas from algae was proposed in the early 1950s, and

received a big impetus during the energy crisis of the 1970s, when projects were initiated to produce gaseous fuels (hydrogen and methane). From 1980 to 1996 the US Department of Energy supported the Aquatic Species Program (ASP), a relatively small effort (about $25 million over almost 20 years) with the specific goal of producing oil from algae [21, 22].

2.2 Why Algae?

The events of the past few years have made it clear that the world can no longer ignore the threats to its economy, climate and national energy security rising from its dependence on petroleum fuel.

Social and political instability in or near major oil producing regions has led to frequent price spikes, hampering an already-slow economic recovery as consumers are forced to spend more of their limited income on gas. Major spills in waterways like the Gulf of Mexico provide dramatic reminders of the risks inherent in oil exploration and production and their effect on our natural environment [23].

Though we have plenty of natural gas resource, Bangladesh spends lots of money for importing petroleum fuels (eg. petrol, diesel etc.) that needs for transportation, irrigation and other purposes.. It is crucial to our national energy security that we develop long-term, domestic sources of fuels. We must reduce our greenhouse gas emissions and protect and preserve our land, water, air and soil by developing renewable and sustainable energy sources. Fortunately, there is a solution to these incredible challenges. It comes in the form of the Earth's oldest organisms – algae – which can help address all of these major issues.

2.2.1 Benefits of algae-based biofuels

Algae are emerging to be one of the most promising long-term, sustainable sources of biomass and oils for fuel, food, feed, and other co-products. What makes them so

attractive are the large number and wide variety of benefits associated with how and where they grow. Here are few reasons why algae are a promising new source of fuel and other products:

1) Algae Grow Fast

Algae have the potential to produce a volume of biomass and biofuels many times greater than that of our most productive crops.

2) Algae Can Have High Biofuels Yields

Algae store energy in the form of oils and carbohydrates, which, combined with their high productivity, means they can produce from 2,000 to as many as 5,000 gallons of biofuels per acre per year.

3) Algae Consume CO_2

Like any other plant, algae, when grown using sunlight, consume (or absorb) carbon dioxide (CO_2) as they grow, releasing oxygen (O_2) for the rest of us to breathe. For high productivity, algae require more CO_2, which can be supplied by emissions sources such as power plants, ethanol facilities, and other sources.

4) Algae Do Not Compete With Agriculture

Algae cultivation uses both land that in many cases is unsuitable for traditional agriculture, as well as water sources that are not useable for other crops, such as sea-, brackish- and wastewater. As such, algae-based fuels complement biofuels made from traditional agricultural processes.

5) Macroalgae Can Be Grown in the Sea

Macroalgae (seaweeds) are grown in the sea, or even on land with seawater, and their sugars can be converted into biofuels and chemicals.

6) Algae Can Purify Wastewaters

Algae thrive in nutrient-rich waters like municipal waste waters (sewage), animal wastes and some industrial effluents, at the same time purifying these wastes while producing a biomass suitable for biofuels production.

7) Algal Biomass Can Be Used as an Energy Source

After oil extraction, the remaining algal biomass can be dried and "pelletized" and used as fuel that is burned in industrial boilers and other power generation sources.

8) Algae Can Be Used to Produce Many Useful Products

Algae can be cultivated to produce a variety of products for large to small markets: plastics, chemical feedstocks, lubricants, fertilizers, and even cosmetics.

9) The Algae Industry is a Job Creation Engine

Like most other biodiesels, algae are also essentially carbon-neutral. While it does emit carbon while it's burned, it absorbs carbon as food during its growing cycle (like all plants), which means that its net carbon figure is zero. Algae are estimated to have a greenhouse gas footprint that is 93 percent less than conventional, petroleum-based diesel [23-25].

2.2.2 The cons of algae-based biofuels

While algae-based biofuels may use far less land and have a higher energy yield than other biodiesel crops, its production also requires more energy and water (albeit not necessarily fresh water) than plant sources such as corn. It also has higher greenhouse gas emissions. The reason is that the production of the final product is more complex and therefore more energy intensive. While many kinds of algae are easy to cultivate, the species of the plant that contain the most fats are most suitable for biodiesel, and these specialized lipid-producers are a bit fussier than ordinary algae. Whereas algae biodiesel product is largely carbon neutral, critics point out that it still requires carbon, and that carbon is derived from petroleum-based sources, which means that algae cultivation is still essentially dependent on petroleum. Ironically, algae biofuels could actually be a

victim of its own success: were it to supplant petroleum-based fuels on a large scale, it would its most important source of carbon dioxide required for production.

The cultivation of algae (like the cultivation of most other plants) requires large amounts of phosphorus as a fertilizer, and while it's not an oft-discussed topic, the world is currently on the brink of a peak of availability of Earth's finite phosphate resources. "Peak phosphorus," as it's called, is the point in time at which the maximum global phosphorus production rate is reached. According to some researchers, Earth's phosphorus reserves are expected to be completely depleted in 50 to 100 years, and peak phosphorus will be reached by the year 2030. (This is a fairly scary prospect for global agriculture, not just for algae production). To succeed, large scale algae production will need to reduce its use of phosphorus and find ways of reusing what it does require. The need for phosphorus in cultivation has been called by Forbes "The Achilles Heel" of algae biofuel.

Finally, many critics point out that the world simply can't produce enough algae through natural photosynthesis to sustain the world's need for fuel. Natural photosynthetic algae can produce about 2,000 gallons of fuel per acre per year today: far short of world demand and far short of what scalable biofuel production requires being economically feasible. For this reason, new production methods are being sought by the companies trying to make algae biofuel work better [24, 25].

2.3 Raw Chemistry

After collection, production of biodiesel from algae can be mainly divided into 2 main phases. First one is extraction of oil from algae and the second one is transesterification of extracted oil to produce biodiesel.

2.3.1 Oil extraction from algae

Most microalgae strains are relatively small and have a thick cell wall, meaning harsh conditions are required to rupture the cells for lipid extraction [26]. Lipids for fuel production can be extracted from algal biomass using two groups of methods: mechanical

methods where pressure is used to rupture the cells and squeeze the oil from the cells and chemical methods where solvents are used to extract the lipids. A combination of both can also be used, which has been shown to most effective in some cases [27].

1) Mechanical methods

Mechanical methods involve placing dried algae cells under pressure, forcing the cells walls to rupture and release the contained lipids. The simplest method is mechanical crushing. When algae are dried it retains its oil content, which then can be "pressed" out with an oil press. Different strains of algae warrant different methods of oil pressing, including the use of screw, expeller and piston. The most frequently used mechanical method is the screw press, which works in a similar way to an extruder for polymer processing. Dry algae are conveyed by a rotating screw along a horizontal annulus whose cross section decreases along the length. The ruptured cells and oil emerge through a nozzle and are easily separated. Although the simplest technology, mechanical methods are very energy intensive, both in the requirement for dry input material and the energy involved in creating sufficient pressure for cell rupture. [28]

Osmotic shock is a sudden reduction in osmotic pressure, this can cause cells in a solution to rupture. Osmotic shock is sometimes used to release cellular components, such as oil. Ultrasonic extraction, a branch of sonochemistry, can greatly accelerate extraction processes. Using an ultrasonic reactor, ultrasonic waves are used to create cavitation bubbles in a solvent material. When these bubbles collapse near the cell walls, the resulting shock waves and liquid jets cause those cells walls to break and release their contents into a solvent. Ultrasonication can enhance basic enzymatic extraction. The combination "sonoenzymatic treatment" accelerates extraction and increases yields [29].

2) Chemical extraction

Several solvents have been used successfully including hexane, ethanol and hexaneethanol mixtures, obtaining up to 98% extraction of lipids. Although ethanol is a very good solvent, it does not selectively extract lipids, extracting also sugars, amino

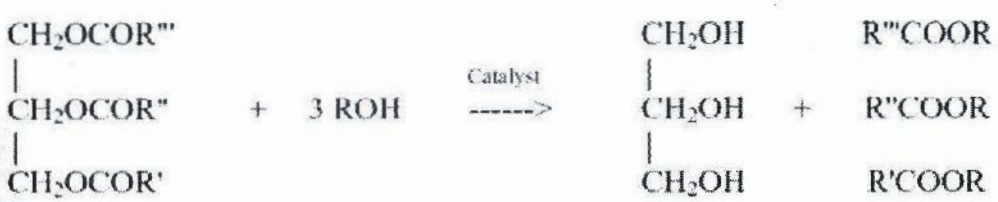
acids, salts and pigments. This is not desirable for biodiesel production, where the purpose of extraction is just the lipids and fatty acids and the use of ethanol as a solvent reduces the amount of energy that can be recovered from residues via anaerobic digestion or other processes [30]. In a study aimed at optimising the analytical determination of lipid content in lyophilized microalgae, compared the effectiveness of a number of organic solvents and organic solvent blends [31]. The study found a 1:1 blend of chloroform and methanol to be most effective for lipid extraction from Chlorella vulgaris, using a technique involving vortex mixing and separation of solvent and aqueous layers by centrifugation.

Lipid extraction and partitioning are performed simultaneously in the Bligh and Dyer (1959)method. The Bligh and Dyer method is one of the widely practised methods for lipid extraction. This procedure is performed by extracting lipids from homogenized cell suspension using 1:2 (v/v) chloroform/methanol [32]. The lipids from the chloroform phase are then extracted and processed by various procedures. The above gravimetric method is still widely used for the estimation of lipids by algal technologists, and the same procedure is also followed for pilot-scale and large-scale extraction processes. In order to improve the basic method, many modifications have been adopted by researchers. The most common modification is the addition of NaCl instead of water, to prevent binding of acidic lipids to denatured lipids [33].

2.3.2 Transesterification

Biodiesel is technically defined as the fatty acid methyl ester or mono-alkyl esters derived from vegetable (plant) oils or animal fats and other biomass-derived oils that meet certain quality specifications [34]. The chemical conversion of plant oils to mono-alkyl esters is achieved through a process called transesterification. There are several methods available: acid-catalyzed, alkaline-catalyzed, enzymecatalyzed, or non-catalyzed [35]. However, the use of alkaline catalyst is the most widely used method due to its short reaction time. An excess of methanol is normally required to complete the reaction above the stoichiometric ratio of 3 parts methanol to one part plant oil.

During the process of transesterification, an alcohol (such as methanol) reacts with the triglyceride oils contained in plant oils, animal fats or recycled greases to form fatty acid alkyl esters (biodiesel) and glycerin. The reaction requires heat and a strong base catalyst such as sodium hydroxide or potassium hydroxide. The simplified chemical reaction is shown below. Some types of feedstocks require pretreatment before they can go through the transesterification process. Feedstocks with less than 4% free fatty acids such as most plant oils and some food-grade animal fats do not require pretreatment. However, feedstocks with more than 4% fatty acids require pretreatment using an acid esterification process. These include inedible animal fats and recycled greases. In this pretreatment step, the feedstock is reacted with an alcohol (like methanol) in the presence of a strong acid catalyst (like sulfuric acid) to convert the free fatty acids into biodiesel. The remaining triglycerides are then converted to biodiesel through the usual transesterification reaction.



Vegetable oil **Alcohol** **Glycerin** **Biodiesel**

Figure 2.2: Transesterifiction reaction of biodiesel

The conversion of triglycerides to simple alkyl esters with various alcohols reduces the high viscosity of oils and fats. As a result, they improve the injection process and ensure better atomization of the fuel in the combustion chamber. It has been shown that base catalysis of the transesterification with reagents such as sodium hydroxide is faster than acid catalysis [36]. Transesterification is a reversible reaction. For example, the transesterification of soybean oil with methanol or 1-butanol proceeded with pseudo-first order or second order kinetics, depending on the molar ratio of alcohol to soybean oil

(30:1 pseudo-first order, 6:1 second order; NaOBu catalyst) while the reverse reaction was second order [37].

Methyl esters are the most commonly used esters as alternative fuel. One reason is the low price of methanol compared to other alcohols. The effect of the possible polymerization reaction is also decreased. Another advantage of the esters is more benign emissions, for example, with the removal of glycerol (which is separated from the esters) the formation of undesirable acrolein may be avoided.

It was shown that in homogeneous catalysis, alkali catalysis is a much more rapid process than acid catalysis in the transesterification reaction. At 32^{0}C, transesterification was 99% complete in 4 h when using an alkaline catalyst (NaOH or NaOMe). At 60^{0}C and a molar ratio alcohol:oil of at least 6:1 and with fully refined oils, the reaction was complete in 1 h to give methyl, ethyl, or butyl esters. The reaction parameters investigated were molar ratio of alcohol to vegetable oil, type of catalyst (alkaline vs. acidic), temperature, reaction time, degree of refinement of the vegetable oil, and effect of the presence of moisture and free fatty acid. Although the crude oils could be transesterified, ester yields were reduced because of gums and extraneous material present in the crude oils [38, 39].

2.4 Properties of Biodiesel

Biodiesel from algae is a liquid which varies in color -between light/dark green to brown- depending on the production feedstock. It is immiscible with water, has a high boiling point and low vapor pressure. The major properties of biodiesel are heating value, flash point, viscosity, density, etc.

Heating value or calorific value is the amount of heat produced from the complete combustion of a specific amount of fuel. It is measured in units of energy per unit of the substance, usually mass, such as: kJ/kg, kJ/mol, kcal/kg, Btu/m^3. Heating value is commonly determined by use of a bomb calorimeter. The heating value or energy

value of biodiesel is 8800-9600 Kcal/Kg. The heating value or calorific value is determined by 'oxygen bomb calorimeter'.

The lowest temperature at which the fuel will ignite if exposed to a flame is referred to as its flashpoint. ASTM (American Society for Testing and Materials) certified B100 must have a flashpoint greater than 130°C (266°F). Flashpoint will vary due to different feedstocks and other factors. Some biodiesel has had flashpoints greater than 300°F. The flash point is determined by 'flashpoint tester, type-00-ESR'.

Table 2.1: ASTM standard of fuel

Physical Property	**Astm Standards**	**Diesel**	**Oil**	**Biodiesel**
Density(g/cc)	800	826.5	911	875
Specific Gravity	0.8	0.8265	0.911	0.875
Kinematic Viscosity	2.5-7.5	2.049	**4.209**	**2.808**
Diesel Index	Min 45	47.731	49.78	49.75
Aniline Point	---	38.83	84.11	59.5
Flash Point	Min 38°	78	**>300**	**162**
Fire Point	Min 42°	82	**>300**	**170**
Saponification Value	Min 180	224	202	202
Iodine Value	Max 135	102	104	104
Cetane Number	Min 45	47.73	49.78	49.73
Calorific Value (MJ/Kg)	Min 33	42.57	41.2	41.2

Viscosity, by definition, is oil's resistance to flow and shear. It is the single most critical physical property of the oil as it affects both the wear rate and the fuel efficiency. Diesel fuels with high viscosity tend to form larger droplets on injection which can cause poor combustion, increased exhaust smoke and emissions. Kinematic viscosity at 40°C of biodiesel is 3.7 to 5.8 centistokes. Kinematic viscosity is determined by 'SAYBOLT/REDWOOD viscometer bath'.

Density is defined as the ratios of the mass of the fuel to the volume of the fuel Oils that are denser contain more energy. For example, petrol and diesel fuels give comparable energy by weight, but diesel is denser and hence gives more energy per liter. Density of biodiesel is 0.87 to 0.89 g/ml [40, 41].

2.5 Current Status of Algae Bioenergy in Some Countries

In November 2006, the U.S. Green Energy Technology Company and Arizona Public Service Company co-operate to establish a microalgae production system in Arizona State. Microalgae production system can be connected to the flue gas from electricity power plant which has 1040-megawatt generating capacity. This business system is to use flue gas which contains carbon dioxide to culture microalgae in a large-scale and to convert microalgae to biofuel. The yield of biofuel reached 5,000-10,000 gallon per acre per year [42]. In 2007, The National Energy Board of the United States launched "Mini-Manhattan Project". The aim was to help the United States to get rid of the dilemma which was heavily dependent on imported oil from abroad. In 2010, The U.S. could achieve microalgae production industrialization and produced microalgae oil reached about millions of barrels per day [43].

In 2010, the Department of Energy in the U.S. allocated $ 6 million funds to the Arizona State University to establish sustainable algal biofuel consortium (SABC). Also, $ 9 million U.S. dollars have been allocated to University of California (San Diego) from the Department of Energy for creating algae biofuel commercialization consortium (CABC). Cellana LLC consortium of Kailua-Kona and Hawaii have also gotten $ 9 million fund from the Department of Energy. The U.S. government strongly supported algae development on finance.The president Barack Obama has done a speech at the University of Miami. At that time, Obama said that the renewable energy positioning in 2012 is algae bioenergy. He declared that his government will invest $ 24 million in biofuel development, and claimed that "Whether you believe it or not, we can rely on the United States home-grown biofuel to replace 17% of imported petroleum fuels". China is also

paying attention to the microalgae bioenergy development. In the large-scale microalgae cultivation field, Chinese Academy of Sciences has successfully developed a large "S" shaped pipe of closed photo biological reactor. In 2008, Shandong University of Science and Technology used the thermal power plant and chemical plant flue gas as sources of carbon dioxide to supply microalgae cultivation in the tower dimensional cultivation reactor. After then, mature microalgae are transferred 21 to energy conversion equipments to product bio-oil. This research results improved microalgae production technology [42].

In 2010, the Chinese Academy of Sciences and China Petroleum Chemical Cooperation held a meeting about "Microalgae Biodiesel Technology Projects". Co-operate developing microalgae biodiesel technology had been decided in this meeting. Till now a pilot study has been done, and outdoor middle scale of microalgae energy plant will be established in 2015 [43]. Hainan Greenbelt microalgae Biotechnology Company has been built a microalgae cultivation experimental base in Ledong County, the microalgae oil content can reach to 28% - 32% .This Company planned to invest $ 29.8 million to establish dry microalgae project in Hainan province. This project will have 30 million tons of annual production capacity of biodiesel. The new Austrian technology company is brewing a huge plan. They invested to microalgae ecological base in the Daqi of Inner Mongolia. 280 hectares of microalgae base will be completed in 2013. Microalgae ecological base will be officially reached industrialization in 2014 [44-46].

Chapter Three

Materials and Methods

This research work was carried out in Bioprocess Technology laboratory of Biotechnology and Genetic Engineering discipline, Khulna University, Khulna, Bangladesh.

3.1 Materials

Following chemicals and equipments were used for biodiesel extraction from algae.

3.1.1 Source material

- Algae (*Chara vulgaris*)

3.1.2 Chemicals

- Chloroform (Loba Chemie, Mumbai, India)
- Sodium Chloride (Merck, Mumbai, India)
- Methanol (Merck KGaA, Darmstadt, Germany)
- Sodium hydroxide (Merck, Mumbai, India)
- Distilled water

3.1.3 Equipments

- Electric balance
- Water heater
- Oxygen bomb calorimeter

3.2 Methodology

Total lipid extraction from algae performed by chemical extraction methods, relying on the chloroform-methanol solvent system, based on the Bligh and Dyer (1959) method [32, 33]. A slight modification of this process was done to facilitate the lipid extraction from *chara vulgaris*. Lipid found from these step were then used for the esterification reaction to produce biodiesel. The procedure of making biodiesel follows several steps.

Drying of algae biomass: Paste of *Chara vulgaris* was prepared by using mortar and pestle as much as possible, and then ground algae dried in the incubator at 80°C for 30 minutes.

Oil extraction: 12 gm of dry algae biomass was mixed with chloroform and methanol, shake vigorously for 10 minutes. Then NaCl solution added in the mixture and shake for another 10 minutes. Resulted mixture was filtered to remove the algal biomass. The clear solution kept for a while to form two phase. The upper phase which contains water and methanol are separated from the lower chloroform-oil phase and discarded. The lower chloroform-oil phase was air dried for 24 hours and the remaining solution was used for the further treatment.

Mixing of alcohol and catalyst: The catalyst sodium hydroxide, at an amount of 0.8 % of algal oil is dissolved in the methanol at an amount of 25% of algal oil by hand shaking and whirling.

Reaction: The alcohol catalyst mix is then charged into a conical flask and the oil is added. The algal oil is heated at 50°C temperature before mixing with alcohol-catalyst mixture. The system from here on is totally closed o the atmosphere to prevent the loss of alcohol. The reaction mix is kept just below the boiling point of the alcohol to speed up the reaction and the reaction takes place. The reaction is held under constant temperature around 55-65°C on the water heater. Reaction time varies from 1 to 8 hours.

Separation: Once the reaction is complete, two major products exist- biodiesel and glycerin. Each has a substantial amount of excess methanol. The glycerin phase is much more dense then the biodiesel. The glycerin that has formed is separated by gravity separation am the biodiesel is ready for further processing. The glycerin separation steps are usually accomplished by gravity settling or with a centrifuge.

Alcohol Removal: After the biodiesel is separated from the glycerin, it contains 3% to 6% methanol and usually some soap. The methanol is removed by vaporization. Methanol tends to act as a co-solvent for soap in the biodiesel, so at higher soap levels the soap will precipitate as a viscous sludge when the methanol is removed.

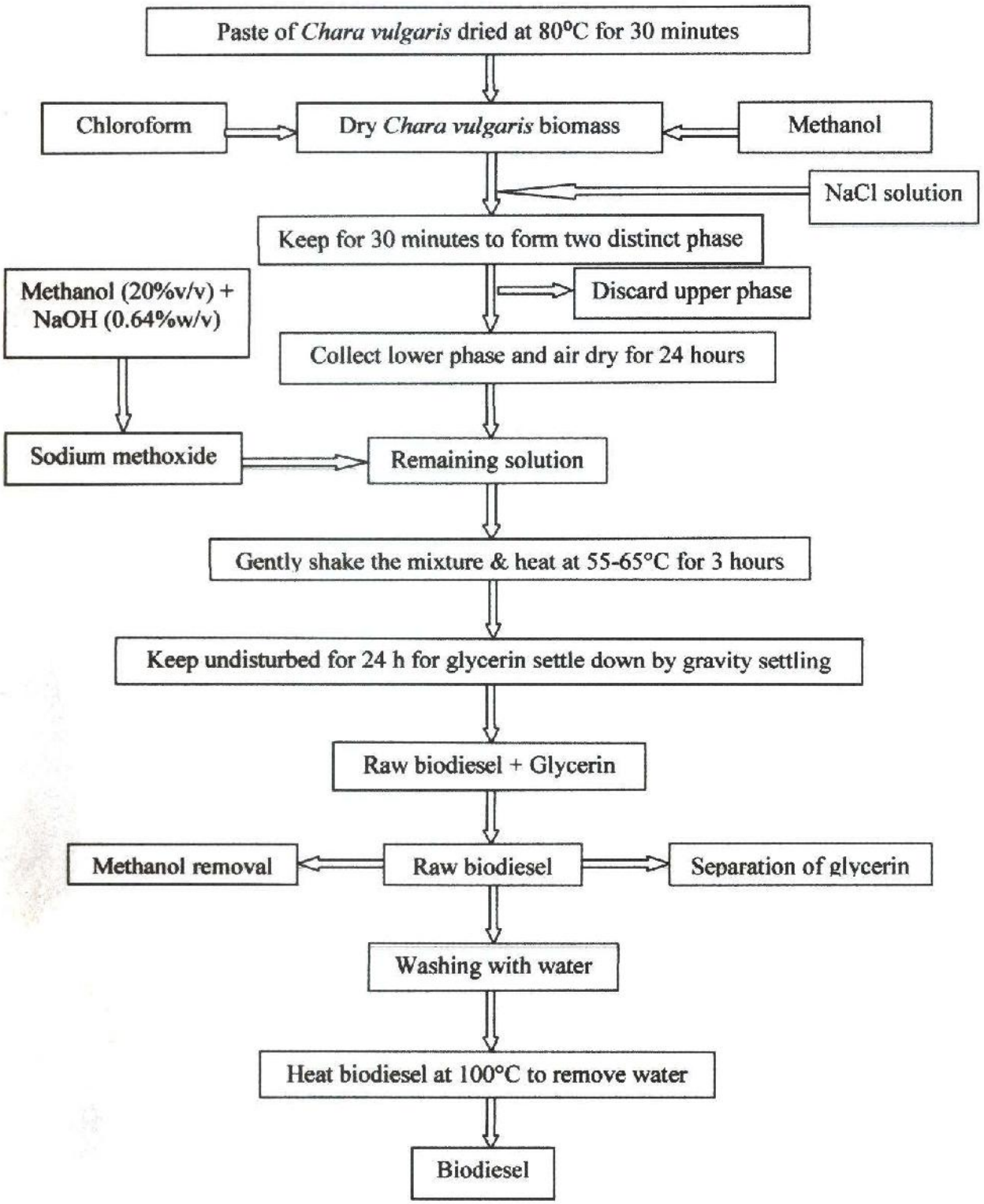


Figure 3.1: Flow chart of algae biodiesel.

Methyl Ester Wash: After the methanol has been removed, the biodiesel needs to be washed to remove residual free glycerin, methanol, soaps and catalyst. This is done by liquid-liquid extraction by mixing water with the biodiesel and gently agitating and the soap is separated by gravity separation. The washing process is done 3-4 times until the wash water no longer picks us soap.

Methyl Ester Drying: Remaining water present in the biodiesel is removed by heating system at 100°C for 10 minutes. Finally usable 100% pure biodiesel is extracted.

3.3 Experimental Design:

3.3.1Box-Behnken design:

The Box-Behnken design is one type of response surface methodology (RSM) based on central composite design (CCD). It is an independent quadratic design in that it does not contain an embedded factorial or fractional factorial matrix. In this design the treatment combinations are at the mid-points of edges of the process space and at the center. These designs are rotatable (or near rotatable) and require three levels of each factor. Table.3.1 represents structure of Box-Behnken design. These designs have limited capability for orthogonal blocking compared to the central composite designs (Box and Behnken, 1960).

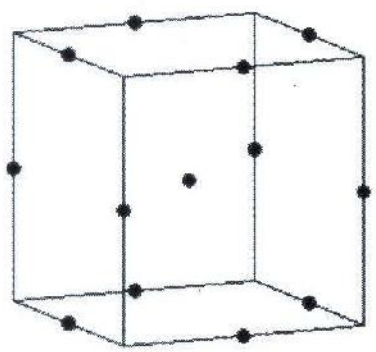


Figure 3.2: Box-Behnken designs for three factors.

The geometry of this design suggests a sphere within the process space such that the surface of the sphere protrudes through each face with the surface of the sphere tangential to the mid-point of each edge of the space. For three factors, the Box-Behnken design offers some advantage in requiring a fewer number of runs [15, 16].

Table: 3.1 Structure of Box-Behnken design

Replication	X_1	X_2	X_3
1	-1	-1	0
1	1	-1	0
1	-1	1	0
1	1	1	0
1	-1	0	-1
1	1	0	-1
1	-1	0	1
1	1	0	1
1	0	-1	-1
1	0	1	-1
1	0	-1	1
1	0	1	1
1	0	0	0
1	0	0	0
1	0	0	0

The second order polynomial equation for three responses:

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_4X_1X_2 + A_5X_1X_3 + A_6 X_2X_3 + A_7X_1^2 + A_8X_2^2 + A_9X_3^2 \quad \ldots\ldots\ldots\ldots (1)$$

Figure 4.1: (a) General view and (b) Microscopic view of collected specimen. (c) General view and (d) Mmicroscopic view of reference *chara vulgaris* [47, 48].

4.2.2 Oil extraction from algae

Algae biomass contains lipids, protein, carbohydrates, ash, and moisture [49]. Lipid preserved in the algal biomass was extracted using chemical method. We used Bligh and Dyer oil extraction method as a pedestal method [32, 33]. Our source material was collected from local area. Amount of extracted oil was minimal for further process. For this purposes we slightly modified the algae oil extraction method of Bligh and Dyer.

4.2.2.1 Drying of algae biomass

Two different techniques were applied to prepare algal biomass. In the first technique fresh algae was sun dried and grounded using mortar and pestle. In The second technique paste of fresh algae *Chara vulgaris* was prepared by using mortar and pestle as much as

possible, and paste of algae was dried in the incubator at 80°C for 30 minutes. Quality of ground algae produced from second technique was good than the first one, hence we took the second technique one for algal biomass production.

4.2.2.2 Oil extraction

12 gm of dry algae biomass was mixed with chloroform and methanol, shake vigorously for 10 minutes. Then NaCl solution added in the mixture and shake for another 10 minutes. Resulted mixture was filtered to remove the algal biomass. The clear solution kept for a while to form two phase. The upper phase which contains water and methanol are separated from the lower chloroform-oil phase and discarded. The lower chloroform-oil phase was air dried for 24 hours and the remaining solution was used for the further treatment.

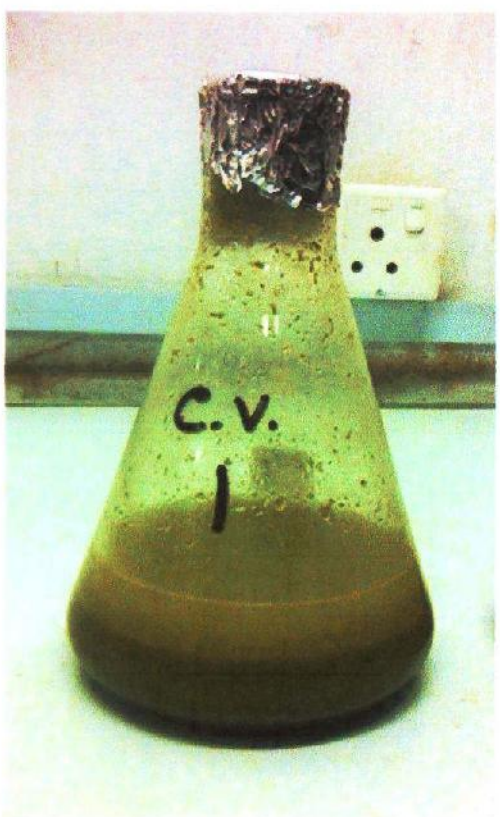


Figure 4.2: Two phase solution algal biomass

4.2.3 Conversion of algae oil into biodiesel

Solutions found from the previous steps were used in the biodiesel production. Sodium-methoxide solution were mixed with the algal solution and heated at 55-65°c temperature. After the reaction was complete, two major products exist- biodiesel and glycerin. The glycerin phase was much more dense then the biodiesel. The glycerin that has formed was separated by gravity separation and the biodiesel was ready for further processing.

4.2.3.1 Washing of raw biodiesel

Washing was done by liquid-liquid extraction by mixing water with the biodiesel and gently agitating and the soap is separated by gravity separation. The washing process was done 7-8 times until the wash water no longer pick us soap.

4.2.3.2 Processed biodiesel

After washing, remaining water present in the biodiesel was removed by heating system at 100°C for 15 minutes. Finally usable 100% pure biodiesel was extracted.

4.2.4 Properties of algae biodiesel

The physical properties of biodiesel produced from algae had the values present within the limit of the physical properties of biodiesel.

Table 4.1 Physical properties of algae biodiesel

Properties	Algae biodiesel	Biodiesel (ASTM)
Viscosity	5.003cst	3.7-5.8 cst.
Flashpoint	133°C	>130 °C.
Calorific value	9255.106 kcal/kg	8850-10000 kcal/kg
density	0.871g/ml	0.87 to 0.89 g/ml

4.3 Production Process Optimization

We intended to optimize the parameters of algae biodiesel production process. We used Box–Behnken design as the statistical tool to optimize this procedure.

4.3.1 Process optimization by Box-Behnken method

The important factors for the production were chloroform, NaCl and temperature. Hence these factors were considered as the independent variables and their effects on calorific value and yield of algae biodiesel were studied using Box- Behnken design of Response surface methodology (RSM).

Table 4.2: Coded levels of independent variables in the experimental design

Variables	Coded levels		
	+1	0	-1
Chloroform (ml)	118.8	158.4	198
NaCl (%)	0.70	0.75	0.80
Temperature (°C)	55	60	65

Where, each of chloroform and NaCl concentration is given for 12gm of dry algae biomass.

The range and levels of experimental variables investigated in this study were presented in Table 4.2. The central values (zero level) chosen for experimental design were: chloroform-X_1 (158.4 ml i.e. 13.2 ml chloroform/ gm of wet algae biomass), NaCl-X_2 (0.75%) and temperature-X_3 (60°C). Chloroform level was calculated at the zero level by maintaining the 2:1 ratio of chloroform: methanol.

The results of Box-Behnken design experiments for studying the effects of three independent variables, viz., chloroform, NaCl and temperature on calorific value is presented in Table 4.3. These values were used for analysis of regression where Microsoft office excel tool "data analysis" was used. 95% confidence level was kept. The

calculation of regression gives the coefficients (A_0 to A_9 of Eq.1). For each response different set of these coefficients were obtained.

Table 4.3: The Box-Behnken design matrixes employed for three independent variables (chloroform, NaCl and temperature) with observed calorific values and yield for algae biodiesel.

Run No.	Chloroform (X_1) (ml)	NaCl (X_2) (%)	Temperature(X_3) (°C)	Calorific value (c) (kcal/kg)	Yield (y) (ml)
1	118.8	0.70	60	9167.173	3.0
2	198	0.70	60	9153.926	3.4
3	118.8	0.80	60	9108.31	2.9
4	198	0.80	60	9219.738	3.4
5	118.8	0.75	55	9188.192	3.1
6	198	0.75	55	9242.961	3.6
7	118.8	0.75	65	9132.539	3.0
8	198	0.75	65	9255.106	3.5
9	158.4	0.70	55	9158.281	3.2
10	158.4	0.80	55	9151.03	3.1
11	158.4	0.70	65	9143.935	3.2
12	158.4	0.80	65	9174.205	3.2
13	158.4	0.75	60	9169.471	3.3
14	158.4	0.75	60	9169.471	3.3
15	158.4	0.75	60	9169.471	3.3

Where, each of chloroform and NaCl concentration is given for 12gm of dry algae biomass.

Table 4.4: Regression coefficient and corresponding probability values (*p*-values) for specific responses (calorific value and yield) for algae biodiesel.

Parameter (coefficient)	Calorific value (c)		Yield value (y)	
	coefficient	*p*-value	coefficient	*p*-value
Constant (A_0)	9680.969	0.01485	-19.25	0.078605
$X_1(A_1)$	- 20.1792	0.004058	- 0.00347	0.803372
$X_2(A_2)$	11836.46	0.069057	66.5	0.010884
$X_3(A_3)$	- 114.098	0.051354	- 0.0775	0.620817
$X_1 X_2(A_4)$	15.74179	0.009388	0.012626	0.363217
$X_1 X_3(A_5)$	0.085604	0.076392	−2.6E-18	1
$X_2 X_3(A_6)$	37.521	0.272441	0.1	0.363217
$X_1 X_1(A_7)$	0.012962	0.050241	1.12E-19	1
$X_2 X_2(A_8)$	- 11004.2	0.017777	- 50	0.004867
$X_3 X_3(A_9)$	0.59609	0.118613	2.25E-17	1

Table 4.4 represents the ANOVA analysis of design variables where values of coefficients and *p*-values were represented for the three responses. The *p*-values are used as a tool to check the significance of each coefficient, which also indicate the interaction strength between each independent variable. A *p*-value less than 0.05 indicate that the factor interacted significantly with the response. It was observed that all the *p* values are smaller than 0.05, indicates that all linear factor, cross product factor and quadratic factor significantly related to calorific value except one cross product factor and one quadratic factor like temperature. It is seen that chloroform-NaCl cross product were significantly related to the response (c). Yield did not show significant relation with chloroform and temperature, but was significantly related to the linear factor and quadratic factor like NaCl.

From regression analysis all nine coefficients were used in making the response equation, though not all factors were significant ($p < 0.05$). The second-order polynomial equations for the three responses were found as follows:

$$Y(c) = 9680.969 - 20.1792X_1 + 11836.46X_2 - 114.098X_3 + 15.74179X_1X_2 + 0.085604X_1X_3 + 37.521\ X_2X_3 + 0.012962X_1^2 - 11004.2X_2^2 + 0.59609X_3^2 \quad \ldots\ldots\ldots\ldots (2)$$

$$Y(y) = -19.25 - 0.00347X_1 + 66.5X_2 - 0.0775X_3 + 0.012626X_1X_2 - (2.6E\text{-}18)X_1X_3 + 0.1\ X_2X_3 + (1.12E\text{-}19)X_1^2 - 50X_2^2 + (2.25E\text{-}17)X_3^2 \quad \ldots\ldots\ldots\ldots\ldots (3)$$

Where Y(c) is calorific value and Y(y) yield value, X_1, X_2 and X_3 are coded values for chloroform, NaCl and temperature respectively.

Table 4.5: R^2 values for the ANOVA analysis of the two response output

R^2 values	Calorific value (c)	Yield (y)
	0.947005	0.976563

From the ANOVA analysis, we found the predicted values of the response based on Equation (2) and (3). The calculation of regression analysis also gives the value of the determination coefficient R^2 represented in table 4.5. Table 4.6 shows the experimental value of Calorific value and yield of biodiesel which was produced from algae. Also show the predicted value after regression analysis.

4.3.2 Response surface analysis

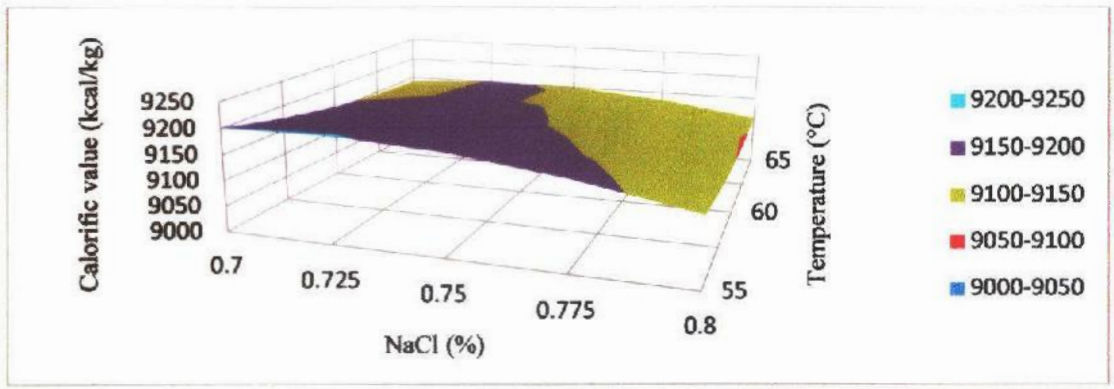
The effect of three variables on the response was seen by holding one factor constant while the other two factors were varied. The values of calorific value and yield were predicted by using equation (2) and (3) and all the 3-D response surface plots are shown in Figure 4.3 and 4.4. Due to three coded levels and the three coded variables total nine combinations were possible for each response. The plots were created with the aim to observe optimum condition from predicted values.

Table 4.6: Predicted and experimental values for two responses (calorific value and yield)

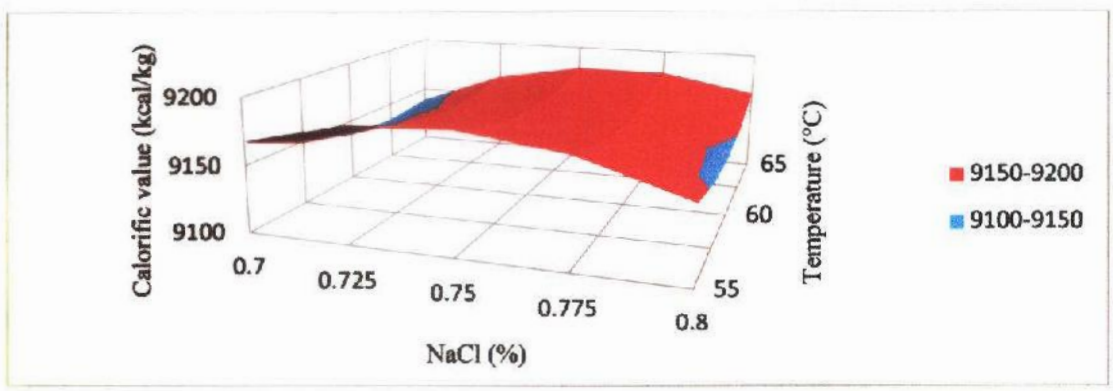
Run no	Calorific value (kcal/kg)		Yield (ml)	
	Predicted value	Experimental value	Predicted value	Experimental value
1	9155.27	9167.173	2.9875	3.0
2	9161.812	9153.926	3.4125	3.4
3	9100.424	9108.31	2.8875	2.9
4	9231.641	9219.738	3.4125	3.4
5	9191.544	9188.192	3.075	3.1
6	9226.525	9242.961	3.55	3.6
7	9148.976	9132.539	3.05	3.0
8	9251.754	9255.106	3.525	3.5
9	9166.832	9158.281	3.2375	3.2
10	9155.563	9151.03	3.1375	3.1
11	9139.402	9143.935	3.1625	3.2
12	9165.654	9174.205	3.1625	3.2
13	9169.471	9169.471	3.3	3.3
14	9169.471	9169.471	3.3	3.3
15	9169.471	9169.471	3.3	3.3

4.3.2.1 Analysis of calorific value

The effect of different temperature and NaCl concentration on calorific value was observed by keeping the chloroform constant at three level 118.8, 158.4 and 198 ml respectively (figure 4.3-A). Chloroform kept constant at low level of 118.8 ml in the first 3-D plot of figure 4.3-A, where it can be seen that calorific value is higher at the low level of temperature and NaCl concentration. But in the second plot, higher responses observed at the middle position of NaCl concentration. When constant chloroform level increases to 198 ml, better response observed at highest level at 0.80% NaCl and 65°C temperature. By comparing the three curves at different chloroform level it was observed that good responses found at the highest level of NaCl concentration and temperature.



(a)



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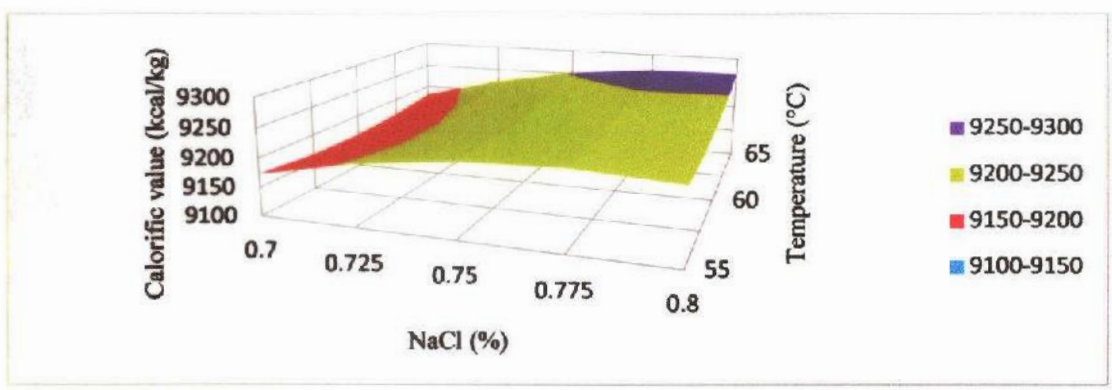
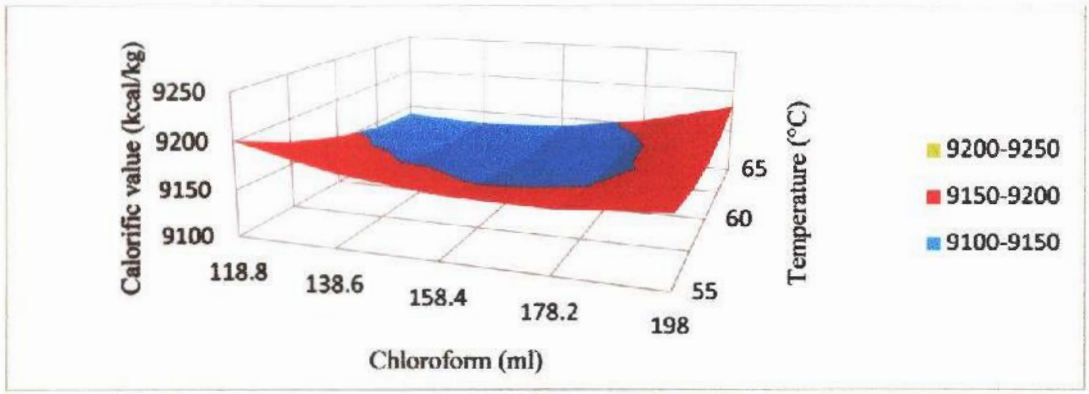
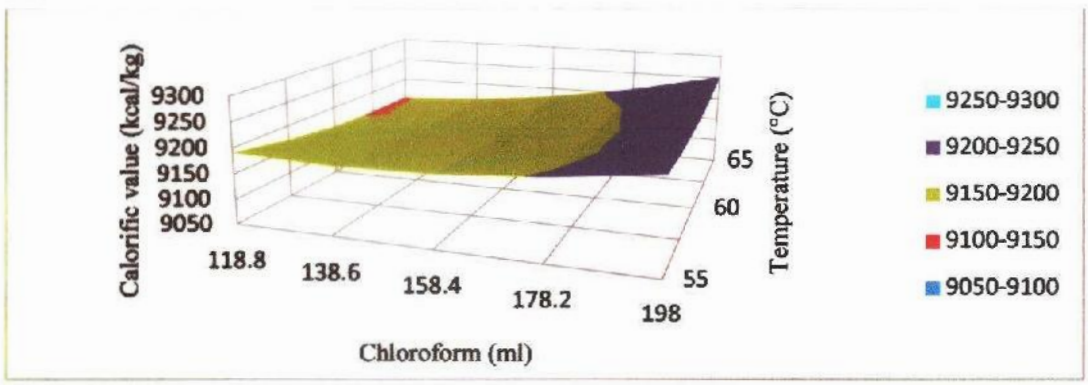


(c)

Figure 4.3-A: Different chloroform concentrations- (a) Effects of temperature and NaCl on calorific value at constant 118.8 ml chloroform (b) Effects of temperature and NaCl on calorific value at constant 158.4 ml chloroform (c) Effects of temperature and NaCl on calorific value at constant 198 ml chloroform.



(a)



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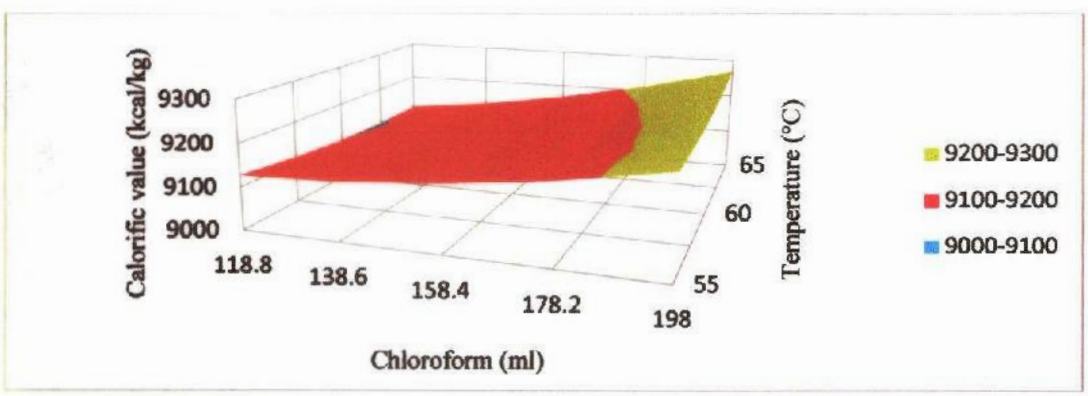
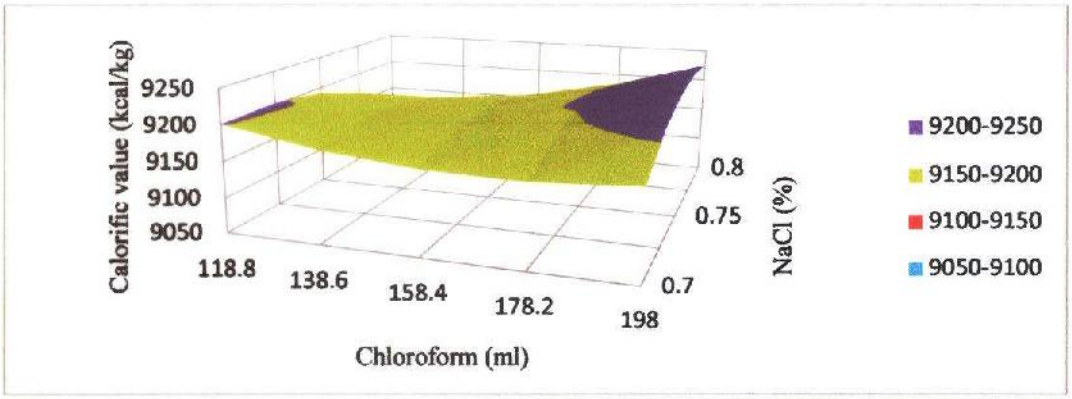
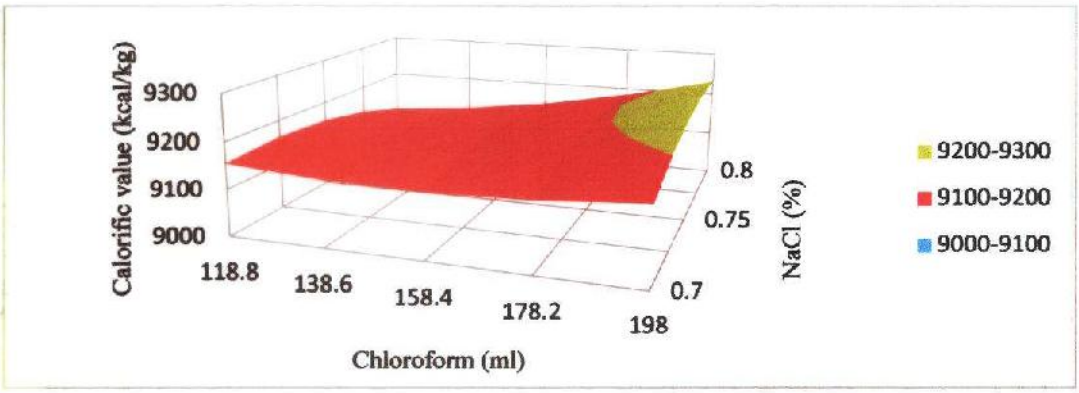


(c)

Figure 4.3-B: Different sodium chloride concentrations- (a) Effects of temperature and chloroform on calorific value at constant 0.70% NaCl (b) Effects of temperature and chloroform on calorific value at constant 0.75% NaCl (c) Effects of temperature and chloroform on calorific value at constant 0.80% NaCl.



(a)



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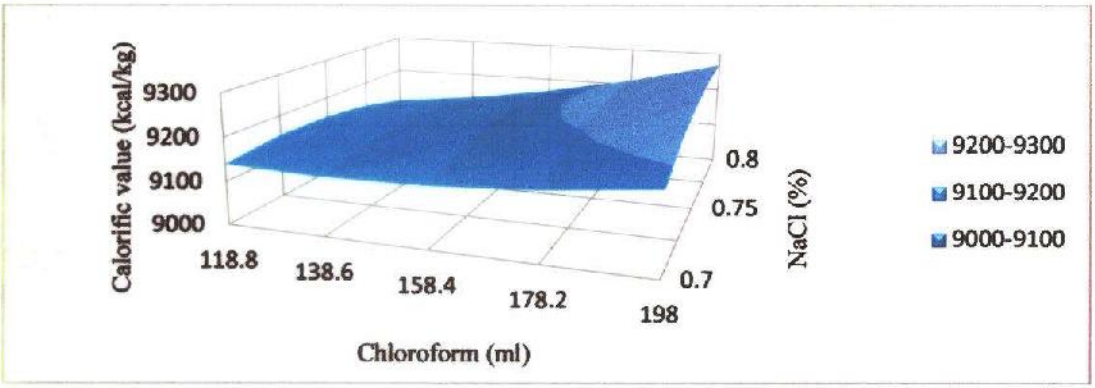


(c)

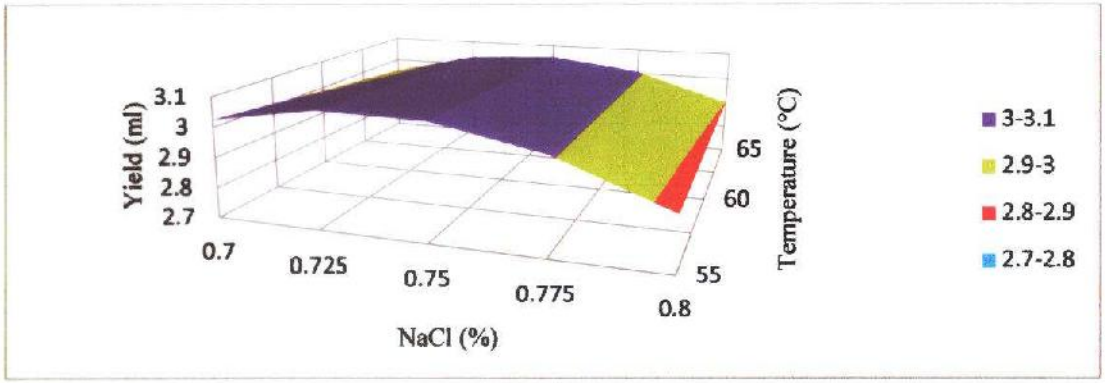
Figure 4.3-C: Different temperatures - (a) Effects of chloroform and NaCl concentrations on calorific value at constant 55°C temperature (b) Effects of chloroform and NaCl concentrations on calorific value at constant 60°C temperature (c) Effects of chloroform and NaCl concentrations on calorific value at constant 65°C temperature.

Figure 4.3-B shown the effect of different temperature and chloroform on calorific value at NaCl concentration constant. Three level of NaCl concentration 0.70%, 0.75% and 0.80% was shown in figure (a), (b) and (c) respectively. In the first 3-D plot of figure 4.3-B NaCl concentration kept constant at 0.70%, where it can be seen that calorific value is low in the middle of the plot at 158.4 ml chloroform and 60°C temperature and high at the both end of curve at low and high level of temperature and chloroform. The second plot shown that though the response is still low in the middle position, but higher responses observed at the high level temperature and chloroform than the low level of temperature and chloroform. Whereas, different scenario found in the third plot of figure 4.3-B. better response observed at highest level of chloroform and 65°C temperature at 0.80% NaCl concentration. By comparing the three curves at different chloroform level it was observed that good quality responses found at the peak level of chloroform and temperature.

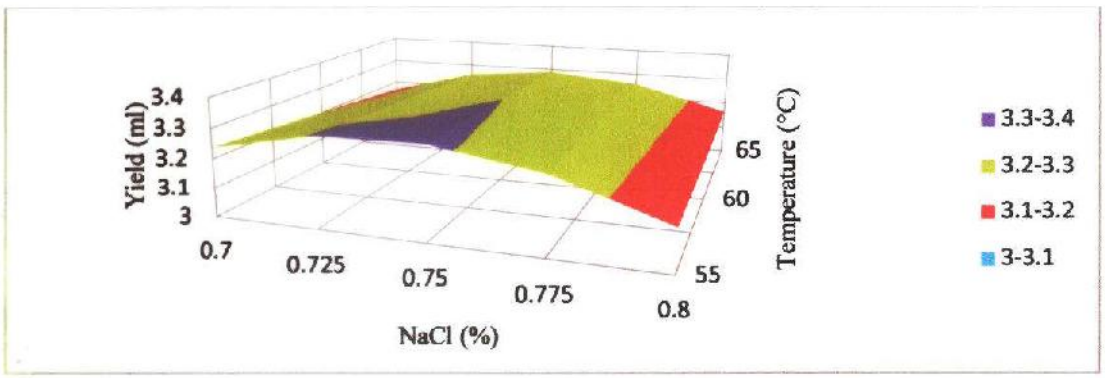
In figure 4.3-C the effect of different chloroform and NaCl concentration on calorific value at constant temperature was shown. Effect of three temperatures constant level 55°C, 60°C and 65°C was shown in figure (a), (b) and (c) respectively. In the first 3-D plot of figure 4.3-C temperature was kept constant at 55°C, where it can be seen that calorific value is slightly low in the middle of the plot at 158.4 ml chloroform and 0.75% NaCl concentration and high at the both end of curve at low and high level of chloroform and NaCl concentration. The second plot shown that though the response is still low in the middle position, but higher responses observed at the high level chloroform and NaCl concentration than the low level of chloroform and NaCl concentration at 60°C temperature constant. But at 65°C temperature constant, improved response observed at highest level of chloroform and 0.80% NaCl concentration. By comparing the three curves at different temperature level it was observed that fine responses found at the maximum level of chloroform and NaCl concentration.

4.3.2.2 Analysis of yield value

The effect of different parameter on yield can be observed from the surface plot diagram shown in figure 4.4.



(a)



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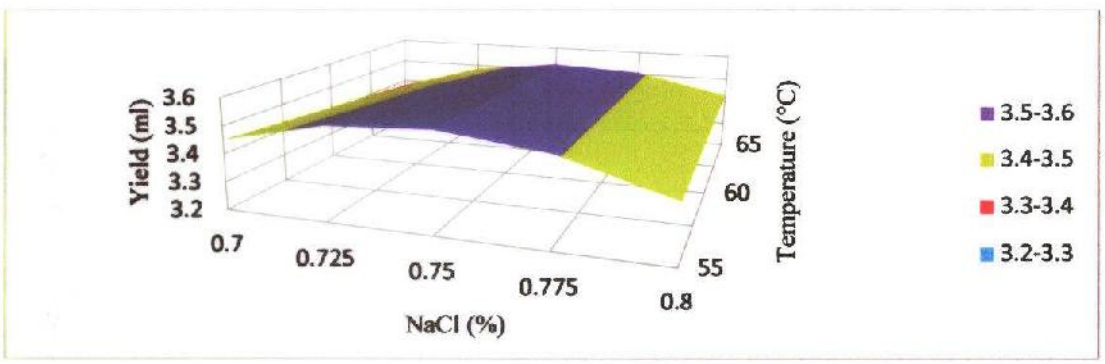
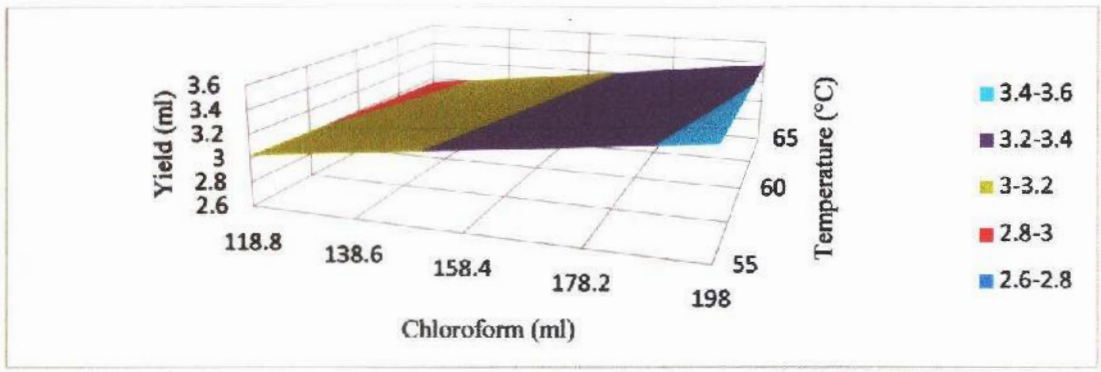
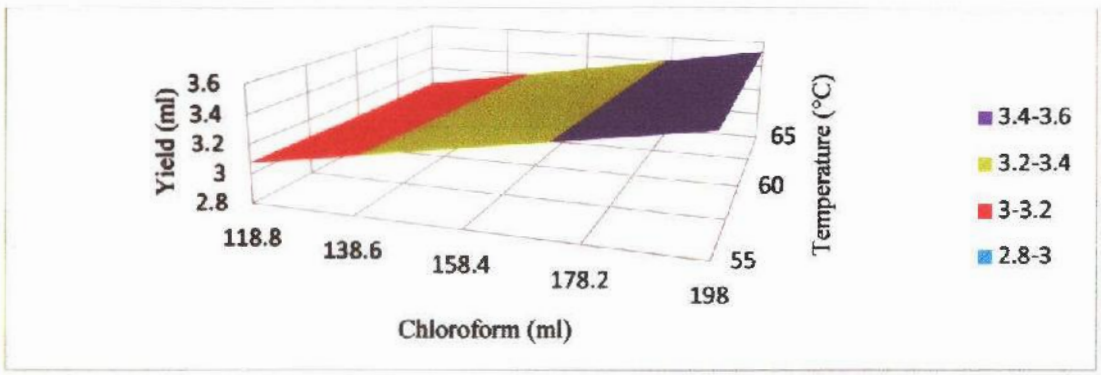


(c)

Figure 4.4-A: Different chloroform concentrations- (a) Effects of temperature and NaCl on yield at constant 118.8 ml chloroform (b) Effects of temperature and NaCl on yield at constant 158.4 ml chloroform (c) Effects of temperature and NaCl on yield at constant 198 ml chloroform.



(a)



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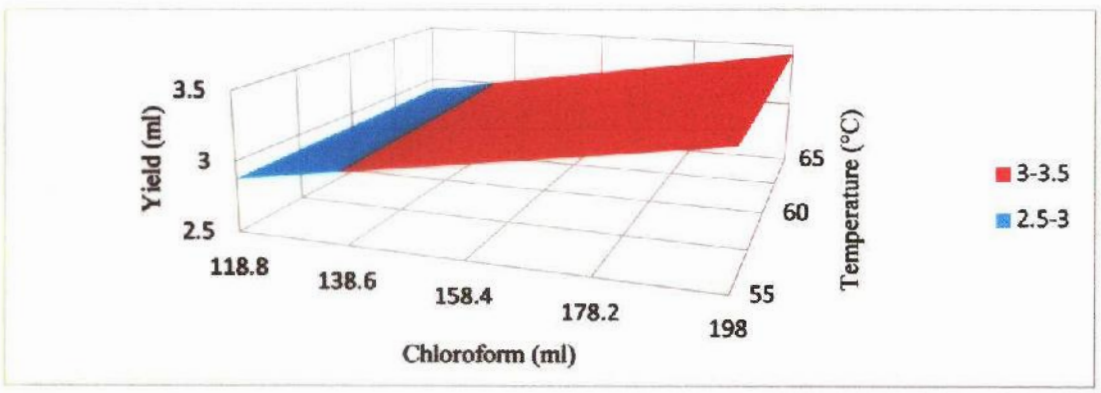
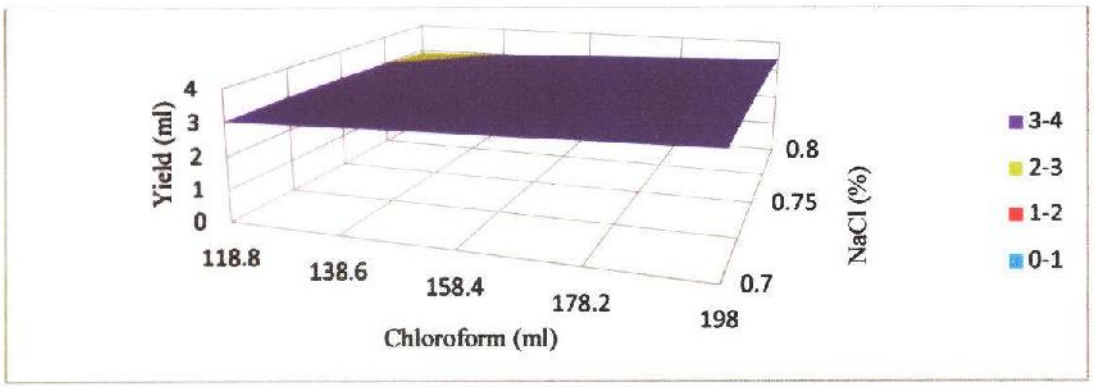
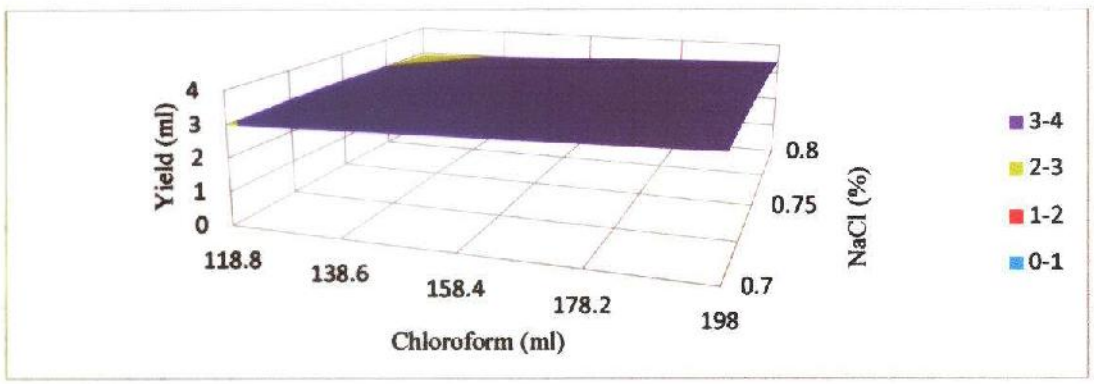


(c)

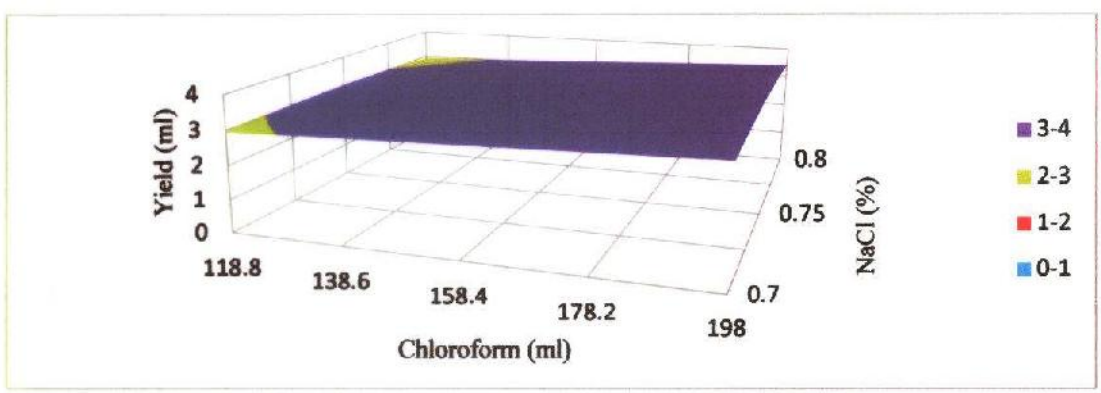
Figure 4.4-B: Different sodium chloride concentrations- (a) Effects of temperature and chloroform on yield at constant 0.70% NaCl (b) Effects of temperature and chloroform on yield at constant 0.75% NaCl (c) Effects of temperature and chloroform on yield at constant 0.80% NaCl.



(a)



(b)



(c)

Figure 4.4-C: Different temperatures - (a) Effects of chloroform and NaCl concentrations on yield at constant 55°C temperature (b) Effects of chloroform and NaCl concentrations on yield at constant 60°C temperature (c) Effects of chloroform and NaCl concentrations on yield at constant 65°C temperature.

The consequence of different temperature and NaCl concentration on yield was observed by keeping the chloroform constant at three level 118.8, 158.4 and 198 ml respectively (figure 4.4-A). Chloroform kept constant at low level of 118.8 ml in the first 3-D plot of figure 4.4-A, where it can be seen that yield is higher at middle of the plot at 0.75% NaCl concentration and low at low and high level of NaCl concentration. Similarity found in the second plot, high responses observed at the middle position of NaCl concentration and temperature. As constant chloroform level increases to 198 ml, better response observed at mid level at 0.75% NaCl and 65°C temperature. By comparing the three curves at different chloroform level it was observed that good responses found at the mid level of NaCl concentration and temperature.

Figure 4.4-B shown the effect of different temperature and chloroform on calorific value at NaCl concentration constant. Three level of NaCl concentration 0.70%, 0.75% and 0.80% was shown in figure (a), (b) and (c) correspondingly. In the first 3-D plot of figure 4.4-B NaCl concentration kept constant at 0.70%, where it can be seen that response level increases as the level of temperature and chloroform increases. The second plot shown similar responses, higher responses observed at the high level of temperature and chloroform than the low level of temperature and chloroform. Whereas, in the third plot of figure 4.3-B, enhanced response observed at highest level of chloroform and 65°C temperature at 0.80% NaCl concentration. By comparing the three curves at diverse chloroform level it was observed that excellence responses found at the peak level of chloroform and temperature.

In figure 4.4-C the effect of different chloroform and NaCl concentration on calorific value at constant temperature was exposed. Effect of three temperatures constant level 55°C, 60°C and 65°C was shown in figure (a), (b) and (c) respectively. In the first 3-D plot of figure 4.3-C temperature was kept constant at 55°C, slight increase of response was observed with increasing level of chloroform and NaCl concentration. The second and third plot also showed the similar effect for 60°C temperature constant. By comparing the three curves at different temperature level it was observed that there is little effect of temperature difference on responses, though fine responses found at the maximum level of chloroform and NaCl concentration.

4.3.3 Contour plot analysis

The contour plot was produced by plotting NaCl concentration (y-axis) against chloroform (x-axis) for a series of predicted value at a constant temperature of 65°C. Figure 4.5 and 4.6 stands for the contour curve for calorific value and yield correspondingly where different values expressed a diverse zone.

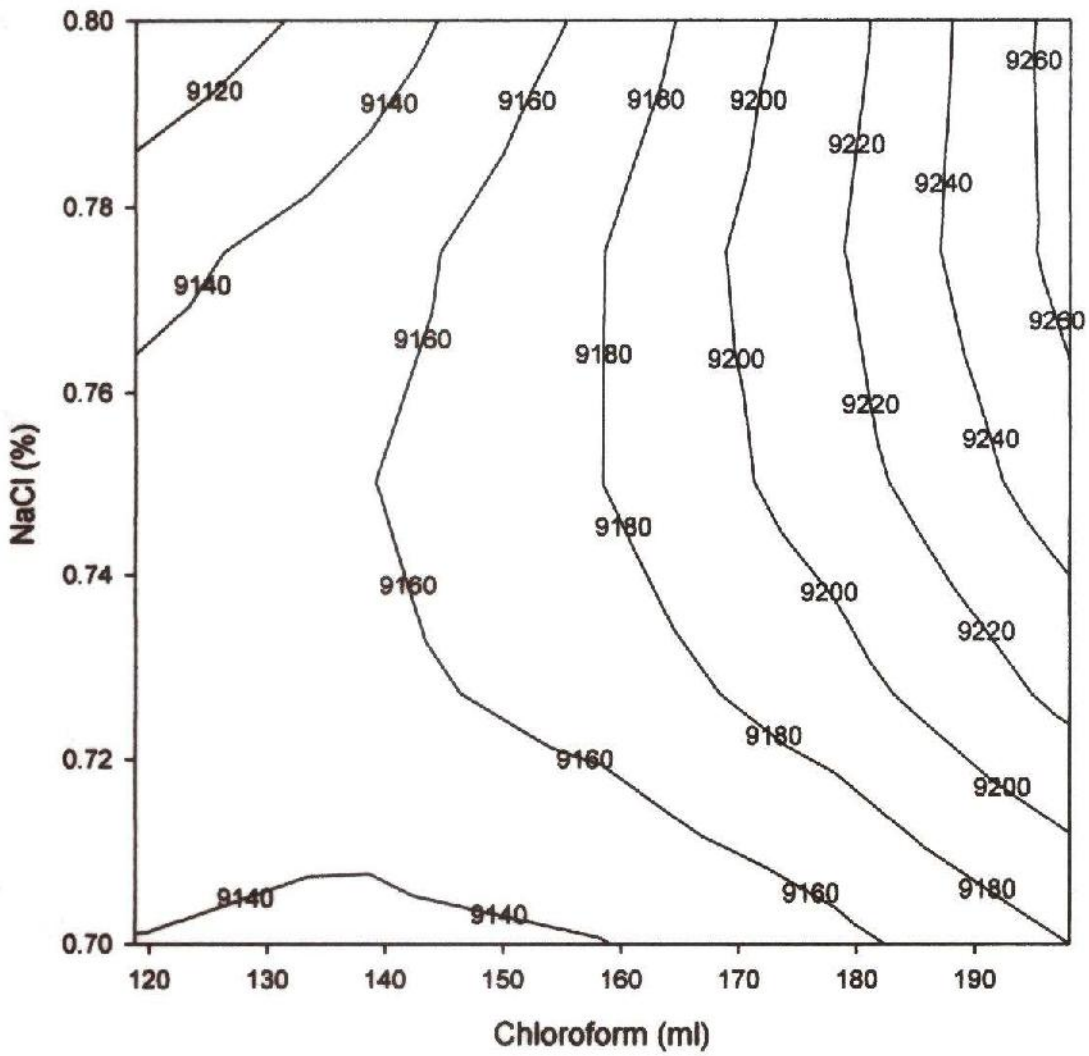


Figure 4.5: Contour plot showing calorific value at various NaCl concentrations and chloroform at a constant temperature of 65°C.

Experimentally, only one combination produced maximum calorific value (Table 2). From the curve, it was found that several combinations of NaCl concentrations and chloroform were likely to produce the same line. It can be observed that a extremely smaller portion on the curve in right corner consists of high calorific value (9260 kcal/kg) zones and smaller lower calorific value (9120 kcal/kg) zone at the left corner. It was found that a higher calorific value zone could be obtained using a NaCl concentrations range of 0.76-0.80% and chloroform range of 196-198 ml. while a lower calorific value zone resulted from a NaCl concentrations range of 0.78-0.80% and chloroform range of 120-130 ml. This curve signifies that high calorific value is more responsive to NaCl concentrations than to chloroform when temperature is kept constant.

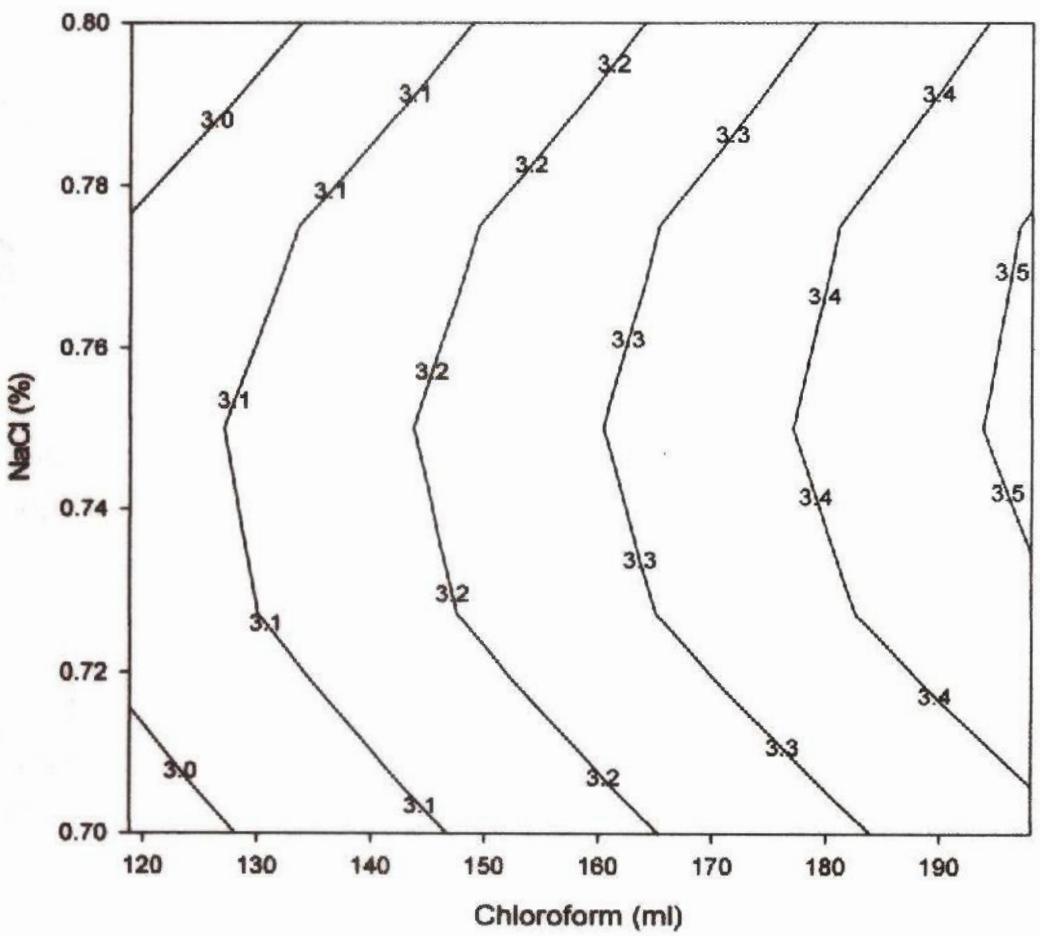


Figure 4.6: Contour plot showing yield at various NaCl concentrations and chloroform at a constant temperature of 65°C.

From the curve, it was found that a number of combinations of NaCl concentrations and chloroform were possible to generate the same line. Whereas, experimentally only one combination produced maximum yield (Table 2). It can be observed that a very smaller portion on the curve in right edge consists of high yield (3.5 ml) zones and smaller lower yield (3.0 ml) zone at the lower left corner. It was found that a higher calorific value zone could be obtained using a NaCl concentrations range of 0.73-0.77% and chloroform range of 196-198 ml. while a lower calorific value zone resulted from a NaCl concentrations range of 0.70-0.71% and chloroform range of 120-128 ml. This curve point out that high calorific value is more responsive to NaCl concentrations than to chloroform when temperature is kept constant.

4.4 Overall Discussion

When chloroform kept constant at 198 ml the surface plot for calorific value showed a higher in the corner at 0.75% NaCl (Figure 4.3-A). The above-mentioned state produced a good calorific value, both in experiment and prediction. Because high calorific value indicates better quality biodiesel, we observed the plot on this basis. However, the calorific value was found much lower for the low level of chloroform. Figure 4.3-B can be helpful in understanding the patterns of NaCl concentration. It can be seen that, high calorific value observed at the middle of NaCl concentration with increase Chloroform concentration and temperature. From this curve, it can be observed that the pattern of responses varies with respect to chloroform and temperature. When the responses were varied with three factors the relationship became more complex, that is why low value was observed at 0.70%, high value at 0.75%, and low value again at 0.80% NaCl concentration (Figure 4.3-A and B). From this, it can be concluded that at 0.75% NaCl concentration calorific value is optimum with varying chloroform concentration and temperature. As shown in the Figure 4.3-C similar patterns were found for varying temperature factor. All the patterns show low response at low temperature and increases with temperature. Similar results can be seen from Table 4.2, which contains experimental values. It was also found that when temperature was kept constant at 65°C (fig.4.3), maximum calorific value 9255.106 kcal/kg was observed at 198 ml chloroform

and 0.75% NaCl. This condition was considered optimum and thus used later for further study.

Similar 3-D surface plots were created to see the effect of the three variables on the yield response. From figure 4.4 (A) and (B) it can be seen that yield increases with increasing chloroform. High response was observed at 198 ml chloroform concentration. Whereas high yield found at the middle point of the NaCl concentration at 0.75%, low response found in both low level and high level of NaCl concentration correspondingly at 0.70% and 0.80%. But the effect of different temperature on the response was varied very slightly. From this it can be calculated that NaCl concentration had much more effect on the response rather than chloroform and temperature. Higher response found at 0.75% NaCl concentration, 198 ml chloroform and at 65°C temperature, which is similar found in experimental value. Thus this is the optimum condition for yield of algae biodiesel.

Chapter Five

Conclusion and Recommendation

Biodiesel was produced from macroalgae (*Chara vulgaris*) which are collected from our coastal area. The Box–Behnken design based on response surface methods (RSM) used as the statistical tool to optimize three variables for predicting the best performing conditions (calorific value and yield) of algae biodiesel. The three parameters for production condition were chloroform, sodium chloride concentration and temperature. Optimal conditions were estimated by the aid of statistical regression analysis and surface plot chart. The Optimal condition of biodiesel production parameter was recognized to be 198 ml Chloroform with 0.75% sodium chloride at 65°C temperature where the calorific value of biodiesel is 9255.106 kcal/kg and yield 3.6 ml.

Further research should be done having macroalgae and microalgae to compare the ratio of biodiesel production, chemical analysis and statistical significance. Increasing lipid content of macroalgae by means of genetic engineering can be a great option for future study. The cost of oil extraction from algae using chemicals is not satisfactory, it is needed to investigate how to reduce the cost or develop a cost effective mechanical extraction tool.

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APPENDIX

Appendix 1: Data Input for regression analysis of calorific value for algae biodiesel.

Microsoft office Excel -2007 was used for regression analysis.

Operating procedure:

Start → Microsoft Excel→ data → Data analysis → Input Y range (all values of Y column)→Input X range (all values of X_1 , X_2 , X_3, X_1X_2, X_1X_3, X_2X_3, X_1X_1, X_2X_2, and X_3X_3 columns)→confidence level 95% → Out put range (any place was clicked near input data at the same sheet)→ Residuals.

Following data was used for regression analysis of calorific value:

X1	X2	X3	X1*X2	X1*X3	X2*X3	X1*X1	X2*X2	X3*X3	Y (c)
118.8	0.7	60	83.16	7128	42	14113.44	0.49	3600	9167.173
198	0.7	60	138.6	11880	42	39204	0.49	3600	9153.926
118.8	0.8	60	95.04	7128	48	14113.44	0.64	3600	9108.31
198	0.8	60	158.4	11880	48	39204	0.64	3600	9219.738
118.8	0.75	55	89.1	6534	41.25	14113.44	0.5625	3025	9188.192
198	0.75	55	148.5	10890	41.25	39204	0.5625	3025	9242.961
118.8	0.75	65	89.1	7722	48.75	14113.44	0.5625	4225	9132.539
198	0.75	65	148.5	12870	48.75	39204	0.5625	4225	9255.106
158.4	0.7	55	110.88	8712	38.5	25090.56	0.49	3025	9158.281
158.4	0.8	55	126.72	8712	44	25090.56	0.64	3025	9151.03
158.4	0.7	65	110.88	10296	45.5	25090.56	0.49	4225	9143.935
158.4	0.8	65	126.72	10296	52	25090.56	0.64	4225	9174.205
158.4	0.75	60	118.8	9504	45	25090.56	0.5625	3600	9169.471
158.4	0.75	60	118.8	9504	45	25090.56	0.5625	3600	9169.471
158.4	0.75	60	118.8	9504	45	25090.56	0.5625	3600	9169.471

Where, X_1= Chloroform (ml), X_2= NaCl (%), X_3= temperature (°C) and Y= Calorific value.

Following data was used for regression analysis of yield:

X1	X2	X3	X1*X2	X1*X3	X2*X3	X1*X1	X2*X2	X3*X3	Y (y)
118.8	0.7	60	83.16	7128	42	14113.44	0.49	3600	3
198	0.7	60	138.6	11880	42	39204	0.49	3600	3.4
118.8	0.8	60	95.04	7128	48	14113.44	0.64	3600	2.9
198	0.8	60	158.4	11880	48	39204	0.64	3600	3.4
118.8	0.75	55	89.1	6534	41.25	14113.44	0.5625	3025	3.1
198	0.75	55	148.5	10890	41.25	39204	0.5625	3025	3.6
118.8	0.75	65	89.1	7722	48.75	14113.44	0.5625	4225	3
198	0.75	65	148.5	12870	48.75	39204	0.5625	4225	3.5
158.4	0.7	55	110.88	8712	38.5	25090.56	0.49	3025	3.2
158.4	0.8	55	126.72	8712	44	25090.56	0.64	3025	3.1
158.4	0.7	65	110.88	10296	45.5	25090.56	0.49	4225	3.2
158.4	0.8	65	126.72	10296	52	25090.56	0.64	4225	3.2
158.4	0.75	60	118.8	9504	45	25090.56	0.5625	3600	3.3
158.4	0.75	60	118.8	9504	45	25090.56	0.5625	3600	3.3
158.4	0.75	60	118.8	9504	45	25090.56	0.5625	3600	3.3

Where, X_1= Chloroform (ml), X_2= NaCl (%), X_3= temperature (°C) and Y= Yield.

Appendix 2: Summary output of regression analysis for calorific value of algae biodiesel.

Output of regression analysis for calorific value:

Regression Statistics	
Multiple R	0.973142
R Square	0.947005
Adjusted R Square	0.851614
Standard Error	15.21752
Observations	15

Output of regression analysis for yield:

Regression Statistics	
Multiple R	0.988212
R Square	0.976563
Adjusted R Square	0.934375
Standard Error	0.05
Observations	15

Appendix 3: ANOVAs

ANOVA for calorific value:

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	9	20690.71	2298.968	9.927617	0.010527
Residual	5	1157.865	231.573		
Total	14	21848.58			

ANOVA for yield:

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	9	0.520833	0.05787	23.14815	0.001477
Residual	5	0.0125	0.0025		
Total	14	0.533333			

Appendix 4: Coefficients of X variables

Parameter (coefficient)	Calorific value (c)	Yield value (y)
	coefficient	coefficient
Constant (A_0)	9680.969	-19.25
$X_1(A_1)$	- 20.1792	- 0.00347
$X_2(A_2)$	11836.46	66.5
$X_3(A_3)$	- 114.098	- 0.0775
$X_1 X_2(A_4)$	15.74179	0.012626
$X_1 X_3(A_5)$	0.085604	– 2.6E-18
$X_2 X_3(A_6)$	37.521	0.1
$X_1 X_1(A_7)$	0.012962	1.12E-19
$X_2 X_2(A_8)$	- 11004.2	- 50
$X_3 X_3(A_9)$	0.59609	2.25E-17

Appendix 5: Determination of predicted value

Coefficients (A_1, A_2, A_3 . . .) and their corresponding X (X_1, X_2, X_3 . . .) variables were used for construction of 2nd order polynomial equation; it was used for determination of predicted values. Total 15 predicted values were obtained for each response. General equation was as follows

$$Y= A_0+ A_1X_1+ A_2X_2 + A_3X_3+ A_4X_1X_2 + A_5X_1X_3+ A_6 X_2X_3+ A_7X_1^2+ A_8X_2^2+ A_9X_3^2 \quad \text{.....(1)}$$

For calorific value:

$$Y(c) = 9680.969 - 20.1792X_1 + 11836.46X_2 - 114.098X_3 + 15.74179X_1X_2 + 0.085604X_1X_3 + 37.521\ X_2X_3 + 0.012962X_1^2 - 11004.2X_2^2 + 0.59609X_3^2 \quad \text{..... (2)}$$

For yield:

$$Y(y) = -19.25 - 0.00347X_1 + 66.5X_2 - 0.0775X_3 + 0.012626X_1X_2 - (2.6E\text{-}18)X_1X_3 + 0.1\ X_2X_3 + (1.12E\text{-}19)X_1^2 - 50X_2^2 + (2.25E\text{-}17)X_3^2 \quad \text{..... (3)}$$

Where Y(c) is calorific value and Y(ŷ) yield value, X_1, X_2 and X_3 are coded values for chloroform, NaCl and temperature respectively.

Appendix 6: Contour plot construction

Sigma plot software was used for construction of contour plot.

6.1 Contour plot showing calorific value at various NaCl concentrations and chloroform at a constant temperature of 65°C.

chloroform	NaCl	C V
118.8	0.7	9139.52
118.8	0.727	9151.466
118.8	0.75	9148.988
118.8	0.775	9133.089
118.8	0.8	9103.435
138.6	0.7	9134.386
138.6	0.727	9154.748
138.6	0.75	9159.438
138.6	0.775	9151.332
138.6	0.8	9129.47
158.4	0.7	9139.416
158.4	0.727	9168.193
158.4	0.75	9180.052
158.4	0.775	9179.738
158.4	0.8	9165.668
178.2	0.7	9154.609
178.2	0.727	9191.802
178.2	0.75	9210.83
178.2	0.775	9218.308
178.2	0.8	9212.03
198	0.7	9179.965
198	0.727	9225.574
198	0.75	9251.77
198	0.775	9267.04
198	0.8	9268.555