PRODUCTION OF BIODIESEL BY LIPASE CATALYSED TRANSESTERIFICATION OF *Acacia nilotica* SEED OIL

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DEPARTMENT OF BIOCHEMISTRY,
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MAY, 2016

DECLARATION

I declare that the work entitled "Production of Biodiesel by Lipase Catalysed Transesterification of *Acacia nilotica* Seed Oil" has been performed by me in the Department of Biochemistry under the supervision of Professor S. Ibrahim, Professor I. A. Umar and Dr. A. B. Sallau. The information derived from the literature has been acknowledged in the text and in the list of references. No part of this thesis was previously presented at any University for another degree.

---------- ---------- ----------

Name of Student Signature Date

CERTIFICATION

This thesis entitled **"PRODUCTION OF BIODIESEL BY LIPASE CATALYSED TRANSESTERIFICATION OF A*cacia nilotica* SEED OIL"** by Auwalu GARBA meets the regulation governing the award of the degree of Doctor of Philosophy (Ph.D) of the Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

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ABSTRACT

Biodiesel is becoming prominent among the alternatives source of energy due to its economic and environmental values. This work investigated the possibility of using calcium alginate immobilized lipase from *Pseudomonas aeruginosa* and response surface methodology to optimize the production of biodiesel from *Acacia nilotica* seed oil. The physicochemical properties of both *A. nilotica* seed oil and the biodiesel produced were assessed using standard procedure. The purified lipase from *P. aeruginosa* was immobilized. Both the purified and immobilized lipases were characterized. The oil was yield (13.5%), specific gravity (0.84), refractive index (1.34), viscosity (4.03 Pa. s) calorific value (29.0) MJ/ kg, cetane number (32.9), iodine value (184.0 g I_2/ 100 g), acid value (6.25) mg KOH/g, saponification value (189.0 mg KOH/ g) and peroxide value (6.47 meq/kg). A maximum lipase production was achieved after 60 hours of incubation with activity of 8.6 U/ml. The lipase purified by ion exchange chromatography and gel filtration had a specific activity of 147.7 U/mg proteins with 29.8 % recovery and purification fold of 4.63. The molecular weight was found to be 32 kDa as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis. The K_m and V_{max} were found to be 0.07 $mmole^{-1}$ and 55.5 µmole/ min respectively. The respective temperature and pH optima of the lipase were in the range of 45°C and 50 °C and pH range of 7.5 and 8.0. The enzyme was activated in the presence of Ca^{2+}, Mg^{2+}, gum arabic, 2-mercaptoethanol, dithiotreitol, *n*-hexane and methanol with the residual activity of (110.0, 92.3, 104.4 , 90.2, 102.2, 112.3 and 103.4%) respectively, but it was inactivated by Mn^{2+}, Ni^{2+}, CTAB, S.D.S, EDTA, tween-80, triton -X100, ethanol, iso-propanol and butanol (23.8, 26.1, 42.2, 44.6, 34.2, 47.5, 62.9, 26.1, 49.9 and 28.1%) respectively. The immobilized lipase had pH and temperature optima in the range of 7.5 to 8.5 and 45 to 55°C, respectively and was stable at pH (7.5 to 8.5) and the temperature range of 45°C to 60°C. The K_m and V_{max} were found to be 0.12 mM and 50.0 µmol/ min respectively. The enzyme was activated in the presence of Mg^2, Ca^{2+} and *n*-hexane, and inactivated by Mn^{2+}, Ni^{2+}, ethanol, iso-propanol, butanol and EDTA. From the analysis of the model developed, the quadratic coefficients of lipase had significant ($p<0.05$) effects on the biodiesel production. All other linear coefficients were not significant ($p>0.05$) except for incubation time. Verification of experiments confirmed the validity of the predicted model and maximum yield of biodiesel (55.9%) was obtained with oil/methanol ratio (1:10), *n*-hexane (1.20 ml), immobilized lipase (64 U/ ml) in 195 minutes. The methyl esters composition indicated the presence of octadecenoic (69.14%), hexadecanoic (15.47%) and (7.92%) octadecanoic acid as dominant fatty acid methyl esters. Other properties of the biodiesel produced include specific gravity (0.84), refractive index (1.34±0.04), viscosity (3.73±0.15 , cetane number (32.81±0.10), calorific value (29.0), flash point (113°C), cloud point (1.06°C) and pour point (4.0°C) as well as iodine value (187.6±0.60), acid value (5.61±0.01), saponification value (189 ±1.00) and peroxide value (6.60±0.05), which were largely in conformity with the ASTM and EN biodiesel standards. The ability of immobilized lipase to be repeatedly used makes the production of biodiesel from *Acacia nilotica* seed oil cost effective.

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LIST OF ABBREVIATIONS

ANOVA----------Analysis of Variance

AOAC----------Association of Analytical Chemists

ASTM----------American Standard of Testing Materials

BSA---------- Bovine Serum Albumin

CCD---------- Central Composite Design

CTAB----------Cetyl trimethyl ammonium bromide

CV---------- Coefficient of Variance

DEAE– ---------- Diethyl amino ethyl

DMSO---------- Dimethyl sulfoxide

E----------Enzyme

EC----------Enzyme Commission

EDTA----------Ethylene diamine tetraacetic acid

EN----------European Standard

FAME----------Fatty acid methyl ester

IU----------International Unit

kDa----------Kilodalton

K_m----------Michaelis constant

n-H----------*n*-Hexane

NOX----------Nitrogen oxides

OMR----------Oil methanol ratio

pI ----------Isoelectric point

PM----------Particulate matter

PMSF----------Phenyl methane sulfonyl fluoride

PNP----------Para nitrophenol

*p*NPL----------Para nitrophenyl laurate

*p*NPP----------Para nitrophenyl palmitate

POME----------Palm oil mill effluent

Rf----------Relative mobility

RSM----------Response Surface Methodology

SDS----------Sodium dodecyl sulphate

SDS-PAGE----------Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SmF----------Submerged fermentation

Sqrt----------Square root

SSF----------Solid state fermentation

T----------Time

T1SS----------Type I Secretory System

T2SS----------Type II Secretory System

TEMED----------Tetramethyl ethylene diamine

TSS----------Total suspended solid

V_{max} ----------Maximun velocity

CHAPTER ONE

1.0 INTRODUCTION

The increase in the demand for energy has led to the search for alternative energy sources**.**The world production of biodiesel increased threefold between 2000 and 2005 with the estimated production of 2.92 million tones in 2005 and indicating that share of biodiesel in global fuel production will increase in future (Mousdale, 2008). Biodiesel, a renewable, biodegradable, non-inflammable and non-toxic fuel is of considerable value as a substitute to petro-diesel (Hoda *et al*., 2014). Renewable energy is an alternative option to reduce the energy crisis, gain energy security and sequester high amounts of carbon in the atmosphere (Arthe *et al*., 2008). Biodiesel has lower sulfur, aromatic content, higher cetane number and flash point than petroleum diesel (Al-Zuhair 2007; Ranganathan *et al*., 2008). It is derived from natural renewable biological sources such as vegetable oil and animal fats and is used for diesel engines and heating systems (Anjana *et al.*, (2000). One of the advantages of biodiesel as compared to other biofuels is that it can be pumped, stored and handled using the same equipment used for petro-diesel fuel (Robles-Medina *et al*., 2009). Biodiesel as an alternative fuel is similar to petro-diesel, therefore it can be used as such or blended with petro-diesel with no need of modification of the existing engines (Bozbas, 2008).

Transesterification is an equilibrium and reversible reaction during which oil is converted to fatty acid alkyl esters and glycerol (Kafuku and Mbarawa, 2010; Ito *et al*., 2012; Balakrishnan *et al.*, 2013; Atapour *et al.*, 2014). In the process an alcohol such as methanol is reacted with the free fatty acids released from triacylglycerols contained in vegetable oils, animal fats or recycled greases, forming biodiesel (fatty acid alkyl esters) and glycerol. The use of a catalyst is necessary

as it improves the solubility of the feed stock and accelerates the rate of transesterification reaction (Atabani *et al.,* 2012). A number of processes have been developed for biodiesel production involving chemical or enzyme catalysis or supercritical alcohol treatment (Kusdiana and Saka, 2004; Warabi *et al.,* 2004). Enzymatic transesterification of triacylglycerol is a good alternative to chemical processes due to its eco-friendliness, selective nature and low temperature requirements (Du *et al*., 2004). Methanolysis catalysed by lipase has become more attractive in biodiesel production since it is considered to be an effective way to overcome the drawbacks involved in the chemical process such as difficulties in removal of by-products and the tendency to form soap (Ha *et al*., 2007). The use of enzymes allows the conversion of free fatty acids contained in the fat or oil to fatty acid alkyl esters completely with recovery of glycerol as by-product (Modi *et al*., 2007). There are many reports on biodiesel production by using free or immobilized lipases (Watanabe *et al.,* 2000; Kamini and Iefuji, 2001). Immobilized lipase is suitable for continuous biodiesel production because of the ease of its recovery from the reaction mixture and reutilization which can reduce the cost of purification and immobilization of enzymes.

A number of feedstocks such as rapeseed oil (Nelson *et al*,. 1996), palm oil (Abigor *et al*., 2000), sunflower oil (Belafi *et al*., 2002), cotton seed oil (Dossat *et al*., 2002) and restaurant kitchen wastes (Hsu *et al*., 2002) have been used for production of biodiesel. In most developed countries biodiesel is produced from rapeseed, sunflower, soy beans, and peanuts which are essentially edible (Samukawa *et al*., 2000). The use of edible oil is not suitable for a resource poor developing countries owing to its effect on food security. More research is targeted towards finding alternative non edible oil based renewable fuel and hence the oil of *Acacia nilotica* seed that is non-edible has been chosen for the present investigation. In this work *Pseudomonas*

aeruginosa a ubiquitous gram negative bacterium that grows in soil, water, plants, animal tissues and other places that contain moisture isolated from oily environment was chosen because the lipase it produces has activity over a wide range of temperature, pH, organic solvents and possesses random specificity. The isolated *P. aeruginosa* was induced to produce lipase which was then partially purified and characterized. The partially purified lipase was immobilized by entrapment on calcium alginate beads and used for transesterification of *A.nilotica* seed oil to produce biodiesel.

1.1 Statement of Research Problem

i. Contemporary biodiesel is produced from edible oil which poses a threat to national and global food security hence, there is the need to look for non-edible sources of oil for biodiesel production.

ii. The use of petro- diesel leads to emission of greenhouse gases which is correlated with global warming.

iii. The energy intensive nature of biodiesel production from vegetable oil by chemical methods makes the use of enzymatic method that does not require much energy a chosen method for this work.

iv. The production of biodiesel from oil by using soluble lipase is capital intensive because of its inactivation by alcohol and glycerol by–product

v. *Acacia nilotica* seed oil has been studied for its chemical and nutrients composition but very little is known about its biodiesel production potential.

1.2 Justification

It is necessary to look for an alternative fuel which can be produced from materials available within the country endowed with arable land that supports the growth of oil producing plants.Biodiesel is less polluting and is safer to handle than petroleum diesel because it is biodegradable and non-toxic, so its usage as alternative fuel may help to address the problems of environmental concerns such as global warming. Thus, biodiesel has the potential of lowering the net gas emissions from the transportation sector; that causes global warming and it could significantly decrease the mass and carcinogenicity of particulate matter emissions (Idris, 2016).The biodegradability of biodiesel can be compared with sugar and its less toxicity with salt.

It is pertinent to have a sustainable way of boosting indigenous economy by applying principles of biochemistry and biotechnology to utilize the immobilized lipase from *P. aeruginosa* which overcomes the drawbacks associated with biodiesel production from readily available raw materials such as *A. niloticas*eed oilby chemical method

1.3. Aims and Objectives

1.3.1 General Aim

The aim of the study was to produce biodiesel by *P.aeruginosa* lipase catalysed transesterification of *A. nilotica* seed oil.

1.3.2 Specific Objectives.

The specific objectives of this work are to;

i. Extract and characterize oil from *A. nilotica* seed

ii. Isolate, purify and characterize lipase from *P. aeruginosa*.

iii. Characterize the immobilized lipase

iv. Optimize biodiesel production by lipase catalysed transesterification of *A. nilotica* seed oil

v Purify and characterize the biodiesel produced.

1.4 Null Hypothesis

Biodiesel cannot be produced by lipase catalysed transesterification of *A. nilotica* seed oil

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 History of Biodiesel

The diesel engine was invented by German engineer Rudolf Diesel (1858-1913). Dr Diesel demonstrated his engine at the exhibition fair in Paris, France in 1898, which stood as an example of Diesel's vision because it was fueled by coal dust suspended in water, heavy mineral oil and vegetable oils. He thought the future of his engine was the utilization of a biomas based fuel and hoped that it would provide a way for the smaller industries and farmers to compete with the monopolizing industries which controlled all energy production at that time, as well as serve as an alternative for the inefficient fuel consumption of the steam engine (Oliver, 2012).

Modern biodiesel which is made by converting triacylglycerols into fatty acid alkyl esters originated from the research conducted in Belgium in 1930's, but today's biodiesel industry was established in Europe in late 1980s. Due to the widespread availability and low cost of petro-diesel, vegetable oil-based fuels gained little attention, except in times of hike in the oil prices and its shortages. Interest in using vegetable oils to fuel diesel engines was intensified during the Second World War and the oil crises of the 1970's. The design of the newer diesel engine does not allow them to run on traditional vegetable oil owing to the higher viscosity of vegetable oil as compared to petro-diesel. A mechanism was therefore needed to reduce the viscosity of vegetable oils so that they could be burnt completely in the diesel engine. In 1937 a Belgian inventor first proposed the use of transesterification reaction to convert vegetable oils into fatty acid alkyl esters which forms the basis for the production of modern biodiesel. Pioneering work in Europe and South Africa by Martin Mittelbach extended the development of the biodiesel fuel industry in the early 1990s with the U.S industry coming on more slowly, due to lower prices for

petro-diesel. The terrorist attacks of 9/11/2001 in the U.S that resulted in high oil prices and increased energy security awareness made the biodiesel industry to become a household name. The production of biodiesel had reached 1.1 billion gallons as at 2005 with most fuel being produced in the European Union though biodiesel projects worldwide have been on the rise due to rising crude oil prices and concerns over global warming (www.history of biodiesel_fuel.htm).

2.2 Biodiesel

Biodiesel has arisen as an alternative to petroleum diesel because of its similarities with it (Fjerbaek *et al*., 2009; Demirbas, 2009). It has many advantages over petroleum diesel such as being non-toxic, biodegradable, renewable, does not contribute to net accumulation of the green house gases (Bajpai and Tyagi, 2006). One of the biggest advantages of biodiesel compared to many other alternative fuels is that it can be used in existing diesel engines without modification, and can be blended in at any ratio with petroleum diesel. Schumacher *et al*., (2001); Demirbas (2009) reported that biodiesel results in a 45% reduction in total hydrocarbon emissions,47% reduction in CO emissions and 66% reduction in particulate matter (PM) emissions. Even though the biodiesel environmental considerations are very positive, it increases nitrogen oxides (NOX) emissions. However, reports show that reduction in (NOX) emission is possible with some modifications in combustion temperature and injection timing (Hess *et al*., 2005). The direct use of vegetable oil has several negative effects such as their high viscosity and low volatility which lead to incomplete combustion in diesel engines and eventual carbon deposition (Basha *et al*., 2009).

2.3 *Acacia nilotica*

A. nilotica (L.) is a thorny wattle native to India, Pakistan and Africa. Nine subspecies are recognised (Brenan, 1983). It belongs to the Family: Leguminosae/ Fabaceae–Mimosoideae and Genus: *Acacia*. It is about 7 to13 m tall with a stem diameter of 20 to 30 cm. The crown is low, spreading and almost symmetrical and can be flattened or a rounded umbrella-shape (in free standing specimens). The bark is very dark brown to black with deep regular vertical grooves in older specimens. The thorns are straight, paired at the nodes of the stem and usually pointing slightly backwards. The leaves are bipinnate 4.5 to 7cm long, with 2 to14 pairs of pinnae. The trees generally lose their leaves during the dry season, though riverine subspecies can be almost evergreen. *A. nilotica* seeds contain proteins, fats, nitrogen freed extract, metabolisable energy, tannin, phosphorus, calcium, magnesium, potassium, silicon, chlorine, copper, zinc, manganese and iron.

In Africa and the Indian subcontinent *A. nilotica* is extensively used as timber and firewood. The bark and seeds are used as a source of tannins (New, 1984). The species is also used for medicinal purposes. Bark of *A. nilotica* has been used for treating haemorrhage, cold, diarrhoea, tuberculosis and leprosy while the roots have been used as an aphrodiasic and the flowers for treating syphilis lesions (New, 1984). The gum of *A. nilotica* is sometimes used as a substitute for gum arabic obtained from *A. senegal* (Gupta, 1970). The species is suitable for the production of paper and has similar pulping properties to a range of other plants (Nasroun, 1979).

2.3.1 Cultivation of *Acacia nilotica*

It grows in areas receiving less than 350 mm of rainfall to areas receiving more than 1,500 mm per annum. The species are reported to be very sensitive to frost, but will grow in areas where the

mean monthly temperature of the coldest month is 16°C (Gupta, 1970). Other sub species are restricted to riverine habitats and seasonally flooded areas. It is also tolerant to saline soil and can be propagated from truncheons, root suckers and cuttings. It is easily established from seed, but it needs scarification (the hard seed coat must be subjected to mechanical abrasion or hot water treatment) especially if the seeds are not fresh. The seedlings need full exposure to sunlight, free draining soil and is moderately long-lived**.** The image of the pods of *A.nilotica* is shown in plate 2.1 below



Plate 2.1 Image of *Acacia nilotica* Pods (Kiran and Bargali, 2009)

2.4 Methods of Oil Extraction

The first step to be taken in the refining process of oil is its extraction. It is extracted from animals, fruits such as olive and palm and oil seeds such as cotton seed, sesame seed, soybean and peanuts. Several methods have been used for extraction of oil from seeds ranging from traditional method, manual method, mechanical presses, sohxlet extraction, ultrasound- assisted oil extraction and supercritical fluid extraction

2.4.1 Traditional Methods

It is the commonest method used for oil extraction from coconut, olive, palm fruit and shea butter. The traditional method of oil extraction from seed oil is performed by pressing a rotating wood which is fitted in the centre of the container and therefore serves as a compressor. During the crushing of the nuts warm water is put into the machine which enables the juice to seep through the bore holes of the container into the basin. The juice is boiled until the water evaporates (Nyanjou, 2008).

2.4.2 Manual Method

In manual method, screw pressers are usually used to extract oil from pre-ground oil seeds. This entails the use of a ghanis which consists of a pestle and mortar that is rotated by animal power or by a motor. Seed is fed into the mortar which breaks the cells and the eventual release of oil due to pressure exerted by the pestle.

2.4.3 Mechanical Extraction

In mechanical extraction of the oil, no chemical is used. The use of conventional hydraulic presser equipment has been replaced by continuous screw-presser (Bargale, 1997). Mechanical extraction of oil is brought about by exerting high pressure on the seed to rupture the cells and allows the extraction of oil from the seed. Extraction is achieved by compressing the material in a container with small perforation to allow the liquid component to be expressed (Antony *et al*., 2011). The extruders in mechanical extraction provide enough pressure and temperature on seeds to deactivate enzymes (Gerpen *et al*., 2004). Inekwe *et al*., (2012) subjected *Jatropha curcas* seeds to mechanical pressing and oils were extracted without application of solvent. Jung and Mahfuz (2009) used a dry extruder with high temperature for extraction of oil and protein.

2.4.4 Soxhlet Extraction

Oil can be extracted from the seeds through repeated washing or percolation using organic solvent such as hexane, petroleum ether etc under reflux in glassware (Ana-Godson and Udofia-Bassey, 2015). The soxhlet extractor is made up of a glass reservoir fixed between a flask at the bottom and a condenser at the top. At the centre of the extractor is a thimble which contains the seed sample for extraction. In solvent extraction of oil from seeds, *n*-hexane is extensively used because of its low vapourization temperature, high stability, low corrosiveness and low greasy residual effects (Seth *et al.,* 2007). Kovo and Bawa (2007) extracted oil from *A. nilotica* seed by soxhlet extraction with a combination of different solvents: petroleum-ethanol, ether-acetone, chloroform–methanol. Tissouras *et al*., (2013) extracted oil from *A.nilotica* seed by soxhlet extraction using n-hexane. Myriam *et al.,* (2012) extracted oil from soybean seeds with *n*-hexane in a fixed bed extractor.

2.4.5 Ultrasonic-Assisted Extraction

Ultrasound assisted extraction allows better yield and a lower extraction temperature as compared to the conventional method. The ultrasound/microwave extraction technique reduces the extraction time and solvent amounts and produces higher extraction efficiency with less environmental impact (Cravotto *et al.,* 2008). Luthria *et al.,* (2007) used ultrasonication technique for oil extraction from soybean. Lou *et al*. (2010) reported the use of ultrasonication method to extract oil from Chilean chickpea.

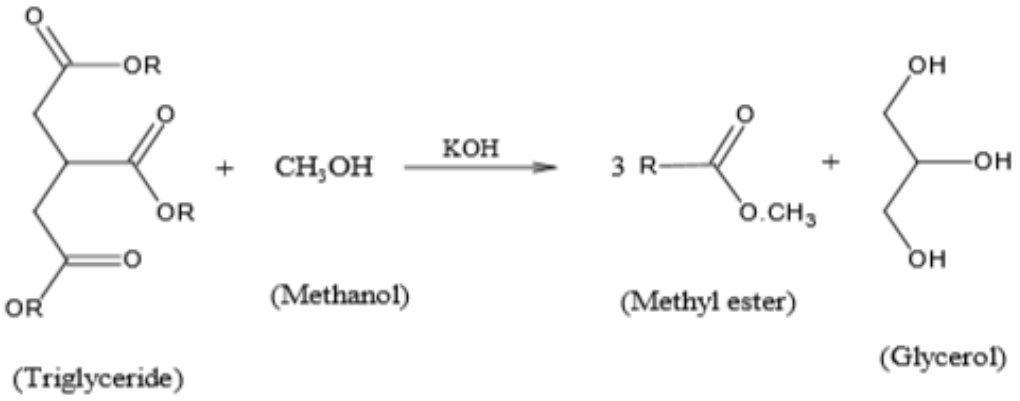
2.4.6 Supercritical Fluid Extraction

In supercritical fluid extraction, supercritical carbon dioxide is utilized to extract oil from seeds where temperature and pressure are the main factors affecting supercritical fluid extraction (Salgin, 2007). Zaidul *et al*. (2007) used supercritical carbon dioxide for extraction of oil from palm kernel. Salgin (2007) used supercritical CO_2 and supercritical ethanol mixture for oil extraction from *Jojoba* seeds.

2.5. Transesterification of Vegetable Oil

Transesterification is the method used for the transformation of triacylglycerol into biodiesel. It entails the reaction between triacylglycerol and an acyl-acceptor (Fan, 2012). The acyl-acceptor could be either carboxylic acids, alcohols or another ester. Transesterification produces glycerol when alcohol is used as acyl-acceptor or triacylglycerol when ester is used as acyl group donor (Robles-Medina *et al*., 2009). Dubé, *et al*., (2007) reported that stoichiometrically for each mole of triacylglycerol three moles of alcohol are required. A higher molar ratio of alcohol is used in order to achieve maximum biodiesel production (Chew, 2008). Methanol, ethanol and propanol

are the most commonly used alcohols. The yield of biodiesel is independent on the type of alcohol used and selection of alcohol used in biodiesel productionis dependent on itscost (Sharma *et al*., 2008). Typical transesterification reaction scheme is shown in Equation 2.1 (Eze *et al.*, 2010)



--------(2.1)

2.6 Feedstock for Transesterification

A number of feed stocks have been utilized for transesterification processes and the commonly used feedsocks are given below

2.6.1 Vegetable Oils and Animal Fats

All types of vegetable oils and animal fats can be used as feedstock in the transesterification process. Vegetable oils and animal fats contain triacylglycerols which are useful in the transesterification process (Sharma *et al*., 2008). Vegetable oils and animal fats that are used for the transesterification process are divided into edible and non-edible. The most common edible vegetable oils and animal fats used in the transesterification process are soybean oil (Liu *et al.*, 2008), sunflower oil (Granados *et al*., (2007), palm oil (Jitputti *et al*., 2006) and coffee oil (Oliveira *et al*., 2008). The non-edible oils that are commonly used in the transesterification

process are waste oil (Ozbay *et al*., 2008), Jatropha oil (Tamalampudi *et al.*, 2008) and algae oil (Christi, 2007).

2.6.2 Alcohol

Primary alcohols such as methanol and ethanol are commonly used in the transesterification reaction as a result of their better reactivity in comparison with higher order alcohols. However, higher order alcohols such as propanol and butanol could also be used depending on their suitability and compatibility (Sharma *et al.*, 2008). Royon *et al.*, (2007) reported the use of higher order alcohols not only as feedstock but also as solvent which could enhance the transesterification process.

2.7 Types of Transesterification Reaction

There are basically two methods of biodiesel production by transesterification (Haas *et al*., 2006) The first is the chemical method which is alcoholysis of oil by methyl or ethyl alcohol in the presence of strong acid or strong base (Fukuda *et al.*,2001*)* and the enzymatic method which requires the use of lipases.

2.7.1Chemical Transesterification

Chemical transesterification entails the use of a strong acid or strong base and is efficient in terms of reaction time. However, it is pertinent to select an appropriate catalyst

2.7.2 Base Catalysed Transesterification

A base catalyst has a pH value greater than 7.0 with ability to accept electrons. Sodium hydroxide (NaOH), potassium hydroxide (KOH) and sodium methoxide (CH_3ONa) are the commonly used homogeneous base catalysts for transesterification (Helwani *et al*., 2009). The ability to be carried out at low temperature and pressure with high conversion (98%) within a short time makes a base catalyzed process to be commonly used (Fukuda *et al*., 2001). The presence of free fatty acids promotes soap formation which consumes the catalyst, lowers the yield and results in difficult down stream processing and product purification (Al-Zuhair, 2007).

2.7.3 Acid Catalyzed Transesterification

Sulphuric, sulphonic, phosphoric and hydrochloric acids are the most commonly used acid catalysts for transesterification (Meher *et al*., 2006). Acid catalyzed transesterification reaction is not as popular as the base-catalyzed transesterification because of the corrosive nature of strong acids and the reaction proceeds at slow rate.Akoh *et al*., (2007); Al-Zuhair *et al*., (2009) reported that acid catalyzed transesterification reactions may be 4000 times slower than the base catalysed reactions. The advantage of acid catalyzed processes is that it is not influenced by free fatty acid content of the feed stock because there is no soap formation due to the presence free fatty acids, hence biodiesel can be produced from low cost feedstocks (Zhang *et al*., 2003).

2.7.4 Enzyme Catalysed Transesterification

Enzymes are biological catalysts which allow many chemical reactions to occur within the homeostasis constraints of living system. Large scale application of enzymes have been reported in the production of detergents, drinks, textiles, starch hydrolysis, fructose production, semi synthetic penicillin, leather, pulp, baking and lipase based reactions (Kudli-Shrinivas, 2007).

Enzymatic approach for biodiesel production has not received much commercial attention except in China with the first industrial scale lipase catalysed biodiesel production with the capacity of 20000 tons annually. The use of enzyme to produce biodiesel has the advantage of having low operating conditions, high product purity and can be carried out at 35°C to 45°C (De-Paola *et al*., 2009). The slow rate of reaction and enzyme inactivation by organic solvents are the major disadvantages of enzymatic transesterification reaction (Jegannathan *et al.,* 2008). Annapurna*et al*., (2009) reported enzymatic transesterification of *Jatropha* oil using immobilized lipase from *Enterobacter aerogenes.* Chien-Hung *et al*., (2012) reported biodiesel production by enzymatic transesterification catalyzed by *Burkholderia* lipase immobilized on hydrophobic magnetic particles. Transesterification of mahua oil with immobilized lipase from *Pseudomonas cepacia* was reported by Kumari *et al*., (2007).

2.8 Lipases: An Overview

Lipases (EC 3.1.1.3) belong to the family of hydrolases that liberate fatty acids and glycerol in aqueous environment upon their actions on the carboxyl ester bonds present in triacylglycerols (Gupta and Rathi, 2004). They are carboxylesterases that catalyze the hydrolysis, esterification and transesterification of acylglycerols with acyl chains having more than ten carbon atoms at oil-water interface (Ranjitha *et al*., 2009) ; (Treichel *et al.,* 2010). Lipases have an α/β-hydrolase fold, a conserved catalytic triad Ser, His, Asp/Glu and around it, lies a consensus sequence Gly-x-Ser-x-Gly (Gupta and Rathi, 2004). The catalytic triad is completely buried by a short helical segment or lid. When lipase comes in contact with an interface, the lid opens up to create an electrophilic region around the serine residue by restructuring of the lipase which results in

exposing the hydrophobic residues, burrying the hydrophilic ones.and eventual stabilization of the transition state intermediate during catalysis (Stamatis *et al*., 1999).

2.8.1 Nomenclature of Lipase

Lipases (EC 3.1.1.3) being a member of group of hydrolases are placed in enzyme class 3.1 according to the classification recommended by the Enzyme Commission of the International Union of Biochemistry. Lipases which hydrolyze esters of fatty acids are named triacylglycerol; carboxyl ester hydrolases and classified as 3.1.1. They are further classified according to the substrates upon which they act. Most of the lipases act on esters of glycerol which is the molecular backbone of most of the lipid material occurring in nature. Glycerol ester hydrolases are classified as EC 3.1.1.3 (Nelson and Cox, 2005).

2.8.2 Sources of Lipases

In animals, lipases are mainly obtained from pancreatic tissues of hogs, pigs and fore-stomach tissue of calves or lambs. The presence of trypsin in pig pancreatic lipases which results in bitter tasting amino acids and the presence of residual animal hormones or viruses limits the use of animal lipases (Vakhlu and Kour, 2006). The yield and the processes involved have limited commercial use of plant lipases. Microorganisms serve as important source of lipases owing to their technical and economical advantages coupled with their high yield (Srivastava, 2008). Microorganisms are cultivated on inexpensive medium containing appropriate nutrient composition under controlled conditions. The strain, the composition of the growth medium, cultivation conditions, pH and temperature are among the factors that influence microbial lipase production (Treichel *et al*., 2010).

2.9 Lipase Producing Micro Organisms

Lipase producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, oilseeds, decaying food, compost heaps, coal tips and hot springs (Sztajer and Maliszewska, 1989). The oily environment may provide a good environment for isolation of lipase producing microorganisms suggesting that a tremendous potential for identification of new sources of lipases with novel properties has been offered by nature. Many microorganisms are known as potential producers of extracellular lipases, including bacteria, yeast and fungi (Abada, 2008). Extracellular lipase has been isolated from many different bacterial species including *Bacillus* (Ertuğrul *et al*., 2007) and *Pseudomona s*sp. (Wang *et al*., 2009). The most lipase producing fungi genera are *Candida cylindracea, Aspergillus, Rhizopus, Penicillium, Mucor, Geotrichum* and *Fusarium* (Salihu *et al.,* 2012); (D'Annibale*et al*., 2006). Ertugrul *et al*. (2007) isolated 17 bacterial strains capable of growing on media based on olive mill waste water and selected strain of *Bacillus sp.* as the best lipase producer by screening on tributyrin agar medium and obtained the lipase activity of 168 UL^{-1}. Kiran *et al*., (2000) isolated 57 heterotrophic bacteria from the marine sponge of which 37% produced a clear halo around the colonies on tributyrin agar plates for lipase production with the strain *Pseudomonas* MSI057 exhibiting larger clear zones around the colonies with maximum lipase activity of 750 Uml^{-1}obtained. Abada (2008) produced lipase from a strain of *B. stearothermophilus* AB-1 isolated from air and obtained a maximum lipase activity of 1585 U ml^{-1} after 48 hours of fermentation. Alkan *et al*., (2007) produced an extracellular lipase by *B. coagulans* and obtained a maximum lipase activity of 149 U gs^{-1} after 24 hours of fermentation. Vishnupriya *et al*., (2010) investigated lipase production by *Streptomyces grisesus* and obtained

maximum enzyme activity of 51.9 U/ml. Cihangir and Sarikaya (2004) isolated a strain of *Aspergillus sp*. from soil samples and obtained activity of 17 U ml^{-1}..

2.10 *Pseudomonas aeruginosa*

It is a gram negative, rod shaped monoflagillated bacterium about 1-5µm long and 0.5-1.5µm wide. It is an obligate aerobic organism that can also respire anaerobically on nitrate or other alternative electron acceptors. It can degrade a wide range of organic molecules which makes it ubiquitous. It is found in environments such as soil, water, human, animals, plants, sewage and hospitals (Lederberg *et al*., 2000). The genus *Pseudomonas* produces a number of extracellular enzymes including lipase which synthesis and secretion is influenced by a variety of environmental factors such as ions, carbon and nitrogen sources. Lipases from *P. aeruginosa* have a wide spectrum of substrate utilization, high stability towards extremes of temperature, pH, organic solvent and posses a random positional specificity (Gaur *et al*., 2008).

2.11 Secretion of Lipase by Bacteria

Bacterial lipases may be intracellular, membrane bound or extracellular. The production of intracellular lipase has been reported only in A.*B. clausii* strain (Lee and Park, 2008). Ertugrul *et al*., (2007) observed the production of both intracellular and extracellular lipase in *Bacillus* sp. Boekema *et al*., (2007) reported the production of extracellular lipase as consequence of secretion of accumulated intracellular lipase by membrane-bound chaperones. Different types of secretory systems are involved in the secretion of bacterial lipase into the external medium. The type I secretory system (T1SS) is coupled with an energy driven exporter complex comprising of three protein subunits. The type II secretory system (T2SS) has two components: the general

protein secretion (Sec-dependent pathway) and twin-arginine translocation (Tat-dependent pathway). In bacteria, lipases are secreted in the unfolded state into the periplasmic space through the Sec-dependent pathway where folding takes place with the help of chaperone called lipase specific foldase (Lif). The folded lipase is then transported into the external mediumby a transporter complex (Angkawidjaja and Kanaya, 2006: Buist *et al*., 2006).

2.12 Lipase Production by Solid State Fermentation

Solid state fermentation (SSF) entails the use of residues and by-products of agro industries as nutrient sources to support the microbial growth in the absence of water. The use of by products as substrates for lipase production due to their low cost may reduce the final cost of the enzyme production. Solid state fermentation allows the best reproduction of the natural microbial environment. It is more cost-effective, has lower water consumption, has reduced waste water treatment costs and lower energy consumption i.e no need to heat up water (Durand, 2003; Pandey, 2003). Menoncin *et al*., (2008) reported the production of lipase from *Penicillium simplicissimum* by solid state fermentation on babassu cake (a residue of the babassu oil production) supplemented with 6.25% (w/w) sugar cane molasses.

2.13 Lipase Production by Submerged Fermentation

Many studies have been carried out to define the optimal culture and nutritional requirements for lipase production by submerged fermentation. Submerged fermentation is ideal for the growing of unicellular organisms such as bacteria or yeasts. However, production of lipase needs large space, complex media, equipment and control systems to reduce the risk of contamination. Veerapagu *et al., (*2013) produced lipase from *Pseudomonas gessardii* by submerged

fermentation with peptone (0.2% w/v), $NH_4H_2PO_4$ (0.1% w/v), NaCl (0.25% w/v), $MgSO_4.7H_2O$ (0.04% w/v), $CaCl_2.2H_2O$ (0.04% w/v), olive oil (2.0 ml), 1-2 drops tween-80 as emulsifier. Salihu *et al.*, (2012) produced lipase from *Candida cylindracea*on palm oil mill effluent based medium containing POME sample (1.0%w/v) total suspended solids (TSS) supplemented with olive oil (0.2% v/v), tween-80 (0.65% v/v) and peptone (0.45% w/v), adjusting the initial pH to 6.0. Lipidic carbon sources seem to be essential for obtaining a high lipase yield. However, in the absence of fats and oils, a good yield has also been reported (Shimada *et al*., 1992).

2.14 Classification of Bacterial Lipases

Bacterial lipases are classified into eight families and the largest family is subdivided into six sub-families as reported by Arpigny and Jaeger, (1999). The conserved sequence motifs and biological properties of lipases formed the basis for their classification. Family I are considered as true lipases which comprise of *Pseudomonas*, *Bacillus* and *Staphylococcus* lipases. The lipases in family 1 have the catalytic pentapeptide Gly-Xaa-Ser-Xaa-Gly. Family II lipases have Gly-Asp-Ser-Leu motif at the active site which comprise of esterases of *Streptomyces*, *Aeromonas* and *Salmonella*. Family III comprises of lipases of *Streptomyces* sp which are also extracellular lipases. Family IV have similarity with mammalian hormone sensitive lipases. Family V comprises lipases from mesophilic bacteria like *P. oleovorans* and *Haemophilus influenza*. Family VI lipases are the smallest esterases and are dimeric. Family VII lipases are large esterases with amino acid sequence homologous to the eukaryotic acetylcholine esterases. Family VIII lipases are similar to β-lactamases. Some few lipases could not be grouped into any of the eight super families as such they are arbitrarily classified as new family 9 and 10. De-

Pascale *et al*., (2008) reported a cold active lipase to belong to a novel lipase family due to the fact they could not fit into the traditional classification.

2.15 Screening of Lipase Production

Several methods have been proposed for screening of lipase production. The methods either make use of the microorganism under study (Nair and Kumar, 2007) or measure the activity of lipase in the crude or purified sample (Singh *et al*., 2010). In plate detection method agar plates containing lipid substrate is used and lipase production is observed as clear halos or opaque zones around the well containing culture or enzyme preparation. Singh *et al*., (2006); Nair and Kumar (2007) reported the use of plates containing chromogenic substrates with pH indicators such as phenol red and Victoria blue to screen for lipase production. Kim and Hou (2006) reported the use of rhodamine B plates which indicate lipolysis by the formation of fluorescent orange halos around the colonies. The colorimetric methods are based on measuring the complexes formed between the released free fatty acids and a divalent metal ion, usually copper (Rahman *et al*., 2005). Spectrophotometric method is the widely used method which entails the use of synthetic substrates (paranitrophenyl esters of fatty acids) to assay for lipase activity by measuring the amount of paranitrophenol released upon hydrolysis of the substrate (Wang *et al*., 2009). The titrimetric method employs the neutralisation of the free fatty acids released in order to determine the extent of lipolysis from the volume of the base consumed to titrate the released fatty acids (Shukla *et al*., 2007).

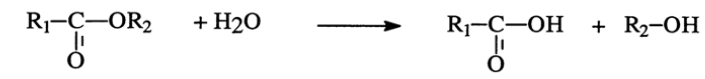
2.16 Reactions of Lipases

In addition to the hydrolytic reactions catalyzed by lipases, they can catalyze synthetic reactions such as esterification and transesterification in form of acidolysis, alcoholysis and inter esterification in the presence of only a small amount of water. Shimada *et al*., (2005) reported the activation of lipases at oil-water or air-water interfaces. Divakar and Manohar (2007) grouped the reactions catalyzed by lipases into three important types:

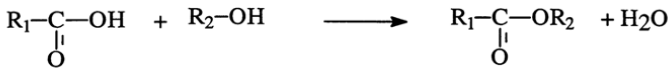
Hydrolysis: In hydrolysis the dominant reaction is the cleavage of ester bonds which occurs in high water conditions and is employed in the production of fatty acids, monoacylglycerols, diacylglycerols, flavouring agents for dairy products, detergents for laundry and household uses. Esterification occurs under low water conditions and results in high yield of the esterified products. Reactions such as the formation of geranyl and menthyl esters from butyric acid and geranol or lauric acid and menthol involve esterification (Marlot *et al*., 1985).

Transesterification occurs when two or more compounds exchange acid moiety. The reaction is called acidolysis when a free acid is the donor of the acyl group and interesterification if the acyl donor is an ester. It is called alcoholysis when the acyl group acceptor is a nucleophilic alcohol (Macrae, 1985). Examples of some reactions catalyzed by lipase in aqueous and non-aqueous conditions is shown in Equation 2.2 (Villeneuve, *et al.,* 2000).

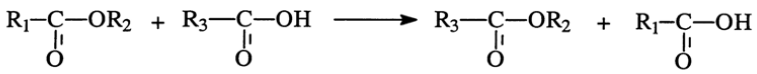
Hydrolysis :



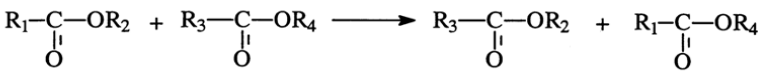
Ester synthesis :



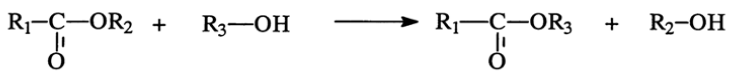
Acidolysis :



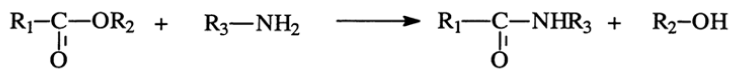
Interesterification :



Alcoholysis :



Aminolysis :



----------Equation 2.2:

2.17 Lipase Specificity

The specificity of lipase refers to its regio selectivity for specific position on the molecule of triacylglycerol. Based on the preference for acyl group positions in triacylglycerols lipases are classified into three: 1,3-specific that hydrolyze ester bonds in positions R1 or R3 of triacylglycerol, 2-specific that hydrolyze ester bond in position R2 of triacylglycerol and nonspecific or random specific that do not distinguish between positions of esters. Fjerbaek *et al*., (2009) reported that for biodiesel production from triacylglycerol, lipases should be non stereo-specific where all triacylglycerols, diacylglycerols and monoacylglycerols can be

converted to fatty acids methyl esters. The only 2- specific lipase is *Gotrichum candidum* lipase which is not commonly used for transesterification (Macrae, 1985).

2.18 Purification of Lipases

In order to achieve the maximum catalytic activity of enzyme for efficient industrial application, a certain degree of purity is desirable. Through the process of purification the three dimensional structure of enzymes and their structure-function relationships can be understood (Saxena *et al*., 2003). For purification of enzymes to homogeneity, a number of purification steps may be involved (Aires-Barros *et al*., 1994). A rapid, cheap, high yielding and amenable to large scale operations purification strategies is desired by production industries (Saxena *et al*., 2003).

2.18.1 Purification Strategies of Lipases

The removal of cell debris either by centrifugation or filtration aids in recovering lipases of microbial origin by virtue of being extracellular. The methods used for purification of lipases are nonspecific techniques, some of which are extraction, precipitation, hydrophobic interaction, chromatography, gel filtration, crystallization and ion exchange chromatography. Abbas *et al*., (2002) precipitated proteins from culture medium by adding ammonium sulfate up to 75% (w/v) saturation at 0^{o}C. Rakesh *et al.,* (2012) precipitated lipase from *B. pumilus* up to 90% saturation with ammonium sulphate followed by gel filtration and ion exchange chromatography on DEAE-cellulose. Neerupma *et al.,* (*2*006) precipitated lipase from *Bacillus sp* up to 70% saturation at 4^{o}C with ammonium sulphate followed by hydrophobic interaction chromatography on phenyl sepharose. Yossef and Neomi (1992) carried out acetone precipitation of lipase from *P. aeruginosa*at at 4°C followed by hydrophobic interaction chromatography using fractogel TSK-

isobutyl 650. Ponnuswamy *et al.*, (2011) purified lipase by fractionation up to 60% saturation with ammonium sulphate followed by ion exchange chromatography on Diethyl-aminoethyl cellulose (DEAE-cellulose) and then a reverse phase-high performance liquid chromatography (RP-HPLC). Chartrain *et al*., (1993) purified lipase from *P.aeruginosa* MB5001 by a three-step procedure: concentration by ultrafiltration was followed by ion exchange chromatography and gel filtration. Lee and Rhee (1993) used ion exchangechromatography and gel filtration to purify lipase from *P. putida* 3SK**.**

2.19 Characterization of Lipases

In order to have a better understanding of the properties, nature of the purified enzyme and its environmental as well as industrial application, the purification steps are followed by characterization for substrate specificity, pH, temperature stability, the effects on its activity by modulators, surfactants, cations and organic solvents.

2.19.1 Substrate Specificity of Lipases

Different lipid substrates have been used for studying the substrate specificity of lipases. *Pseudo alcaligenes* F-111 lipase showed preference for C_{12} aryl and C_{14} acyl groups when *p*-nitrophenyl esters were used as substrates (Lin *et al*., 1996). Ren *et al*., (2010) reported that lipase from *P. aeruginosa* CS-2 preferentially catalyzed fatty acids with longer chain such as *p*-nitrophenyl palmitate. Lipases from *Pseudomonas S5* and *P. cepacia* also preferentially hydrolyzed substrates with longer chain fatty acids (Rahman *et al*., 2005). Redondo *et al*., (1995) reported that lipases from *Candida rugosa* lip A and B showed a preference for caprylate (C_8), laurate (C_{12}) and C_{18} (cis-Δ9).

2.19.2 Temperature and pH

Lipases have shown a remarkable temperature and pH stability. Sharma *et al*., (2002) purified and characterized lipase from thermophilic *Bacillus sp.* RSJ-1 with maximum activity at 50°C, pH of 8.0–9.0 and retained more than 90% of its activity for two hours and stable at pH range of 8.0–9.0. Meenakshi *et al*., (2010) reported that the optimum temperature for the activity of lipase from *P. aeruginosa* was 50°C -60^{o}C when tween-20 was used as substrate. The NU-2 lipase had a pH optimum of 5.2 and temperature optimum of 37^{o}C. Rakesh *et al*., (2012) reported that lipase from *Bacillus pumilus* RK31 was stable at 40°C, 50°C and 60°C and pH 6 to 8. Messias *et al*., (2009) reported the optimum temperature of 55^{o}C and pH 8.0 in *Botryosphaeria ribis* lipase.

2.19.3 Activators and Inhibitors

Certain compounds have stimulatory effect with others having inhibitory effect on lipases. Benattouche and Aboun (2012) showed that $Ca^{2+}$and Mg^{2+}were found to stimulate lipase activity in *Pseudomonas sp.* While Zn^{2+}, $Ni^{2+}$and Cu^{2+}inhibited it. The presence of Ca^{2+}, Mg^{2+}, Co^{2+}, $Mn^{2+}$and Na^{+} enhanced the lipase activity in *Mucor* sp. and Ag^{+}, Fe^{2+}, Cu^{2+}, Ba^{2+} and Hg^{2+} were found to decrease its activity (Abbas *et al*., 2002). Shabtai *et al.,* (1992) observed tween-80 and tween-20 to enhance the activity of lipase in *P.aeruginosa* YS-7. Tween-80, triton X-100 and SDS were reported to have activating effect in lipase from *Ganoderma lucidum* (Haq-nawaz and Faiza, 2013). Ren *et al*., (2010) reported that polyvinyl alcohol 124, triton X-100, tween-20, tween-80 and CTAB inhibited organic solvent-tolerant lipase activity from *P. aeruginosa* CS-2 with gum arabic and polyvinyl alcohol 124 enhancing its activity.Hesham *et al*., (2005) reported that DMSO activated lipase activity in *P. aeruginosa* px-s with EDTA and PMSF having inhibitory effects.

2.19.4 Organic Solvents

Ether, benzene and spirit have been reported to enhance the activity of lipase in *Ganoderma lucidum* with petroleum ether, ethanol, propanol, *n*-hexane and acetone having inhibitory effects (Haq-nawaz and Faiza, 2013). Yu *et al.*, (2007) reported that the activity of *Candida antarctica* lipase type B was reduced only by ethanol and hexane. Lipase from *P. chrysogenum* was reported to be stable in the presence of up to 50% (v/v) hexane and 1,4-dioxane, but unstable in the presence of xylene (Bancerz*et al.,* 2005).

2.19.5 Kinetic Parameters

Reactions catalyzed by lipase obey Michaelis-Menten kinetics. Reyhameh *et al.,* (2013) reported the K_m and V_{max} values of 0.77 mM and 49.5 µmol /ml for lipase from *Pseudomonas sp*. The K_m and V_{max} values of 0.2353 mM and 161.2nmol/min/ml respectively were reported by Muhammad *et al*., (2012) for lipase from *B. stearothermophilus* using *p*-NPP as substrate. Kambourova *et al*., (2003) reported a lipase from *B. stearothermophilus* that had a K_m and V_{max} value of 0.33 mM and 188 µM/ min/ mg. Horchani *et al*., (2009) reported a lipase from *Staphylococcus aureus* that had K_m and V_{max} value of 14.53 mM and 1485 µM/min/mg with triolein as substrate. Ramani *et al*., (2010) reported K_m and V_{max} value of 0.66 mM and 0.29mmol/min respectively in *P. gessardii* with olive oil as substrate. Shu *et al*., (2007) reported K_m and V_{max} value of 7.37 mM and 25.91 µmol/min/ mg for lipase from *A. niger* F044 with *p*-NPP as substrate.

2.19.6 Molecular Weight

Reyhameh *et a*l., (2013) reported a molecular mass of 43 kDa by native-PAGE for lipase from *Pseudomonas sp*. A molecular weight of 54.97 kDa was reported by Hesham *et al*., (2005) for

lipase from *P. aeruginosa* px-s. Lipases from *P. flourescens* had a molecular weight of 55 kDa (Kumura *et al.*,1993). Abdou (2003) reported molecular weight of 57 kDa in *S. grimisii* lipase.

2.20. Industrial Aplication of Lipases

Lipases have a wide range of industrial application ranging from food, dairy, bioremediation processes, detergency, cosmetics and perfumery.

2.20.1. Lipases in Food and Food Processing Industries

Lipases are applied in food industry as in cheese ripening and flavour development (Saxena *et al.*, 1999). They are used to produce flavours and for modification of structure by inter or transesterification to increase nutritional value of the products (Reetz, 2002). The free fatty acids generated by the action of lipases on milk fat endow many dairy products, particularly soft cheeses with their specific flavour characteristics. Low caloric triacylglycerols can be produced from cheap oils through modification mediated by lipases (Gupta *et al*., 2003). *Rhizomucor mieghei* lipase was used for transesterification reaction that replaced the palmitic acid in palm oil with stearic acid (Xu and He, 1995). Lipases from *Rhizomucor mieghei* and *Candida antarctica* were used for the synthesis of short chain flavour thioester (Shin *et al*., 1997). Natural esters can be transesterified by lipases to form butter and triacylglycerols (Rajendran *et al*., 2009). Macedo *et al.*, (2003) reported the use of lipases in food to modify flavour by synthesis of esters of short chain fatty acids and alcohols

2.20.2 Lipases in Bioremediation

Lipases from different sources can be used for cleaning wastes from lipid processing factories and restaurants (Pandey *et al.*1999). With respect to the bioremediation of polluted soils lipase producing strains are very critical. Anonymous (1995) reported the use of lipase in the degradation of polyester waste, removal of biofilm deposits from cooling water systems and purification of waste gases from factories. Lipases have been used to handle spillage of oil in refineries, shore sand and processing factories (Nakamura and Nasu, 1990).

2.20.3 Lipases in Cosmetics and Perfumery

The ability of **l**ipases to act in surfactants and in aroma production have been applied in cosmetics and perfumeries (Metzger and Bornscheuer, 2006). In cosmetics and perfume industries the surface active agents used are monoacylglycerols and diacylglycerols which are produced by lipase mediated esterification of glycerols. Maugard *et al.*, (2002) reported the production of water soluble retinol derivatives with great potentials in cosmetics and pharmaceuticals such as skin care products by immobilized lipase.

2.20.4 Lipase in Detergency

Detergents are made environmentally friendly with enhanced ability to remove tough stains by their formulation using enzymes. The lipases used in detergent formulation must be stable over a wide range of pH and temperature (6-10 and 30-60^{o}C), must have broad specificity, it must withstand damaging surfactants and enzymes such as the linear alkyl benzene sulfonate and protease which are components of detergent (Khoo and Ibrahim, 2003). Gerritse *et al*., (1998)

reported the use of an alkaline lipase from *P. alcaligenes* M-1 in the removal of fatty stains under conditions of a modern washing machine.

2.20.5 Lipases in Organic Synthesis

One of the striking properties of lipase that is widely used in the resolution of racemic mixtures of organic acid by esterification and transesterification reactions in immiscible system is its stereo-specific nature (Klibanov, 1990). Lipases from *Candida cylindracea*and*C.antarctica* have been used to resolve the enantiomers of flurbiprofen, naproxen, ibuprofen and suprofen (Mertoli *et al*., 1996). Thioesterification processes have been reported in resolution of racemic mixture catalyzed by lipases from *C.antarctica*and *R. mieghei* (Weber *et al*., 1999). An important non steroidal anti inflammatory drug Profen (2-aryl propinoic acid) is pharmacologically active in the (S)-enantiomer form (Hutt and Caldwell, 1984). The pure (S)-ibuprofen is obtained by lipase catalyzed kinetic resolution via hydrolysis thus proving its usefulness in resolution of racemic mixture (Lee *et al.*, 1995).

2.21 Immobilization of Lipases for Biodiesel Production

Immobilization means retaining enzymes in a proper geometry for economic reusability under stabilized condition by associating it with an insoluble matrix. The operational and temperature stability of lipases could be enhanced by immobilization. Immobilized enzymes have a number of advantages amongst which are the availability of the product in greater purity and greater control of enzymatic reaction (Hartmier, 1988). It induces change in the conformation of enzyme which allows substrate access to the active site. Adsorption of the open form of lipases by interfacial activation which mimicks the lipophilic substrate is favoured by hydrophobic supports

(Séverac *et al*., 2011). Methods of enzyme immobilization can be classified as adsorption, covalent bonding, entrapment and cross-linking. A very important factor for obtaining an efficient lipase activity is achieved by proper selection of method and support material. The material used for immobilization should be readily available, biodegradable, toxic free and of low cost.

2.21.1 Immobilization of Lipases by Adsorption

Adsorption is the adhesion of lipase on the surface of the adsorbent by weak interactions such as Vander Waals, ionic and hydrophobic interactions (Jegannathan *et al.*, 2008). Simple mixing of an aqueous solution of enzyme with the carrier material followed by washing away the excess enzyme from the immobilized enzyme on the carrier is what is needed to immobilize lipase by adsorption (Costa *et al.*, 2005). The pH, temperature and ionic strength determine the level of adsorption. The advantages of adsorption is mild and easy condition of preparation, low cost, there is no need for chemical modification, the carrier can be recovered for repeated use (Fukuda *et al*., 2001). Acrylic resin, celite, polypropylene and textile membrane are broadly used carriers. Du *et al*., (2004) used acrylic resin to immobilize *C. antartica* lipase for biodiesel production from soybean oil. Naranjo *et al.,* (2010) reported the use of granular activated carbon for adsorption of *C. antarctica* B lipase to produce biodiesel from palm oil. Li *et al*., (2010) reported the production of biodiesel from crude rice bran by *Candida sp.*99–125 lipase adsorbed on textile membrane. Ji *et al*., (2010) reported the use of *P. aeruginosa* lipase adsorbed on celite for biodiesel production from soy bean oil.

21.2 Lipase Immobilization by Entrapment

Entrapment method is based on capturing of enzymes within a polymer network but allowing substrate and products to pass through (O'Driscoll, 1976). It entails mixing a soluble enzyme with a polymer solution and then cross linking the polymer to form a lattice structure that captures the enzyme (Costa *et al.,* 2005). Entrapment method is fast, cheap and can be carried out under mild condition and has wider industrial applications (Ikeda *et al*., 2002). Entrapment can be divided into three: gel entrapment, fiber entrapment and micro encapsulation. The disadvantage of entrapment method of immobilization is the mass transfer problem due to the action of support as a barrier, so the enzyme is effective only for low molecular weight substrates (Zhang *et al*., 2012). Alginate, celite, carrageenan, resins and acrylic polymers are the most commonly used carriers for entrapment. Devanesan *et al.,* (2007) immobilized *Pseudomonas fluorescens* Mtcc 103 lipase on alginate for biodiesel production from Jatropha oil. Jegannathan *et al.,* (2010) reported the use of *Burkholderia cepacia* lipase encapsulated on *β*-carrageenan for biodiesel production from palm oil. Sawangpanya *et al.,*(2010) reported the production of biodiesel from palm oil by calcium alginate immobilized *C. rugosa* lipase. Nassreddine *et al*., (2008) reported the immobilization of lipase from *C.antarctica* for biodiesel production from sun flower oil. Noureddini *et al*., (2005) reported the production biodiesel from soy bean oil by lipase from *Pseudomonas cepacia* immobilized on hydrophobic sol gel. Macario *et al*., (2009) reported the encapsulation of *R. mieghei* lipase within the micellar phase of a surfactant that is self assembled with silica preserving the mobility and activity of the enzyme.

2.21.3 Lipases Immobilization by Covalent Attachment

In covalent attachment there is chemical reaction between the active functional groups of the carrier and the amino acid residues outside the catalytic and binding site of the enzyme (Cao, 2005). A number of support materials have been used such as natural polymers (agarose, chitin and chitosan), synthetic polymers (hydrophobic polypeptides, nylon fibers) and eupergit (made by copolymerization of N, N'-methylene-bis-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether and methacrylamide for immobilization of lipases by covalent binding (Knezevic *et al.,* 2002). Xie and Wang (2012) reported a technique for the immobilization of *C. rugosa* lipase on magnetic chitosan microspheres for transesterification of soybean oil. A method of covalent binding of *Thermomyces lanuginosus* lipase on polyglutaraldehyde-activated olive pomace powder was reported by Yücel (2011). Mendes *et al*., (2011) reported the immobilization of *Porcine* pancreatic lipase by covalent binding it with chitosan beads for biodiesel production from salicornia oil. Dizge *et al.,* (2009) reported the production of biodiesel from canola oil by *T. lanuginosus* lipase immobilized by covalent attachment on polyglutaraldehyde activated styrene divinyl benzene.

2.21.4Lipases Immobilization by Crosslinking

Crosslinking is the interaction of the three dimensional network within enzyme with coupling reagent and carrier (Zhang *et al.,* 2012). As a result of strong interaction between the enzyme and the carrier its stability is enhanced. However, a lower activity is obtained when the cross-linking conditions are intense (Tan *et al.,* 2010). Immobilization of *C. rugosa* lipase on fine powder of *Scirpus grossus L* by glutaraldehyde cross linked technique was used for biodiesel production from palm oil Kensingh *et al.,* (2010). Loren *et al.,* 2009) investigated the immobilization of

Alcaligenes spp. lipase on poyethylamine agarose, glutaraldehyde agarose crosslinking method for biodiesel production from canola oil.

2.21.5 Whole Cell Immobilization

The high cost of lipases significantly affects their application for bulk production of biodiesel (Fukuda *et al.*, 2008). The easier and cost effective way of production of biodiesel by enzymatic transesterification is the use of intact microorganisms such as fungi, bacteria and yeasts cells containing intracellular lipase instead of extracellular lipases. The use of whole cells improves the operational stability and avoids the need to isolate and purify enzymes (Li *et al*., 2008). Biomass support particles have been used for immobilization of whole cells. Sun *et al.,* (2010) used glutaraldehyde cross-linking treatment on whole cell for methanolysis of soybean oil. Koda *et al.,* (2010) reported the immobilization of *Aspergillus oryzae* expressing r-Fusarium heterosporum lipase immobilized with biomass support particles for biodiesel production from rape seed oil.

2.22 Biodiesel Production

The use of natural oils and fats in the production of biodiesel as an alternative fuel source is environment friendly and therefore reduces the greenhouse effect and other pollutants produced by fossil fuels (Li *et al*., 2009). The conversion of oils to methyl or other short chain alcohol esters can be obtained in a single transesterification reaction using lipases in the presence of organic solvents (Jaeger and Eggert, 2002). Kramer (1995) reported that problems such as glycerol recovery and the need to use refined oils and fats as main substrates that impede the continued growth of chemical method can be overcome by enzymatic transesterification. The

advantages of lipase catalysis over chemical methods in the production of simple alkyl esters include the ability to esterify both glycerol linked acyl fatty acyl chains and free fatty acids using single step method, production of a glycerol side stream with minimal water content and reusability of the biocatalyst especially when immobilized (Haas and Foglia, 2005). Production of biodiesel by enzymatic processes at industrial scale tends to be capital intensive due to the high cost of the biocatalyst, inactivation by substrate contaminants and polar short chain alcohols. Soumanou and Bornscheuer (2003) reported the production of biodiesel from sunflower oil using *R. mighei*, *T. lanuginose* and *Pseudomonas fluorescens* lipase. Biodiesel was produced from waste cooking oil with *Candida sp* lipase (Chen *et al*., 2009), from rice bran oil by *Criptococcus SP-2* lipase (Kumari *et al*., 2009), from waste cooking palm oil by *C. antartica* lipase (Kamini and Iefuji, 2001), from soybean oil by *C. rugosa* lipase (Halim *et al.*, 2009), from cotton seed oil by *P. fluorescens* lipase (Rodrigues *et al*., 2010) and from rapeseed oil by *C. Antarctica* lipase (Watanabe *et aL.*, 2001).

2.23 Variables Affecting Transesterification Process

A number of factors including moisture content, free fatty acid contents, molar ratio of oil to alcohol, co-solvent type, amount of catalyst, reaction time, reactiontemperature and mixing intensity could affect the process of transesterification (Demirbas, 2007). These variables affect transesterification processs differently depending on the method employed for the transesterification.

2.23.1 Moisture and Free Fatty Acids Contents.

Moisture and free fatty acids content of the oil used in the transesterification process usually determine its viability. Sharma *et al*., (2008) reported that in order to avoid undesirable effect on the catalyst and the transesterification reaction, the moisture and free fatty acids content of the oil must be reduced to <0.5% for homogenous process as high moisture and free fatty acids content in the oil could lead to formation of soap and emulsion which makes the separation and purification process of the final product difficult and eventual loss of triacylglycerol and biodiesel (Gerpen, 2005). Al-Zuhair *et al*., 2006) reported that higher moisture content could enhance the enzyme catalysed transesterification process.

2.23.2 Molar Ratio of Alcohol to Oil and Type of Alcohol

Stoichiometrically every mole of triacylglycerol requires three moles of alcohol to produce three moles of fatty acid alkyl esters and one mole of glycerol (Ma and Hanna, 1999). Higher yield of fatty acid alkyl esters is obtained when alcohol in excess of stoichiometric requirement is supplied during the transesterification process. Fukuda *et al*., (2001); Gerpen (2005) considered molar ratio of oil to alcohol 1:6. Ramadhas *et al*., (2005) reported oil to alcohol molar ratio of 1:8. Sahoo *et al*., (2007) reported a molar ratio of 1:9. A molar ratio of 1:5 was reported by Alamu *et al*., (2008). Meher *et al*., (2006) reported a molar ratio of 1:12. Depending on the quality of the oil and method of transesterification employed, higher oil to methanol ratio could be used. The yield of fatty acid alkyl esters could be adversely affected when the ratio of oil to alcohol is too high (Miao and Wu, 2006).

2.23.3 Type and Amount of Catalyst

The quality of oil and transesterification method employed determine the type and amount of catalyst required. Any type of catalyst could be used for the transesterification process of purified oil. The yield of fatty acid alkyl esters generally increases with increasing amount of catalyst (Demirbas, 2007).

2.23.4 Reaction Time and Temperature

It is necessary to limit the reaction time at a certain period for practical and economic feasibility of transesterification reaction. The method of transesterification determines the operating temperature. Enzymatic transesterification process requires moderate temperature but non catalytic transesterification process requires high temperature and pressure to yield the desired product. Soumanou and Bornscheuer (2003) reported 40°C for methanolysis by *R. mieghei* lipase. Xıao *et al*., (2010) reported the temperature of 40°C to be the optimum for biodiesel production from palm oil by immobilized *Aspergillus niger* lipase. Freitas *et al.,* (2009) reported 40°C -50°C as optimum temperature for biodiesel production from waste cooking oil by lipase from *Candida Sp*. Salis *et al*., (2009) reported 40°C as the optimum temperature for *P. fluorescens* lipase catalysed transesterification of soybean oil. In general, increasing the temperature leads to an increase of the reaction rate of biodiesel production.

2.23.5 Mixing Intensity

Due to opposite polarity of the two feedstocks: oil (hydrophobic) and alcohol (hydrophilic), transesterification is usually associated with mass transfer problem. Meher *et al*., (2006) reported that mixing is necessary to promote contact between oil and alcohol in order to enhance the

transesterification reactions. Bouaid *et al.,* (2012) used the impeller speed of 600 rpm for biodiesel production from *Jatropha curcas.* Bouaid *et al*., (2007) tested the impeller speeds between 500 and 1200 rpm and found a stirring speed of 600 rpmto be appropriate to avoid external mass transfer limitation. Sok-joong *et al*., (2007) investigated production of biodiesel from soybean oil by an immobilized lipase (Novozyme 435) based on the impeller speed of 600 rpm

2.23.6 Co-solvent

The use of co-solvent for transesterification ensures a homogeneous reaction mixture alleviating the problems with having the reactants in two phases. It reduces the viscosity of the reaction mixture, increases the diffusion rate and reduces mass transfer problems around the enzyme. Li *et al*., (2008) reported that co-solvent could also function as a reaction medium, stabilizer and binder between triacylglycerol and alcohol to allow contact between the feedstocks. For immobilized enzymes, non-polar solvents might force the residue of water to stay around the enzyme increasing the water activity locally and might help to stabilize enzymes (Park *et al.,* 2008). Royon *et al*., (2007) reported the use of tert-butanol as co-solvent. Wang *et al*., (2006) reported the use of *tert*-pentanol and isooctane as co-solvents. Lu *et al*., (2008) used a wide range of organic solvents for the transesterification of trioleyl glycerol with methanol using lipase from *Candida sp.* The use of several solvents such as *n*-hexane and petroleum ether in the reaction medium has been reported by Ghamguia *et al*., (2004).

2.24 Optimization of Biodiesel Production by Response Surface Methodology

Response surface methodology (RSM) is a set of mathematical and statistical methods for modeling and problem analysis. It is suitable for optimizing complicated systems where the

response is influenced by several parameters (Abd-Rabu *et al.,* 2013). It is particularly useful when several imput variables (independent variables) have influence on the quality characteristics (overal response). Due to the simultaneous effects of some operating variables on the system, the design and application of modeling tools such as the response surface methodology are essential for maximizing the productivity and reducing the production process costs. Prerna *et al.,* (2012) employed response surface methodology to optimize the production of biodiesel from high free fatty acid *Jatropha curcas* oil. Hsiao-Ching *et al.,* (2011) used a 3-level-3-factor Box-Behnken design for optimization of biodiesel production by lipase catalyzed transesterification in a packed-bed reactor. Bouaid *et al.,* (2012) used response surface methodology for optimization of biodiesel production from *Jatropha curcas* oil. Hoda *et al.,* (2014) optimized biodiesel production from waste cooking oil by response surface methodology.

2.25 Purification and Recovery of Biodiesel

The purification of fatty acid alkyl esters from the catalyst, residual alcohol and the separation of glycerol as a major secondary product are generally desirable. Once the transesterification reaction is completed and the glycerol is separated by gravity separation from the methyl ester which must be followed by further processing to remove impurities in order to meet the appropriate specifications. Gonzalo *et al.,* (2010) studied water washing of crude biodiesel obtained from rapeseed. Coêlho *et al.,* (2011) purified the ethyl esters of castor oil by water washing at different temperatures (30°C and 70 °C) and pH values (2 and 5). Tomasevic and Siler-Marinkovic (2003) refined the crude biodiesel from used frying oil with the solution of citric acid in methanol (50% v/v) followed by washing with hot water until the wastewater was neutral. Özgül-Yücel and Türkay (2003) used rice hull ash and a commercial silica gel to purify

methyl esters by adsorption of free fatty acids under atmospheric conditions. Baroutian *et al*., (2012) employed a two-step purification method that combined wet and dry washings. Sokoto *et al.,* (2013) employed the use of gravity separation on biodiesel produced from calabash seed oil and the separated upper layer was placed into an evaporating dish and heated in water bath at 90^{o}C for 30 minutes to remove the residual methanol, then neutralized with dilute phosphoric acid (pH 4.0).

CHAPTER THREE

3.0 MATERIAL AND METHOD

3.1 Materials

3.1.1 Chemicals and Reagents

All chemicals and reagents used were of analytical grade. Tris (hydroxymethyl)-amino methane, bromo phenol blue, phenyl methyl sulfonyl fluoride, para nitrophenol,para nitrophenyl laurate, sodium dodecyl sulphate (SDS), ethylenediamine tetraacetic (EDTA), N,N,N,N tetramethyl ethylene diamine (TEMED), ammonium persulphate, acrylamide, bis acrylamide, sodium acetate, acetic acid, tween-80, sodium alginate, calcium chloride, sodium dihydrogen phosphate, disodium hydrogen phosphate, Coomasie brillant blue G-250, bovine serum albumin (Fluka), n-hexane, cetrimide, peptone, olive oil, agar, yeast extract, $MgSO_4$, DEAE-cellulose A-100, sephadex G-75, glycerol, β-mercaptoethanol, glycine, CTAB, triton X-100 and dithiothreitol.

3.1.2 Equipments

Abbe's refractometer Model AR-001, Electrophoresis power supply by Amershan Pharmacia Biotech, viscosity cup NDJ-5A, centrifuge MIKRO 22R Hettich Zentrifuge Germany, shaker (Orbital Incubator 5150 Stuart Scientific UK), spectrophotometer UV vissible), colorimeter 6057 Jenway, pH meter 3505, water bath Gallenkamp, GC-MS machine GP-2010 Plus Shimudzu Japan, oxygen bomb calorimeter Parr 600 calorimeter, chromatographic column, flash point tester Pensky-Martens test module (model 24100-2)

3.2 Methods

3.2.1 Collection, Identification and Preparation of *Acacia nilotica* Seeds

Acacia nilotica seeds were collected from the botanical garden, Ahmadu Bello University, Zaria and authenticated at the Herbarium of the Department of biological science, Ahmadu Bello University Zaria, Nigeria with the voucher number G01457. The seeds were cracked, washed, the shell removed and the kernels were pulverized for oil extraction.

3.2.2 Collection, Screening and Isolation of Lipase Producing *P.aeruginosa.*

Soil sample was collected from groundnut oil spilled area in Samaru market, Sabon-Gari Local Government Area, Kaduna State, Nigeria in a sterile sealed bottle for isolation of lipase producing *Pseudomonas aeruginosa.* The screening was carried out by using the method of Nakayama (1981). The isolates were grown on cetrimide agar. This medium is selective and formulated to enhance the formation of blue or blue-green pyocyanin pigment by *Pseudomonas aeruginosa*.The pigment diffused into the surrounding growth medium.The organism wasidentified morphologically and characterized biochemically according to the method of Cowan and Steel (2004). Colony capable of utilizing olive oil as sole source of carbon was isolated and streaked on modified lipase assay media containing peptone (1.5% w/v), agar (1.5% w/v), NaCl (0.1%w/v), $CaCl_2$ (0.1% w/v), olive oil (1% v/v) as described by Shukla *et al.*, (2007) with modification. The plates were incubated at room temperature for 72 hours. The formation of white precipitate of calcium salt of fatty acid around the colony was considered as positive colony for lipase secretion.

3.2.3 Extraction of Oil from *A. nilotica* Seed by Soxhlet Method

The oil from *A. nilotica* seed was extracted by soxhlet apparatus according to AOAC (1990). Three hundred millilitre of *n*-hexanebeing non polar and therefore suitable for extraction of triacylglycerol, with low vapourization temperature and stability was poured into a round bottom flask and 100 g of *A. nilotica* seed kernel powder was placed in the thimble which was inserted in the centre of the extractor. The extraction was conducted for two hours. The pre-weighed flask containing the oil was cooled in the desiccator and the resulting mixture containing the oil was heated at 70^{o}C to recover solvent from the oil and the percentage of oil extracted was determined by using the formula below:

$$\text{Percentage of oil} = \frac{\text{Weight of the oil obtained (g)}}{\text{Weight of the seed taken (g)}} \times 100 \text{----------Equation 3.1}$$

3.2.4 Determination of Fatty Acid Profile of *A. nilotica* Seed Oil

Principle

An inert carrier gas (mobile phase) such as nitrogen, helium, hydrogen or argon **is** made to flow through the column. The temperature of the column is maintained high in an oven to keep the compound to be separated in their volatile state. These volatilized compounds get partitioned between the liquid or solid stationary phase and the gaseous mobile phase and hence get separated because of diffrences in their partition coefficients. After leaving the column, the se pareted componds pass through the detector, sensed and recorded by the recorder.

Procedure

The fatty acid profile of *A. nilotica* seed oil was determined by GCMS. Twenty microlitre of the sample was diluted (1000 times) in a 1:1 hexane/ethyl acetate mixture and 2 µl of it was injected into the GC column fitted with a 30 m × 0.25mm × 0.25 µm CP WAX 52 CB in a split injection system with ratio of 1:20. The injector temperature was set at 280^{o}. The column temperature was set at180^{o}C and then programmed to rise at 20^{o}C/ minute up to 220^{o}C, where it was maintained constant for 6 minutes. Hydrogen was used as the carrier gas at a flow rate of 2 ml/minute and the column pressure was maintained at 20 psi. Heptadecanoate was used as internal standard (Vieira *et al.*, 2006).

3.2.5 Determination of Specific Gravityof *A. nilotica* Seed Oil

A clean and dried bottle was weighed (W_0) and 10 ml of oil was poured, stopper inserted and reweighed to give (W_1). The oil was substituted with water and weighed again to give (W_2). The specific gravity was then calculated using theformula below:

Specific gravity $=\frac{W_1 - W_0}{W_2 - W_0}$ ---------- Equation 3.2

3.2.6 Determination of Refractive Index of *A. nilotica* Seed Oil

Refractive index of *A. nilotica* seed oil was determined using Abbe's refractometer. Two drops of the oil sample was placed on the ground surface of the lower prism and closed. The cross wire of the telescope was focused by rotating the eye piece and adjusting the mirror so as to get good illumination. The lower knob of the prism box was turned back and forth until the field of view became coloured fringe, the compensator was rotated by using upper knob until the coloured fringe disappeared and the lighted image showed a sharp edge. The prism box was then rotated

until the sharp edge was in coincidence with the intersection of the cross wires in the telescope. The index of refraction was then read off on the scale through the eye piece.

3.2.7 Determination of Viscosity of *A. nilotica* Seed Oil

Viscosity was determined by using rotary viscometer which operates on measuring the torque moment by the sensor of the viscometer when the rotor is rotated constantly by the variable speed motor. The torque moment is proportional to liquid viscosity because of the liquid viscose hysteresis. The oil sample (10 ml) was put in a beaker and the rotor of the instrument was inserted by lifting the screw and the power switch was turned on. The rotation speed of 30 rpm was selected and the viscosity was measured.

3.2.8. Determination of Calorific Value of *A. nilotica* Seed Oil

Calorific value was determined according to the method of Gerpen (2005) with modification by placing 100 ml of *A. nilotica* seed oil in the sample cup in a steel container which was filled with oxygen at 30 atm pressure and was electrically ignited. The heat produced by the combustion reaction was calculated by recording the rise in temperature of the bomb calorimeter.

3.2.9 Determination of Cetane Number of *A. nilotica* Seed Oil

The Cetane number of *A. nilotica* seed oil was determined according to Krisnangkura (1986) using the formula below:

$$CN = 46.3 + \frac{5458}{SV} - 0.225 \times IV$$----------Equation 3.3

Where CN=Cetane number, SV=Saponification value and IV= Iodine value

3.2.10. Determination of Acid Value of *A. nilotica* SeedOil

Acid value of *A. nilotica* seed oil was determined by titrimetric method. Five gram of filtered *A. nilotica* seed oil was poured into a conical flask and 25 ml of fat solvent (95% ethanol: ether 1:1v/v) was added. The content was shaken, 2 drops of phenolphthalein was added and shaken again. It was titrated against 0.1 M solution of KOH. A blank titration was carried out without the oil sample and the acid value was calculated using the formula below:

$$\text{Acid value mgKOH/g fat} = \frac{\text{Titre value x Nomality of KOH}}{\text{Weight of the sample (g)}} \times 56.1 \text{----------Equation 3.4}$$

56.1 is a multiplication factor because 1 ml of 1N KOH contains 56.1 mg KOH.

3.2.11 Determination of Saponification Value of *A. nilotica* Seed Oil

The saponification value of *A. nilotica* seed oil was determined by. One gram of *A. nilotica* seed oil was dissolved in 3 ml of fat solvent (95% ethanol; ether 1:1v/v) and 25 ml of 0.5 M ethanoic KOH was added. The mixture was refluxed for an hour and allowed to cool at room temperature and then 2 drops of 1% phenolphtalein was added. The content was titrated with 0.5 M HCl till the pink colour disappeared. The blank titration was carried out under the same experimental condition used for the tube without oil sample and the saponification value was calculated using the formula below:

$$\text{Saponification value} = \frac{\text{Titre value}}{\text{Weight of the sample (g)}} \times 28.05 \text{----------Equation 3.5}$$

28.05 is a multiplication factor because 1 ml of 0.5 N KOH contains 28.05 mg KOH

3.2.12 Determination of Iodine Value of *A. nilotica* Seed Oil

The iodine value of *A. nilotica* seed oil was determined by titrimetric method. One gram of *A. nilotica* oil was dissolved in 10 ml of fat solvent (95% ethanol: ether 1:1v/v) in a stoppered flask and 25 ml of 0.1N iodine monochloride solution was added. The flask was kept in dark cupboard for one hour. The content was mixed with 50 ml of water and 10 ml of 5% potassium iodide. The liberated iodine was then titrated with 0.1 M sodium thiosulphate till the content became pale yellow and then 2 drops of 0.1% starch solution was added. The mixture was titrated again with sodium thiosulphate. A blank was prepared by replacing *A. nilotica* seed oil with chloroform and titrated in similar manner as the test sample. The iodine value was calculated using the formula below:

$$\text{Iodine number} = \frac{(b-a) \times 12.7}{1000} \times \frac{100}{\text{Weight of A. nilotica oil (g)}} \quad \text{----------Equation 3.6}$$

Where b= volume of thiosulphate used for blank (ml)

a= volume of thiosulphate used for the sample (ml)

w =weight of the oil sample (g)

One litre of 0.1N iodine solution contains 12.7 g of iodine

3.2.13 Determination of Peroxide Value of *A. nilotica* Seed Oil

The peroxide value of the oil was determined by titrimetric method. One gram of *A. nilotica* seed oil was put into a dried tube and 20 ml of solvent mixture of glacial acetic acid and chloroform 2;1 were added. The content was boiled for one minute and poured into a flask containing 20 ml of 5% potassium iodide. It was then titrated with 0.002 M sodium thiosulphate using 0.1% starch

indicator. The blank titration was also carried out by replacing *A. nilotica* oil with chloroform and titrated in similar manner as done for the test sample. The peroxide value of the oil was then calculated using the formula below:

$$\text{Peroxide value} = b - a \times \text{Molarity of} Na_2S_2O_7 \times \frac{100}{W} \quad \text{----------3.7}$$

Where b= volume of thiosulphate used for blank (ml)

a= volume of thiosulphate used for oil sample (ml)

w =weight of the oil sample (g)

3.2.14 Production of Lipase from *P. aeruginosa* by Submerged Fermentation

The lipase production from *P. aeruginosa* was carried out in 250 ml Erlenmeyer flask containing 50ml of a medium composed of peptone (0.5%), yeast extract (0.2%), NaCl (0.1%), $MgSO_4$ (0.05%) and olive oil (1.0% v/v). The medium was sterilized, inoculated with a single colony of *P. aeruginosa* and incubated for 60 hours. At the end of the incubation period, the fermentation media was centrifuged at 6000×g for 10 minutes and the supernatant was mixed with 50 ml acetone: ethanol (1:4 v/v) for 1hour at 4°C. The mixture was centrifuged again at 6000×g for 10 minute. The precipitate obtained was dissolved in distilled water and used as crude lipase preparation for further purification.

3.2.15 Determination of Protein Content

Protein concentration was determined according to Bradford method (1976) using Coomasie brillant blue (R-250) dye that binds to proteins containing lysyl and arginyl residues. Five

microlitre of the crude enzyme extract was made up to 100 µl with distilled water, 1 ml of the dye was added and allowed to stand for 5 minutes at room temperature. Absorbance was read at 595 nm against the reagent blank.

3.2.16 Assay for Lipase Activity

This was carried out according to the method reported by Gopinath *et al*., (2005) with modification using *p*-nitrophenyl laurate as substrate which 0.001g of *p*NPL was dissolved in 10 ml isopropanol (solution A) and 5 g sodium deoxycholate (an emulsifier) was dissolved in 90 ml of 0.05 M tris HCl buffer pH 8.0 (solution B). The solutions A and B were mixed. A total amount of 2.995 ml of substrate solution was put into each test tube and 5 µl of enzyme solution was added and incubated for 15 minutes. The reaction was stopped by adding 1 ml of chilled ethanol acetone mixture1:1 and the absorbanceread at 410 nm against enzyme free control. One unit of enzyme is defined as the amount of enzyme that converts one micromole of substrate to product per minute under defined condition.

3.2.17. Cold Acetone Precipitation

Crude enzyme was precipitated with acetone according to Yossef (1991). The crude lipase was mixed with cold acetone (pre-cooled on dry ice). The resulting precipitate was allowed to settle, centrifuged at 10000×g for 15 minutes and re-dissolved in 0.05 M tris-HCl buffer pH 8 that is suitable for invivo and invitro analysis. The re-dissolved precipitate was then subjected to enzyme assay and protein estimation.

3.2.18 Ion Exchange Chromatography on DEAE-Cellulose

Ion exchange chromatography was carried out according to method of Abbas *et al.*, (2002) with modification. Five grams DEAE-Cellulose-A100 was swollen in a beaker containing 20 ml of distilled water. The slurry was then poured into column of 50×2.5 cm pre-equilibrated with 0.05 M tris-HCl buffer pH 8.0 and then allowed to settle. Two millilitre of the cold acetone precipitated fraction was added to the column. The elution of the proteins bound to the anion exchange column was achieved using a stepwise gradient of NaCl solution 0.1 mM to 1.0 mM to collect fifty fractions (4 ml) each which were assayed for lipase activity and protein concentration was determined

3.2.19. Gel Filtration on Sephadex (G-75)

Gel Filtration was carried out according to the method of Jakoby (1971). Five grams of sephadex G-75 powder was swollen in 20 ml of distilled water. The slurry was then poured into the column and allowed to settle. Two millilitre of active lipase fraction from the anion exchange column was then loaded into tris-HCl buffer pH 8.0 pre-equilibrated column and then eluted with same buffer. Fifty fractions of 4 ml each were collected and then assayed for lipase activity and protein concentration was determined**.**

3.2.20. Determination of K_m and V_{max} of Partially Purified Lipase from *P.aeruginosa*

This was carried out using initial velocity studies wbich was achieved by varying concentration of substrate *p*NPL (0.01 mM, 0.02 mM, 0.03 mM, 0.04 mM, 0.05 mM and 0.06 mM) initially with lipase to determine its activity as already described in section 3.2.17 to obtain the initial

velocity data needed for Lineweaver Burk plot. Michaelis constant K_m and V_{max} were then determined from the plot by extrapolation.

3.2.21 Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to the method of Laemmli (1970) with modification. Resolving gels were polymerized with 15% acrylamide / bis-acrylamide in 1.5 M tris-HCl, pH 8.8 containing TEMED (0.1 % v/v), SDS (1% w/v) and ammonium persulphate (0.1% w/v). The stacking gel was polymerized from 4% acrylamide/ bis-acrylamide in tris-HC1 (0.5 M) pH 6.8, TEMED (0.1% v/v), SDS (0.1 % (w/v) and ammonium persulphate (0.1 % w/v). The protein sample (100 µl) was diluted with an equal volume of SDS loading buffer tris-HCl (0.1 M) pH 6.8 containing glycerol 2 ml of 80% (v/v), 200 µl of 0.1% (v/v) β-mercaptoethanol, 4% w/v SDS, 0.2 % w/v bromophenol blue and incubated at 95°C for 5 minutes before loading on to gel. The protein sample was loaded into the well along with the pre-stained molecular weight markers and electrophoresed in SDS-PAGE running buffer (0.025 M tris-HCl (pH 8.3), 0.2 M glycine, 0.1 % SDS) at 100 V for the first10 minutes and then at 200 V. The gel was washed with water to remove the SDS and then stained with 0.01 % (w/v) Coomassie brillant blue in 5 % (v/v) 95% ethanol:water (95:5 v/v) and destained with 50% v/v methanol and 10% v/v acetic acid to visualize the protein bands.

3.2.22 Estimation of Molecular Weight of Partially Purified Lipase from *P. aeruginosa*

Lipase molecular weight estimation was based on the existence of linear relationship between the logarithm of the molecular weight of SDS-denatured polypeptide and its relative mobility (Rf). The Rf was calculated as the ratio of the distance migrated by the molecule to that migrated by a marker dye front. SDS-PAGE of the purified lipase was carried out along side standard protein

markers (colour plus pre-stained marker mix). The markers are maltose binding protein (MBP)-β galactosidase (175 kDa), MBP-truncated–β galactosidase (80 kDa), (MBP)-chitin binding domain (CBD) (58 kDa), CBD-Mxe inten-2CBD (46 kDa), CBD-Mxe inten (35 kDa), CBD-Ecoli par (23 kDa) and lysozyme (17 kDa). The Rf values of the protein standards and the purified lipase were determined and log of molecular weight of these markers were plotted against their corresponding Rf values. The molecular weight of the purified lipase was estimated from the plot by extrapolation.

3.2.23 Determination of Optimum pH for the Activity of Lipase from *P. aeruginosa*

The procedure was carried out using the following buffers 0.05 M acetate buffer (pH 3.0 to 6.5), and 0.05 M tris-HCl buffer (pH 7.0 to 10.0) to test the activity profile of lipase as a function of pH using paranitrophenyl laurate as substrate. To determine the effect of pH on K_m and V_{max} the initial velocity studies was carried at the pH range 3-9 and Lineweaver–Burk plot of the values at various pH was used to obtain K_m and V_{max} of each corresponding pH. A graph of log V_{max}/ K_m against pH was plotted to deduce the likely ionisable groups at the active site of the purified lipase.

3.2.24 Determination of pH Stability of Lipase from *P. aeruginosa*

Equal volume of enzyme (0.5 ml) was incubated with buffer at various pH, 0.05 M acetate buffer (pH 3.0 to 6.5) and 0.05 M tris-HCl buffer (pH 7.0 to 10.0) for two hours and residual activity was determined by assaying for activity as described in section 3.2.17. The result was expressed in terms of percentage residual activity.

3.2.25 Determination of the Effect of Temperature on the Activity of Lipase from *P. aeruginosa*

The optimal temperature for lipase activity was determined by incubating the enzyme substrate mixture and the activity was determined at different temperature ranging from 25^{o}C to 70°C. From the data the effect of temperature on the enzyme activity, Arrhenius plot was drawn by plotting log of v_o against the reciprocal of temperature (^{o}K). The activation energy (Ea) of the purified enzyme was extrapolated from the plot.

3.2.26 Determination of Thermostability of Lipase from *P. aeruginosa*

Equal volume of enzyme and 0.5 ml tris-HCl buffer 0.05 M (pH 8.0) 0.5 ml was incubated at various temperature ranging from (25^{o}C to 70^{o}C) for two hours and then residual activity was determined and expressed as percentage as in section 3.2.25 above.

3.2.27 Determination of the Effects of Some Surfactants on Lipase Activity

The effect of gum arabic, CTAB, SDS at 1% w/v, tween-80 and triton X-100 at concentration of 1% (w/v) on the activity of lipase was determined according to the method of Zhang *et al*., (2009) with modification. Equal volumes of enzyme (0.5 ml) and solution of (0.5 ml) surfactants was incubated separately for two hours and the residual activity was determined and expressed as percentage as described in section 3.2.25 above.

3.2.28 Determination of the Effects of Some Cations on Lipase Activity

The effect of Mg^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, Fe^{2+}, Cu^{2+} and Ni^{2+}at final concentration of 1 mM on the activity of lipase was determined by incubating 0.5 ml of enzyme with 0.5 ml various cations

separately for two hours and the residual activity determined using paranitrophenyl laurate as substrate and expressed in terms of percentage as in section 3.2.25.

3.2.29 Determination of the Effects of Some Modulators on Lipase Activity

The effect of phenyl methane solfonyl flouride, EDTA, β-mercaptoethanol and dithiothreitol at 5 mM and 10 mM final concentration each on the activity of lipase was determined according to the method of Zhang *et al*., (2009) with modification. Equal volume of enzyme and solution of the modulators (0.5 ml) each was incubated for two hours and then residual activity was determined and expressed in terms of percentage as in section 3.2.25.

3.2.30 Determination of Stability of Lipase in Some Organic Solvents

The lipase stability in the presence of varying concentrations of some organic solvents 15% (v/v), 30% (v/v) and 45% (v/v) was determined in accordance with the method of Zhang *et al*., (2009) with modification. Lipase (0.5 ml) was incubated with methanol, ethanol, isopropanol, *n*-hexane and butanol at varied concentrations for two hours. The residual activity was then determined by incubating the enzyme with substrate and expressed in terms of percentage as in section 3.2.25.

3.2.31 Preparation of Lipase Immobilized in Calcium Alginate Gel Spheres

The calcium alginate beads immobilization of the purified lipase was carried out according to the method of Bhushan *et al*., (2008) with modification. Exactly 5 g of sodium alginate was dissolved in 30ml distilled water and autoclaved at 121^{o}C for 15 minutes. After cooling at room temperature 20 ml of sodium alginate was mixed with 5 ml of purified lipase and then allowed to

stand for 10 minutes. With the help of a syringe the enzyme/alginate mixture was pumped drop wise in 0.4 M $CaCl_2$ and kept for 1 hour to ensure its complete hardening. The alginate beads were washed with distilled water and finally with 50 mM tris-HCl buffer pH 8.0. The lipase / calcium alginate beads were kept in 0.05 M tris-HCl buffer of pH 8.0 at 4^oC until used.

3.2.32 Determination of Protein Concentration of Calcium Alginate Immobilized Lipase

Protein concentration of calcium alginate immobilized lipase was determined according to Bradfordmethod (1976) with modification. One alginate bead containing 15 µl lipase solution entrapped was put in 285 µl distilled water and 3 ml of the dye was added and allowed to stand for 5 minutes at room temperature. Absorbance was read at 595 nm**.**

3.2.33 Assay of Activity of Calcium Alginate Immobilized Lipase

This was carried out according to the method of Gopinath *et al*., (2005) with modification. One calcium alginate bead containing 15 µl lipase solutions (0.13 mg/ml) were put in 8.905 ml of substrate *p*NPL solution (0.03 mM) and dispensed into each test tube and incubated for 15 minutes. The reaction was stopped by removing the calcium alginate immobilized lipase bead and absorbance was read at 410 nm.

3.2.34Determination of K_m and V_{max} of Immobilized Lipase from *P. aeruginosa*

This was carried out by determining initial velocity studies on immobilized lipase. To achieve this, varying concentration of substrate *p*NPL (0.01 mM to 0.06 mM) were incubated with one bead of calcium alginate immobilized lipase each for this and the activity was determined there

after as described in section 3.2.17 to obtain the initial velocity data needed for Lineweaver-Burk plot. From the plot, $K_m$and V_{max} of the immobilized lipase were determined.

3.2.35 Effect of pH on the Activity of Immobilized Lipase from *P. aeruginosa*

The optimal pH of calcium alginate immobilized lipase activity was determined by incubating one bead in each case with *p*NPL (0.03 mM) at different pH ranging from 5 to 9 for 15 minutes. The reaction was then stopped by removing the calcium alginate immobilized lipase bead and the activity was determined as described in section 3.2.24.

3.2.36 Determination of pH Stability of Immobilized Lipase from *P. aeruginosa*

One mililitre of buffer at various pH (5.0 -9.0) was incubated in each case with three beads of calcium alginate immobilized lipase for two hours and residual activity was determined after incubating one of the beads with *p*NPL for 15 minutes and expressed in terms of percentage as described in section 3.2.25.

3.2.37 Determination of the Effect of Temperature on the Activity of Immobilized Lipase from *P. aeruginosa*

The optimal temperature of calcium alginate immobilized lipase activity was determined by incubating one bead with *p*NPL at different temperatures ranging from 25^o to 70°C for 15 minutes. The reaction was stopped by removing the calcium alginate immobilized lipase bead and the activity was determined as described in section 3.2.17.

3.2.38 Determination of Thermostability of Immobilized Lipase from *P. aeruginosa*

One mililitre of buffer each containing three beads of calcium alginate immobilized lipase was incubated at different temperatures ranging from 25^{o}C to 70^{o}C for two hours. The residual activity of the pre incubated immobilized lipase was then determined by incubating one bead with substrate and expressed in terms of percentage as described in section 3.2.25.

3.2.39 Determination of the Effects of Some Modulators on Immobilized Lipase Activity

The effect of phenyl methane solfonyl flouride, EDTA, β-mercaptoethanol and dithiothreitol at 5 mM and 10 mM each final concentration on the activity of immobilized lipase was determined according to the method of Zhang *et al*., (2009) with modification. Two mililitre of various solutions of modulators was incubated with three beads each of calcium alginate immobilized lipase for two hours. The residual activity was determined by incubating one bead with substrate and expressed in terms of percentage as described in section 3.2.25.

3.2.40 Determination of the Effects of Some Cations on Immobilized Lipase Activity

The effect of Mg^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, Fe^{2+},Cu^{2+} and Ni^{2+}at final concentration of 1 mM on the activity of immobilized lipase was determined by incubating two mililitre of various cations with three beads each of calcium alginate immobilized lipase for two hours. The residual activity was determined as described in section 3.2.40.

3.2.41 Determination of Stability of Immobilized Lipase in Some Organic Solvents

The lipase stability in the presence of some organic solvents 15% (v/v), 30% (v/v) and 45% (v/v) was determined in accordance with the methods of Zhang *et al*., (2009) with modification. Two

millilitre each of the varied concentration of methanol, ethanol, isopropanol, *n*-hexane and butanol were incubated with three beads each of calcium alginate immobilized lipase for two hours. The residual activity was determined as described in section 3.2.40.

3.2.42 Determination of Reusability of Calcium Alginate Immobilized Lipase

The reusability of calcium alginate immobilized lipase was determiined by carriying out several cycles of transesterification of *Acacia nilotica* seed oil with the set of beads. After each period of transesterification, the beads were removed, washed and used to carry out another round of transesterification process as described in section 3.2.46

3.2.43 Optimization of Biodiesel Production by Immobilized Lipase

To design the optimization experiment, four factors (A) oil–methanol ratio (B), *n*-hexane, (C) enzyme concentration and (D) time were chosen as shown in (Table3.1). The effect of the four independent variables and their cross interactive effects on biodiesel yield was evaluated using response surface methodology and central composite design (CCD) provided in the Design-Expert 7.1.4 (Stat-Ease, Inc) software which allowed the investigation of linear, quadratic and cross product effects of the factors on the response. Data were analysed and fitted in a second order model so as to correlate the response to the independent variables. The statistical analysis of the model was done to evaluate the analysis of variance which included the Fisher's f-test (model significance), its probability values and coefficient of determination (R^2). The fitted polynomial equation was expressed in the form of surface plots to show the relationship between the response and the experimental levels of each of the variables. The general form of the second-order polynomial equation is shown in equation below:

$$Y = \beta_o + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1} \sum_{j=i+1} \beta_{ij} x_i x_j + \varepsilon \quad \text{----------} \quad (3.8)$$

Y=βo+∑i=1kβixi+∑i=1kβiixi2+∑i=1∑j=i+1βijxixj+ε

Where Y is the response (dependent variables), β_o the constant coefficient, β_i, β_{ii} and β_{ij} the coefficient for the linear, quadratic and interaction effect, χ_i and χ_j the factors (independent variables) and ε is the error.

3.2.44 Validation of the Predicted Model

The predicted model generated from the design was validated experimentally according to the four variables within the design space. Experiments predicted by the point prediction system of the design expert software were conducted. Eighteen combinations of variables were used to produce biodiesel and the yields obtained from the experimental results were compared with the predicted yield.

3.2.45. Transesterification Reaction

Transesterification reaction was carried out according to the method of Kumari *et al*., (2009) with modification in an Elenmeyer flask. The initial reaction mixture contained (98.55 ml) oil / methanol (1:10), three beads of calcium alginate immobilized lipase, 1.20 ml *n*-hexane which serves a binder and stabilizer. These were incubated for 195 minutes at 45 ºC. At the end of the incubation period the reaction was stopped by removing the calcium alginate immobilized lipase beads and 500 µl was taken from the reaction mixture for analysis.

3.2.46 Recovery and Purification of the Produced Biodiesel

Crude biodiesel was purified by gravity separation according to the method of Gerpen (2005) with modification. The crude biodiesel produced was allowed to stand for four hours in a separating funnel for phase separation to occur. Biodiesel was the top layer while glycerol and the calcium alginate immobilized lipase beads were at the bottom layer. The glycerol and the beads were run off first retaining the biodiesel in the separating funnel. Further purification of the biodiesel was carried out using distilled water at 50^{o}C to wash the biodiesel in the separating funnel three times to remove the residual methanol and glycerol. The biodiesel was also heated at 70^{o}C to remove the remnant of *n*-hexane.

3.2.47 Determination of Percentage Conversion of Fatty Acid Methyl Ester (%)

The percentage (%) conversion of methyl ester was measured by determining the remaining unreacted fatty acid in the reaction mixture by titration with 0.1 M NaOH according to the method of Radzi *et al.*, (2005). The percentage conversion of methyl ester was calculated using the formula below:

$$\text{Conversion of methyl ester (\%)} = \frac{\text{Vol. of NaOH used without enzyme} - \text{Vol. of NaOH used with enzyme}}{\text{Vol. of NaOH used without enzyme}} \times 100 \text{----------}$$

.3.9

3.2.48 Analysis of Methyl Ester by Gas Chromatography-Mass Spectrometry

The Fatty acid ester was analyzed by Gas chromatography- mass spectrometry as described earlier in section 3.2.4 above.

3.2.49 Determination of Specific Gravity of Biodiesel

A clean and dried bottle was weighed (W_0) and 10ml of biodiesel was added, stoppered and reweighed to give (W_1). The specific gravity of the biodiesel produced was determined as described in section 3.2.5 and calculated using the formula below:

$$\text{Specific gravity} = \frac{W_1 - W_0}{W_2 - W_0} \quad \text{---------- Equation 3.1}$$

3.2.50 Determination of Refractive Index of Biodiesel Produced

Refractive index of the biodiesel produced was determined as described in section 3.2.6

3.2.51 Determination of Viscosity of the Biodiesel Produced

Viscosity of the biodiesel produced was determined using the NDJ-85 rotary viscometer as described in section 3.2.7.

3.2.52 Determination of Calorific Value of the Biodiesel Produced

The calorific value of the biodieselproduced was measured according to the method of Gerpen *et al.,* (2005) by placing 100 ml of *A. nilotica* biodiesel in the sample cup in a steel container which was filled with oxygen at thirty atm. and electrically ignited. The heat produced by the combustion reaction was determined by recording the rise in temperature of the bomb calorimeter as a calorie is the amount of heat required to raise the temperature of 1.0 g of water from 14.5^{o}C to 15^{o}C.

3.2.53 Determination of Cetane Number of the BiodieSsel Produced

The cetane number of the biodieselproduced was determined according to Krisnangkura (1986) using the following formula below:.

$$CN = 46.3 + \frac{5458}{SV} - 0.225 \times IV$$ ----------Equation 3.11

Where CN= cetane number, SV=saponification value and IV= iodine value

3.2.54 Determination of Flash Point of the Biodiesel Produced

This was carried out according to the ASTM D 93 (2000) with modification using Clevaland opetha bottom cup. One hundred millilitre of *A. nilotica* biodiesel was put into the brass cup of the machine at room temperature. A thermometer was held vertically by means of a clamp in such a way that the bottom of the bulb was about 1 cm above the bottom of the cup. The electrical heating device was switched on, the thermometer reading was observed at every 5^{o}C rise in temperature and a standard test flame was brought near the surface of the biodiesel to see whether a flash would appear at any point on the surface of the biodiesel. The minimum temperature at which a distinct flash appeared on the surface of the biodiesel in the brass cup was recorded as the flash point.

3.2.55 Determination of Cloud Point of the Biodiesel Produced

The cloud point of the biodiesel produced was determined by pouring 20 ml of the biodiesel into a conical flask and the clamp carrying the thermometer was held tightly. The position of the thermometer was adjusted so that it rested at the bottom for uniformity. The conical flask was placed on ice bath and the set up was inspected after every five minutes for cloud formation. The

temperature at which distinct cloudiness formed at the bottom of the tube was recorded as the cloud point of the biodiesel produced.

3.2.56 Determination of Pour Point of the Biodiesel Produced

The pour point of the biodiesel produced was determined by pouring 20 ml of the biodiesel into a cocked conical flask with an opening through which thermometer was inserted. The conical flask was placed on ice bath and inspected after every five minutes to determine the temperature at which the sample started melting which was recorded from the thermometer as the pour point of the biodiesel produced.

3.2.57 Determination of Acid Value of the Biodiesel Produced

Acid value of the *A. nilotica*biodiesel was determined by titrimetric method. Five grams of *A. nilotica* biodiesel was poured into a conical flask and 25 ml of fat solvent 95% ethanol: ether 1:1 (v/v) was added. The content shaken, 2 drops of phenolphthalein were added and shaken again. It was then titrated against 0.1 M solution of KOH. A blank titration was carried out without the biodiesel sample. The acid value was calculated from the formula below:

$$\text{Acid value mg KOH/g fat} = \frac{\text{Titre value x Nomality of KOH}}{\text{Weight of biodiesel (g)}} \text{ x } 56.1 \quad \text{----------3.12}$$

56.1 is a multiplication factor because 1 ml of 1 N KOH contains 56.1 mg KOH.

3.2.58 Determination of Saponification Value of the Biodiesel Produced

The saponification value of *A. nilotica* biodiesel was determined by titrimetric method. One gram of *A. nilotica* biodiesel was dissolved in 3 ml of fat solvent 95% ethanol: ether1:1 v/v

followed by addition of 25 ml of 0.5 M ethanoic KOH. The mixture was refluxed for an hour and allowed to cool at room temperature. Two drops of 1% phenolphtalein was added and titrated with 0.5 M HCl till the pink colour disappeared. The blank titration of sample without biodiesel was carried out under the same experimental condition used for *A. nilotica* biodiesel. The saponification value was then calculated using the formula below:

$$\text{Saponification values} = \frac{\text{Titre value}}{\text{Weight of the biodiesel (g)}} \times 28.05 \quad \text{---------- 3.13}$$

28.05 is a multiplication factor because 1ml of 0.5 N KOH contains 28.05mg KOH.

3.2.59 Determination of Iodine Value of the Biodiesel Produced

The iodine value of *A. nilotica* biodiesel was determined by titrimetric method. One gram of the biodiesel produced was dissolved in 10 ml of fat solvent 95% ethanol: ether 1:1 v/v in a stoppered flask and 25 ml of iodine monochloride solution was added. The flask was then kept in dark cupboard for one hour. After one hour, 50 ml of water and 10 ml of 5% potassium iodide solution were added. The liberated iodine was titrated with 0.1 M sodium thiosulphate till the content became pale yellow. Afterward two drops of 0.1% starch soltion was added and titrated again with sodium thiosulphate till the blue colour disappeared. A blank was prepared by replacing *A. nilotica* biodiesel with chloroform and titrated in similar manner as the test sample. The iodine value was then calculated using the formula below:

$$\text{Iodine Value} = \frac{(b-a) \times 12.7}{1000} \times \frac{100}{\text{Weight of the biodiesel (g)}} \quad \text{---------- 3.14}$$

Where b= Volume of thiosulphate used for blank (ml)

a= Volume of thiosulphate used for the sample (ml)

w =weight of the biodiesel sample (g)

One litre of 0.1N iodine solution contains 12.7 g of iodine

3.2.60. Determination of Peroxide Value of the Biodiesel Produced

The peroxide value of *A. nilotica* biodiesel was determined by titrimetric method. One gram of *A. nilotica* biodiesel was placed into a dried tube, 20 ml of glacial acetic acid chloroform mixture (2:1) was added and the content was boiled for one minute. The content was thereafter poured into a flask containing 20 ml of 5% potassium iodide solution and then titrated with 0.002M sodium thiosulphate using 2 drops of 0.1% starch indicator. The blank titration was also carried out by replacing *A. nilotica* biodiesel with chloroform and titrated in similar manner as the test sample. The peroxide value of biodiesel was then calculated using the formula below:

$$\text{Peroxide value} = \text{b} - \text{a x Molarity of } Na_2S_2O_7 \text{ x } \frac{100}{W} \quad \text{----------3.15}$$

Where b= volume of thiosulphate used for blank (ml)

a= volume of thiosulphate used for the biodiesel (ml)

w =weight of biodiesel (g)

3.2.61 Statistical Analysis

The results were expressed as mean±SD of three replicates at 95 % confidence interval, the data were analysed by analysis of variance (ANOVA) using SPSS program (version 20.0 SPSS Inc. Chicago IL, USA)

CHAPTER FOUR

4.0 RESULT

4.1. Physicochemical Characterization of *Acacia nilotica* Seed Oil

4.1.1 Some Physical Properties of *Acacia nilotica* Seed Oil

Table 4.1 shows some physical properties of *Acacia nilotica* seed oil. The oil content of *Acacia nilotica* extracted by soxhlet method was found to be 13.5%. It was odourless and amber brown in colour. Specific gravity of the oil was 0.84 and refractive index of 1.34. The oil was liquid at room temperature and its viscosity was 4.03 Pa. s. The calorific value was 29.0 (MJ/kg) and the cetane number was 32.9.

4.1.2 Some Chemical Properties of *Acacia nilotica* Seed Oil

Table 4.2 shows some chemical properties of *Acacia nilotica* seed oil. The iodine value was 187 $gI_2/100\,g$, acid value was 6.25 mgKOH/g, saponification value was 189 mgKOH/g and peroxide value was 6.47 meq/kg respectively.

4.1.3 Fatty Acid Profile of *Acacia nilotica* Seed Oil.

The percentage fatty acid profile of *Acacia nilotica* seed oil is shown in Table 4.3. The fatty acid composition indicated the presence of octadecenoic acid in the highest proportion of (69.14%). Other fatty acids present included, hexadecanoic and octadecanoic acid at concentration of 15.47 and 7.92% respectively. While those present at low concentration included pentaflouropropionic acid (1.90%), eicosanoic acid (1.02%), docosanoic acid (1.11%) and pentadecanoic acid (0.64%) respectively. Others had 2.80%. See chromatogram in Appendix i

Table 4.1 Some Physical Properties of *Acacia nilotica* Seed Oil

Properties	*Acacia nilotica* Seed Oil
Oil content (%)	13.5
Odour	Odourless
Colour	Amber brown
Specific gravity	0.84 ± 0.01
Physical state at room temperature	Liquid
Refractive index	1.34 ± 0.01
Viscosity Pa.S	4.03
Calorific value (MJ/kg)	29.0 ± 0.10
Cetane number	32.9±0.05

The values were expressed as Mean ±SD of three replicates

Table 4.2 Some Chemical Properties of *Acacia nilotica* Seed Oil

Properties	*Acacia nilotica* Seed Oil
Iodine value g I_2/100 g	187±0.53
Acid value (mg KOH/g)	6.25±0.01
Saponification value (mg KOH/g)	189.±0.25
Peroxide value (meq/kg)	6.47±0.01

The values were expressed as Mean ±SD of three replicates

Table 4.3 Fatty Acid Profile of *Acacia nilotica* Seed Oil

Acid Type	**Retention Time**	**Proportion (%)**	**Similarity Index**
Hexadecanoic acid	19.42	15.47	93
Octadecenoic acid	22.63	69.14	92
Ocadecanoic acid	22.92	7.92	92
Pentaflouropropionic acid	25.14	1.90	83
Eicosanoic acid	25.36	1.02	90
Docosanoic acid	27.31	1.11	91
Pentadecanoic acid	29.24	0.64	81
Others		2.80	

4.2 Time Course of Lipase Production by*P.aeruginosa*

The time course of lipase production from *P. aeruginosa* is shown in Figure 4.1. The time at which maximum lipase production occurred was between 48 to 72 hours of incubation, but highest lipase production of 8.20 U/ml) was obtained at 48 hours of incubation.The lipase activity decreased after 48 hours to 7.8 U/ml at 72 hours and continued to 96 hours.

4.3 Purification of Lipase from *P.aeruginosa*

The elution profile of *P. aeruginosa* lipase from DEAE-cellulose ion exchange column is presented in Figure 4.2. From the figure it can be seen that fractions 20 to 25 contained the eluted lipase and had the average lipase activity of 15.07 U/ml. These lipase active fractions were eluted at relatively low concentration of NaCl (0.5 mM). Figure 4.3 shows the elution profile of lipase from sephadex G-75 column. The fractions 29 to 32 were the lipase active fractions and had average activity of 19.20 U/ml and protein concentration of 0.13 mg/ml.

The summary of purification profile of lipase from *P. aeruginosa* is presented in Table 4.4. The crude lipase had the total activity of 1032 U/ml, total protein concentration of 32.4 mg/ml, specific activity of 31.9 U/mg, yield, purification fold of 100% and 1.0 respectively. The cold acetone precipitation reduced the total activity to 570.0 U/ml, total protein to 11.4 mg/ml, yield to 55.2%, increased specific activityand purification foldto 62.3 U/mg and 1.95 respectively. At the ion exchange chromatographic (DEAE-cellulose) stage of purification, the enzyme had a total activity of 361.9 U/ml, total protein concentration of 4.3 mg/ml, specific activity of 83.7 U/mg, yield and purification fold of 35.06% and 2.62 respectively. The gel filtration (sephadex G-75) purified fraction had the total activity of 307.2 U/ml, total protein of 2.08, specific activity of 147.7 U/mg protein, yield and purification fold of 29.8% and 4.63 respectively.

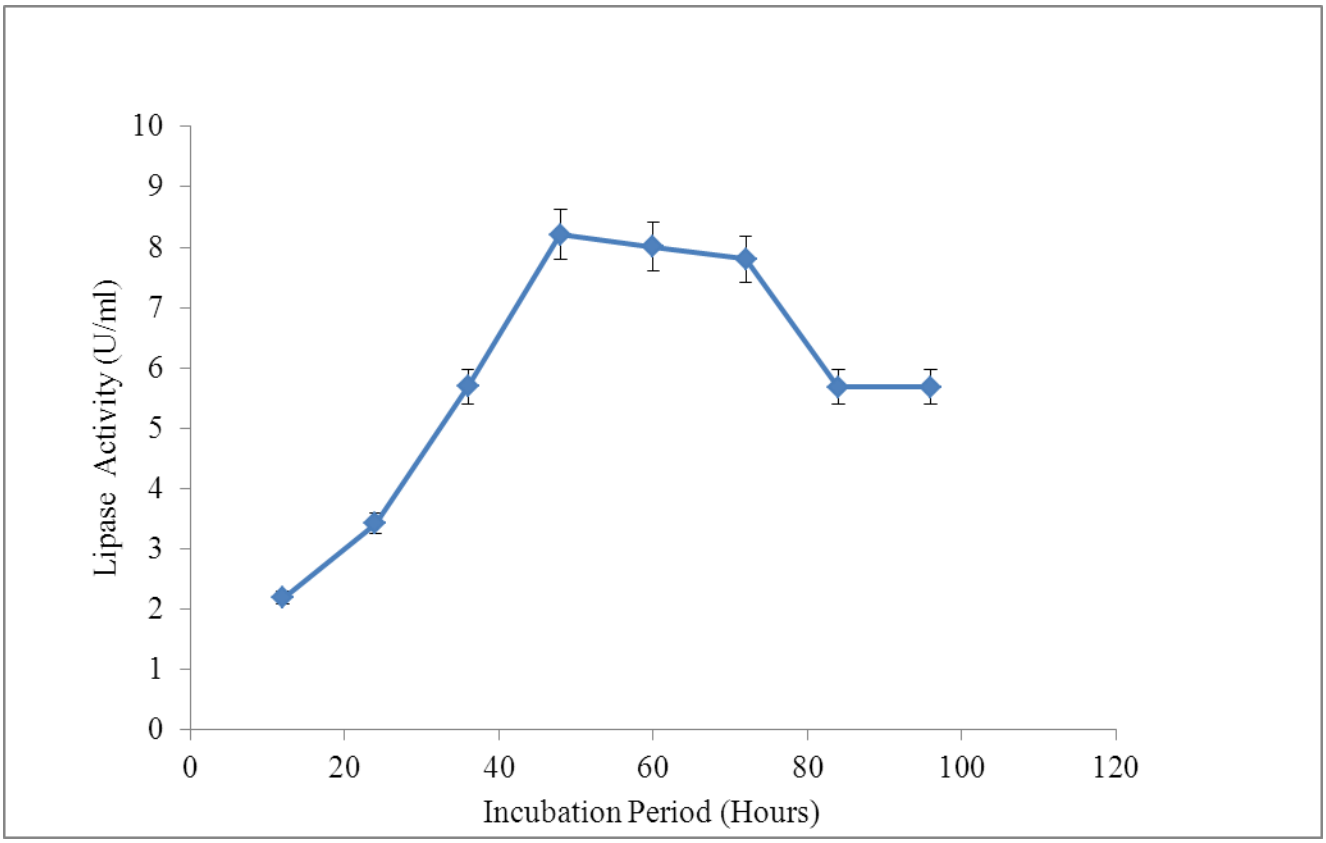


Figure 4.1 Time Course of Lipase Production by *P. aeruginosa*

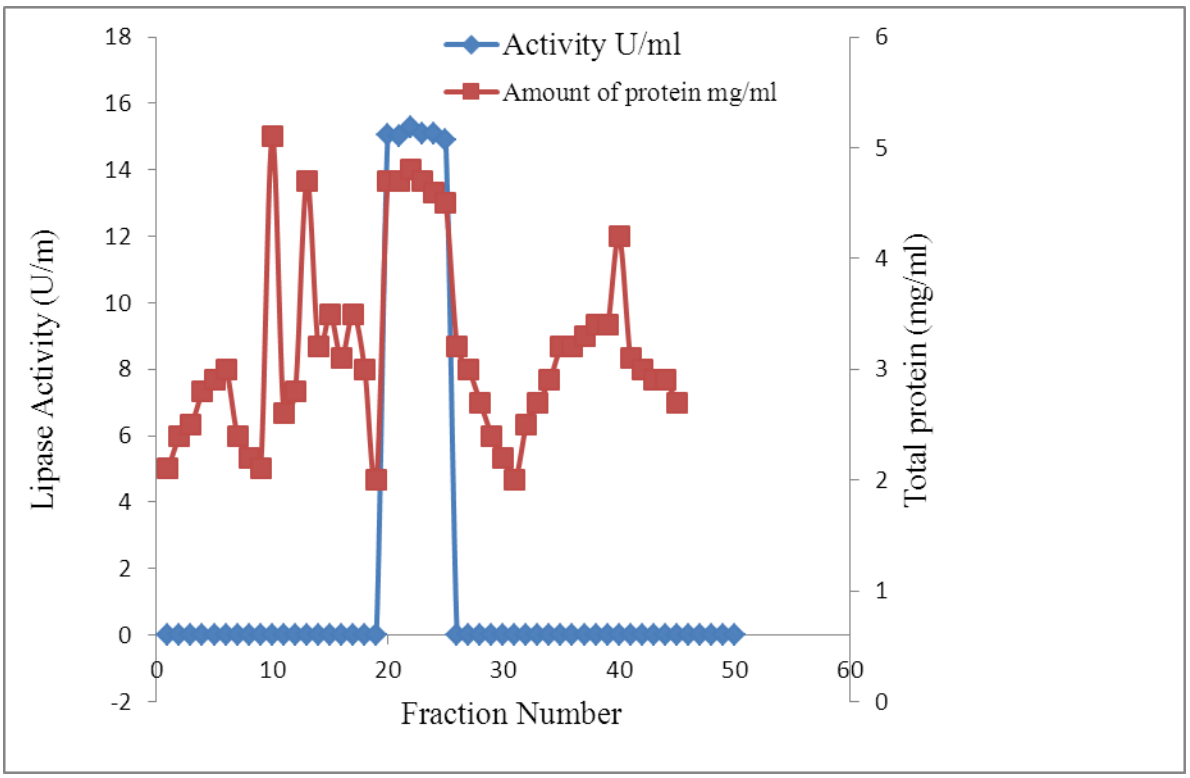


Figure 4.2 Elution Profile of *P. aeruginosa* Lipase from DEAE-Cellulose Column Eluted with Gradient of NaCl (0.1 to 1.0 mM)

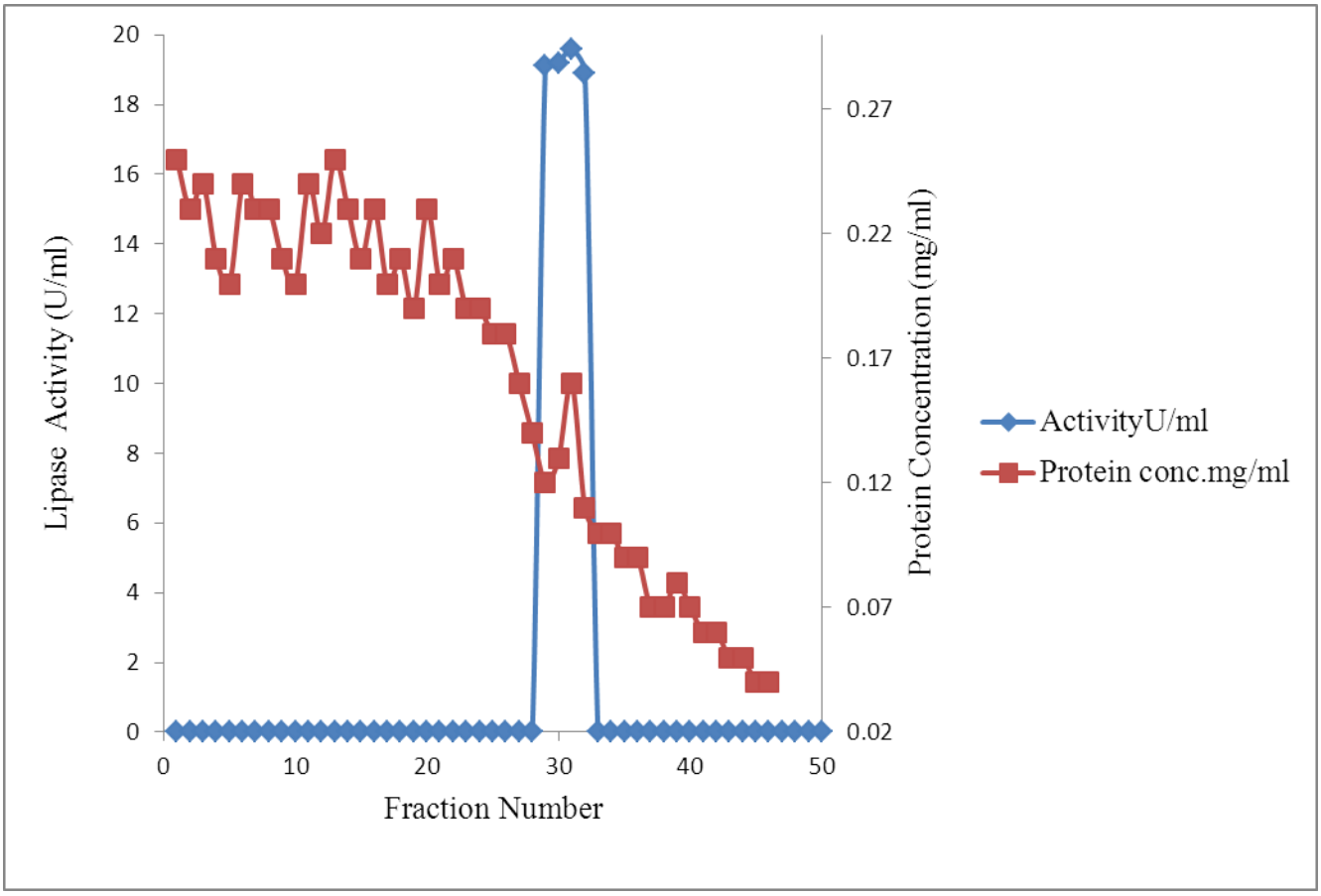


Figure 4.3. Elution Profile of *P. aeruginosa*Lipase from Sephadex G-75 Column Eluted with Tris -HCl Buffer

Table 4.4 Purification Profile of Lipase from *Pseudomonas aeruginosa*

Purification steps	Activity((U/ml)	Total activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)	Yield (%)	Purification Fold
Crude fraction (120 ml)	8.60	1032.0	32.4	31.9	100.0	1.0
Cold acetone precipitation (50 ml)	11.4	570.0	11.4	62.3	55.2	1.95
Ion exchange Chromatography (24 ml)	15.08	361.9	4.34	83.7	35.06	2.62
Gel filtration (16 ml)	19.2	307.2	2.08	147.7	29.8	4.63

4.4 Characterization of Partially Purified Lipase from *P.aeruginosa*

The Lineweaver-Burk plot of lipase obtained using the initial velocity data determined with different substrate concentration shows that free lipase from *P. aeruginosa* had K_m and V_{max} values of 0.07 mM and 55.5 µmol min^{-1} respectively as shown in Figure 4.4.

4.4.1 Effect of pH on the Activity and Stability of Partially Purified Lipase from *P. aeruginosa*

The effect of pH on the activity of *P. aeruginosa* lipase is shown on Figure 4.5. The enzyme had optimum activity of 19.0 U/ml at pH 7.5 as well as 18.03 U/ml at 8.0, but no activity was detected at pH below 5.5 and above 9. The pH stability plot of free lipase is shown in Figure 4.6. At pH 6, (68.4%) of the activity was retained, but the enzyme showed 100% stability in the pH range of 7.5 to 8.5. However, there was a decrease in stability at pH 9.0 although 70% activity was retained.

4.4.2 Effect of pH on k_m and v_{max} of Partially Purified Lipase from *P. aeruginosa*

The effect of pH on the K_m and V_{max} on free lipase is shown on Figure 4.7. The Dixon Webb plot gave the pKa values of 6.50 and 7.0 respectively.

4.4.3 Effect of Temperature on the Activity and Stability of Partially Purified Lipase from *P. aeruginosa*

The effect of temperature on the activity of *P. aeruginosa* lipase is shown on Figure 4.8. The enzyme was active in the temperature range of 30°C-65 °C. The maximum activity of 19.2 U/ml each was achieved at temperature 45°C and 50°C respectively, but the activity decreased to 18.8 U/ml at 55°C with the lowest activity of 17.7 U/ml at 60°C and 65°C respectively. The thermo

stability of lipase is shown in Figure 4.9. The enzyme showed 83.4% and 89.6 % stability at 35°C and 40°C respectively, but showed 100 % stability at 45°C and 50°C retaining 83.8% at 55°C and 80.2% each at 60°C and 65°C respectively,

4.4.4 Activation Energy (Arrhenius plot) of Partially Purified Lipase from *P. aeruginosa*

Figure 4.10 shows the activation energy plot of lipase from *P. aeruginosa*. From the slope of the plot the activation energy of 301.99 Kcal $mol^{-1}$was obtained.

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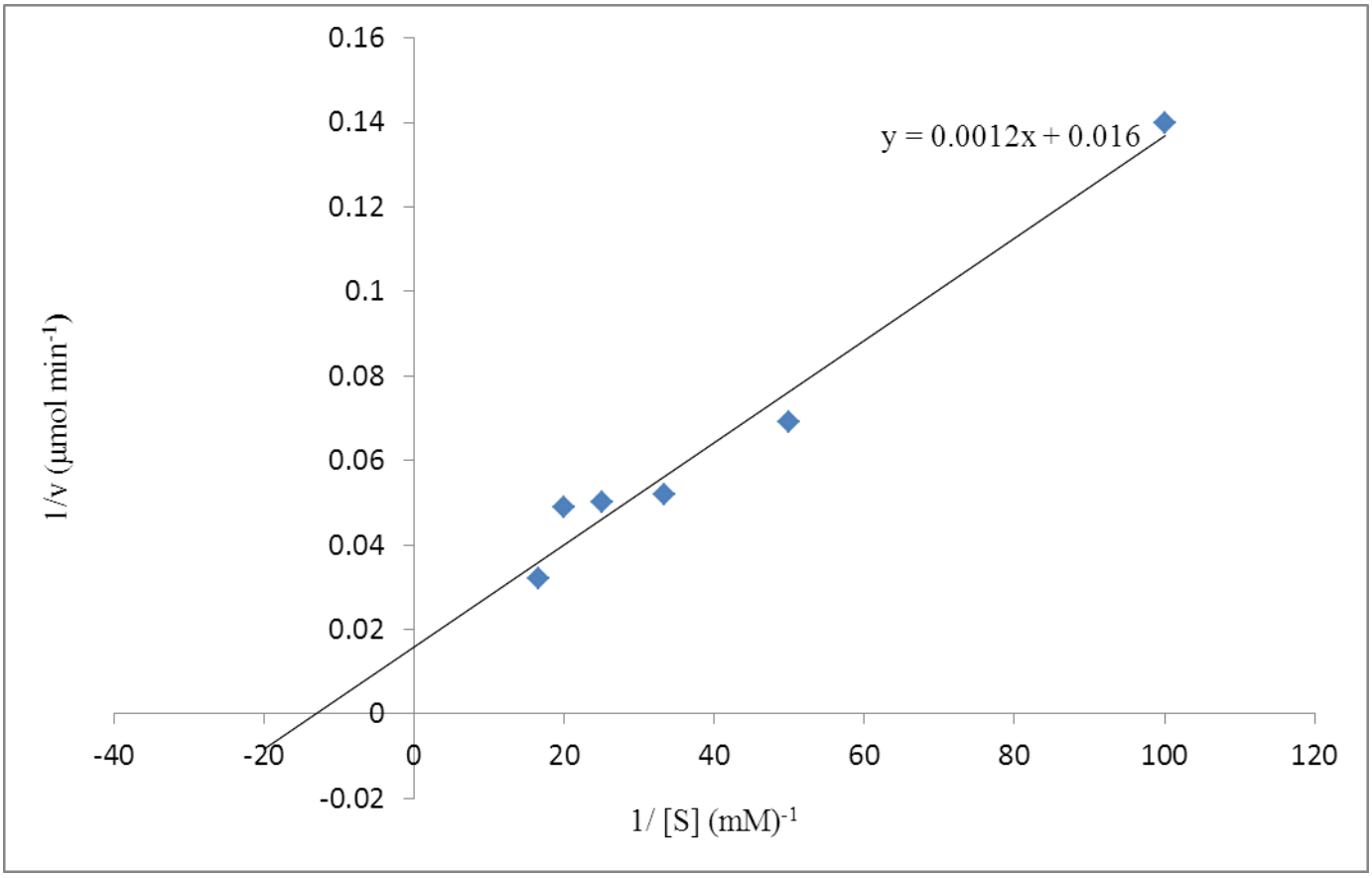


Figure 4.4. Lineweaver-Burk Plot of Partially Purified Lipase from *P. aeruginosa*

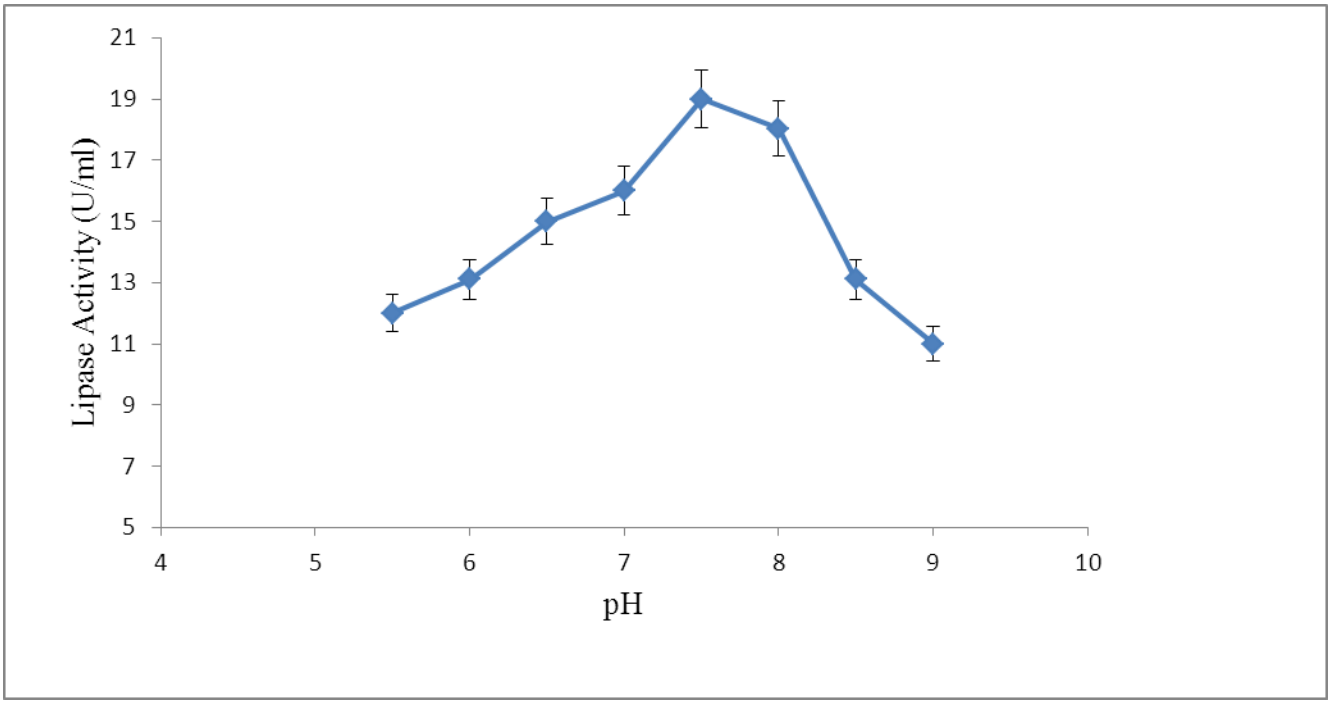


Figure 4.5. Effect of pH on the Activity of Partially Purified Lipase from *P. aeruginosa*

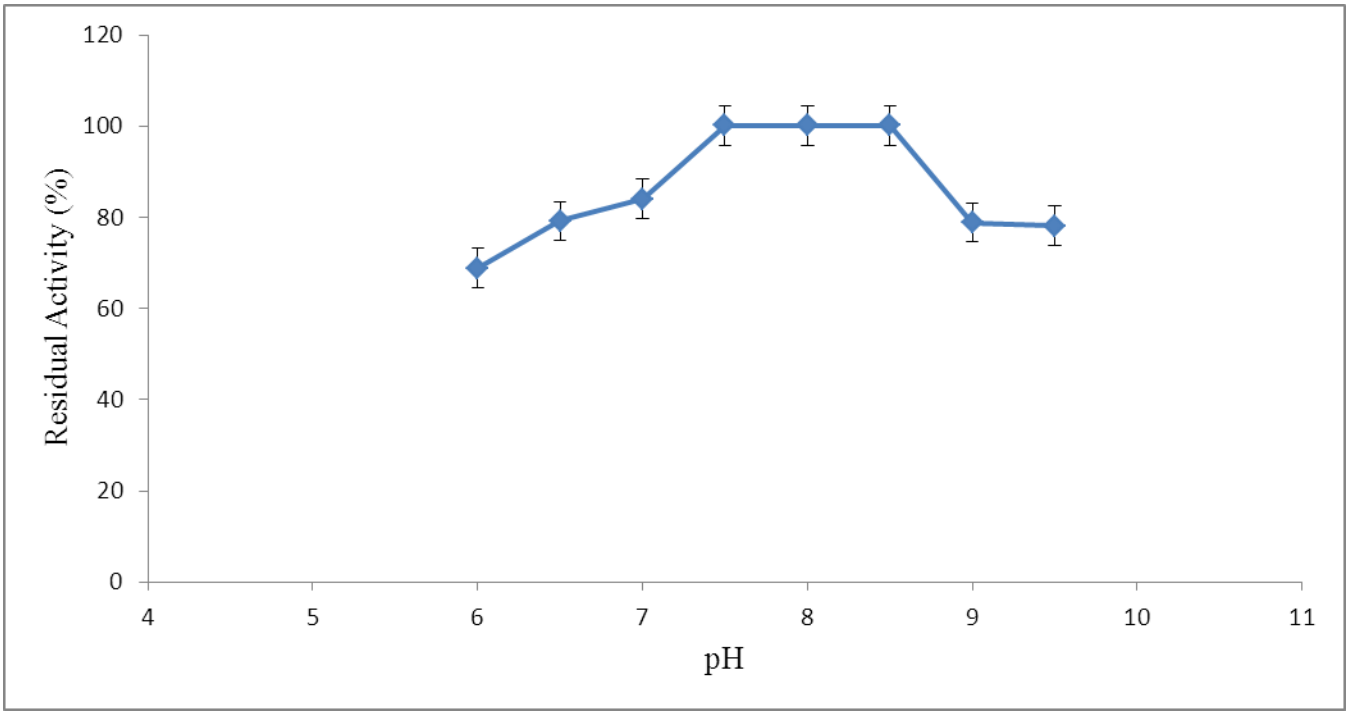


Figure 4.6 Effect of pH on the Stability of Partially Purified Lipase from *P. aeruginosa*

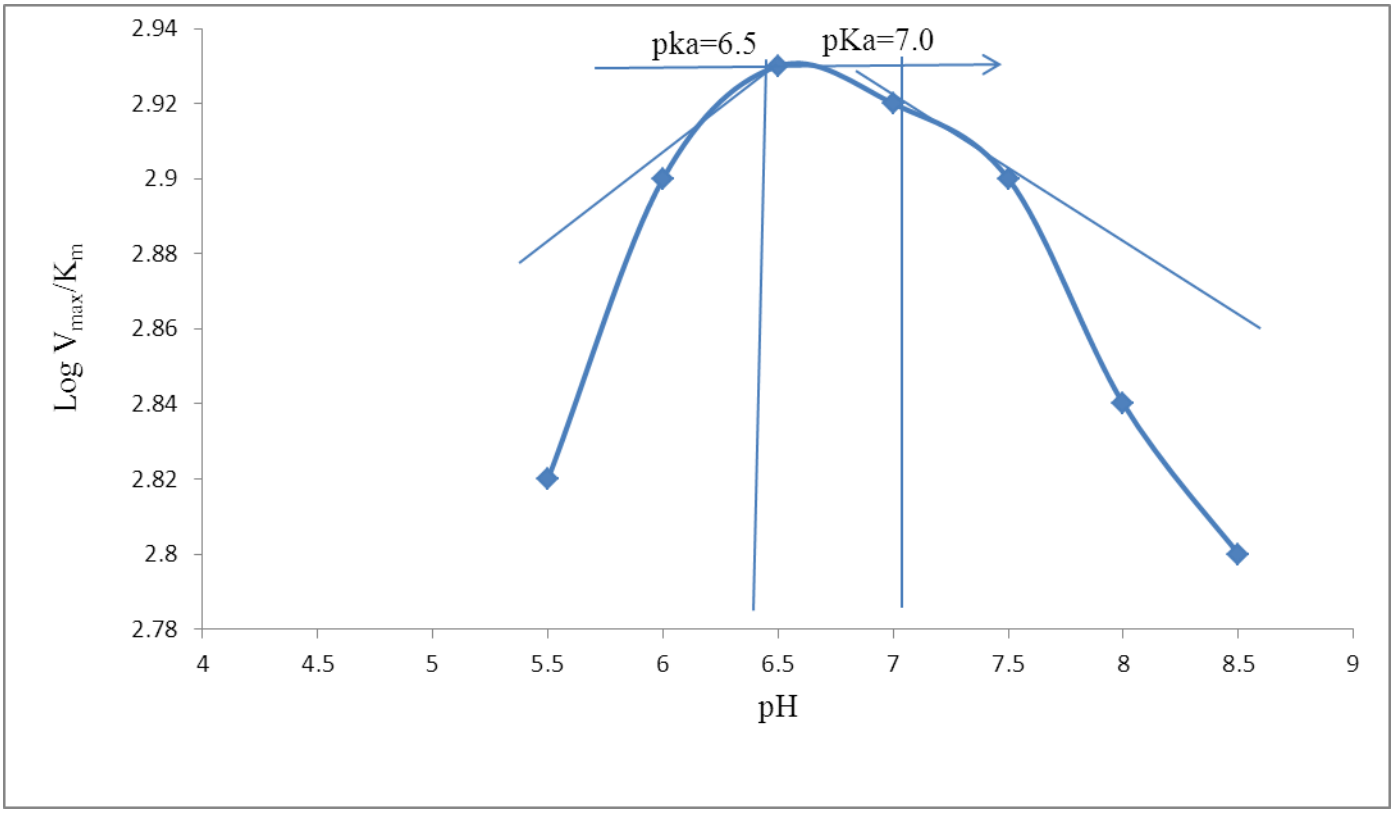


Figure 4.7 Dixon Plot for the Partially Purified *P.aeruginosa* Lipase Indicating the pKas of Amino Acids

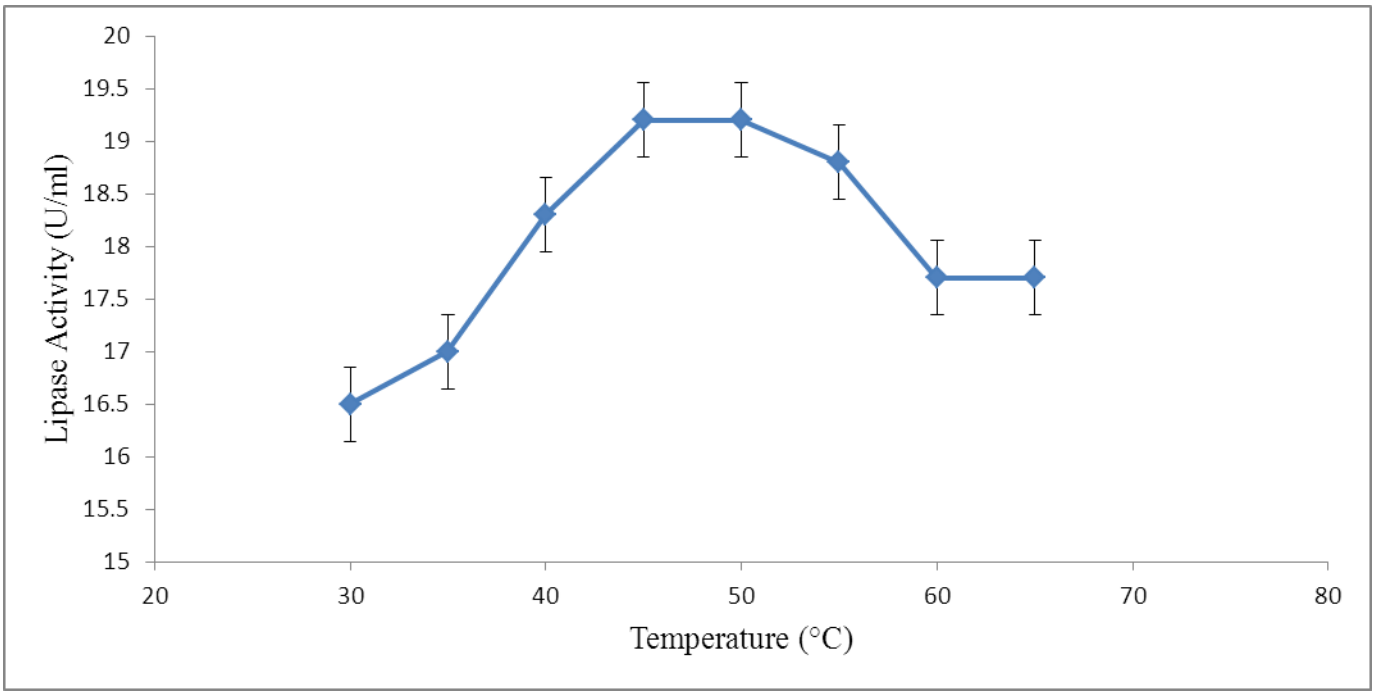


Figure 4.8 Effect of Temperature on the Activity of Partially Purified Lipase from *P. aeruginosa*

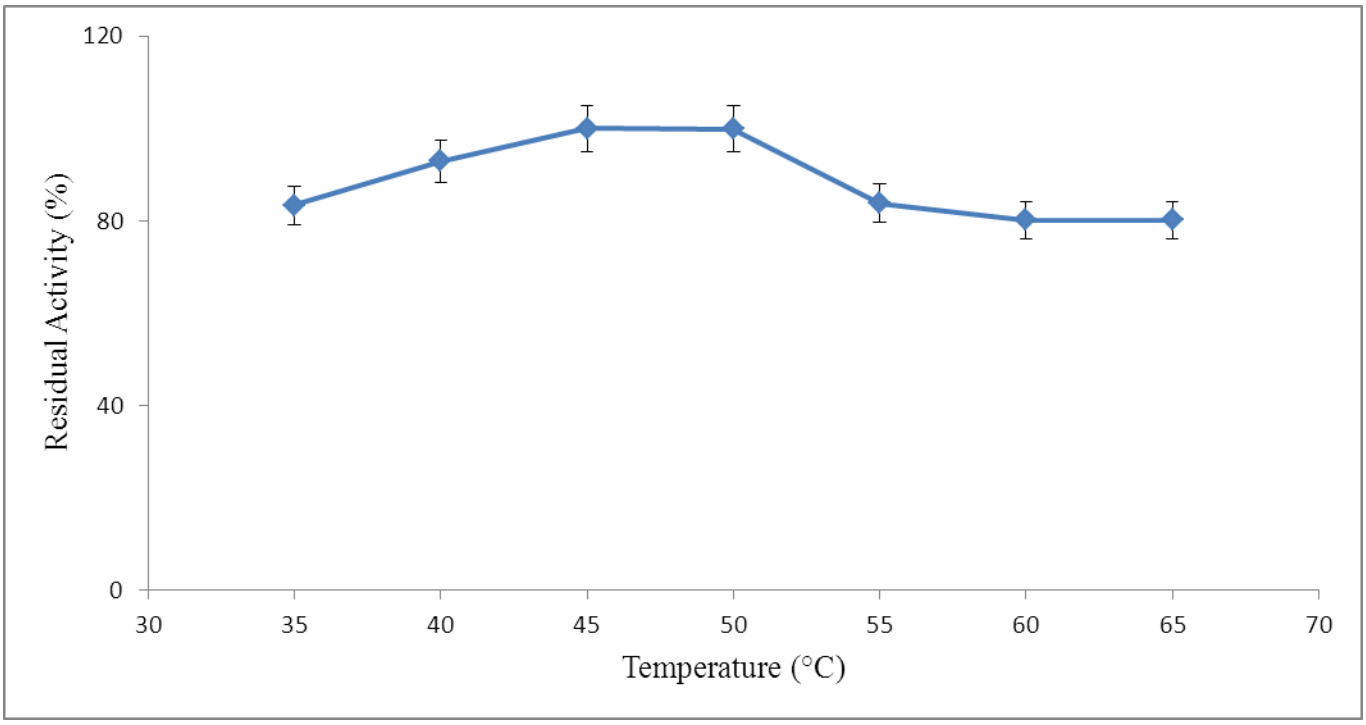


Figure 4.9 Profile of Stability of Partially Purified Lipase from *P. aeruginosa* at varying Temperatures (35^{o}C to 70^{o}C)

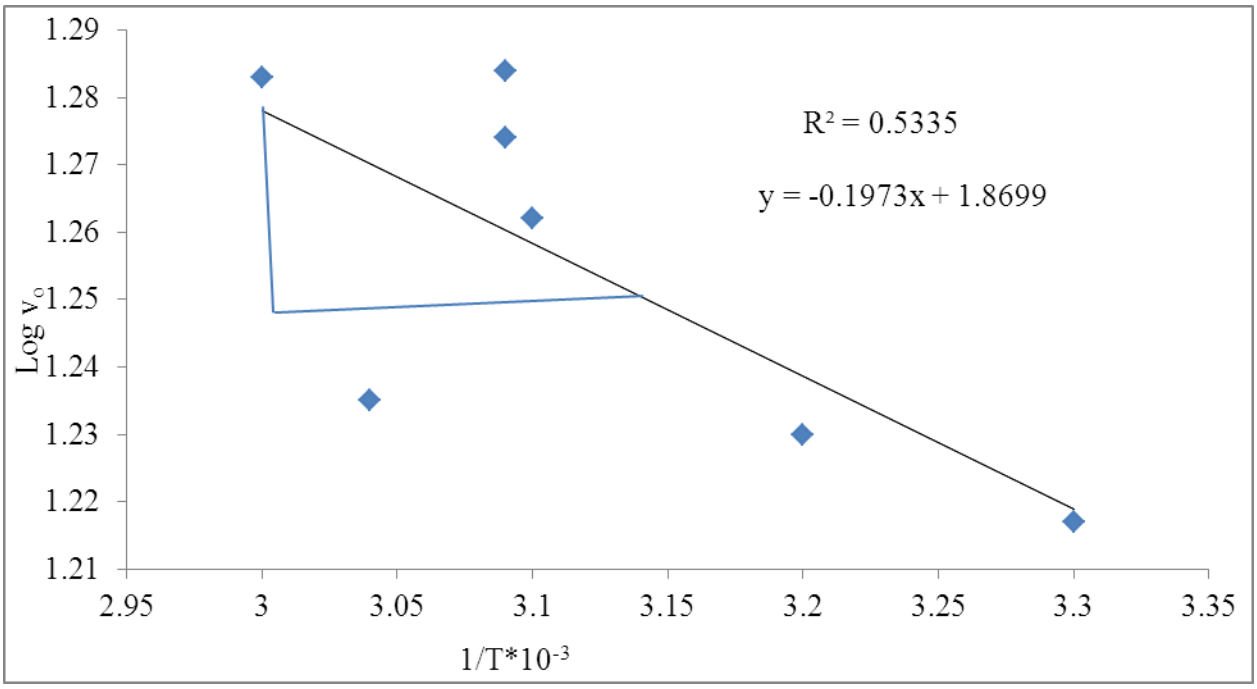


Figure 4.10 Arrhenius Plot of Partially Purified Lipase from *P. aeruginosa*

Plate 4.1 shows the electophoregram of purified *P. aeruginosa* lipase from sephadex G-75 purification step with a single band observed. The purified lipase had an estimated molecular weight of 32.0 kDa as observed from the plot of log.of molecular weights of standard proteins against their relative mobility values and that of purified lipase obtaibed from the electrophoregram as shown in Figure 4.11.

4.4.5 Effect of Some Surfactant on the Activity of Partially Purified Lipase from *P. aeruginosa*

The effect of some surfactants on the lipase activity is shown in Figure 4.12. The activity was enhanced in the presence of gum arabic with the residual activity of 104.0%. The residual activity was found to be 62.9 % in the presence of triton X-100. A low residual activity was found in the presence of CTAB (42.2%), SDS (44.6%) and tween-80 (47.5%) respectively.

4.4.6 Effect of Some Cations on the Activity of Partially Purified Lipase from *P. aeruginosa*

Figure 4.13 shows the dependence of lipase on some divalent cations at 1 mM concentration. The control had 100%, but the enzyme activity was enhanced in the presence of Mg^{2+} and Ca^{2} with the residual activity of 92.4% and 110.0%. The residual activity was found to be 77.2% and 62.9% in the presence of $Co^{2+,}$ Fe^{2+}as well as 64.1%in the presence of Cu^{2+}. A decrease in the enzyme activity was observed in the presence of $Mn^{2+}$and Ni^{2+} with the residual activity of 23.8% and 26.1% respectively.

4.4.7 Effect of Some Modulators on the Activity of Partially Purified Lipase from *P. aeruginosa*

Figure 4.14 shows the effect of some modulators at 5 mM and 10 mM concentrations on the activity of free lipase. The activity retained in the presence of phenyl methane sulfonoyl fluoride

(5 mM and 10 mM) was 90.2% and 83.0% respectively. The enzyme activity was inhibited in the presence of EDTA with the residual activity of 47.1% and 34.2% at concentrations of (5 mM and 10 mM). The residual activity was 102.2% and 94.5% in the presence of 5 mM and 10 mM β-mercaptoethanol respectively. In the presence of 5 mM and 10 mM dithiotrietol the residual activity was 86.1% and 73.7% respectively.

4.4.8 Effect of Some Organic Solvents on the Activity of Lipase

Figure 4.15 shows the effect of some organic solvents at concentration of 15%, 30 % and 45% (v/v) on the activity of *P. aeruginosa* lipase. The enzyme had residual activity of 103.4% in 15% methanol, 50.8 % in 30% methanol and 39.1 % in 45% methanol. The residual activity in the presence of 15% ethanol was 60.5%. In 30% ethanol it was 32.9% as well as 26.1% in 45% ethanol. The residual activity in the presence of 15%, 30% and 45% isopropanol was 80.9%, 59.4% and 49.9% respectively. The enzyme had residual activity of 112.3%, 83.3% and 61.9%in the presence of 15%, 30% and 45% *n*-hexane respectively. The activity retained in 15%, 30% and 45% butanol was 76.0%, 60.5% and 28.1% respectively.

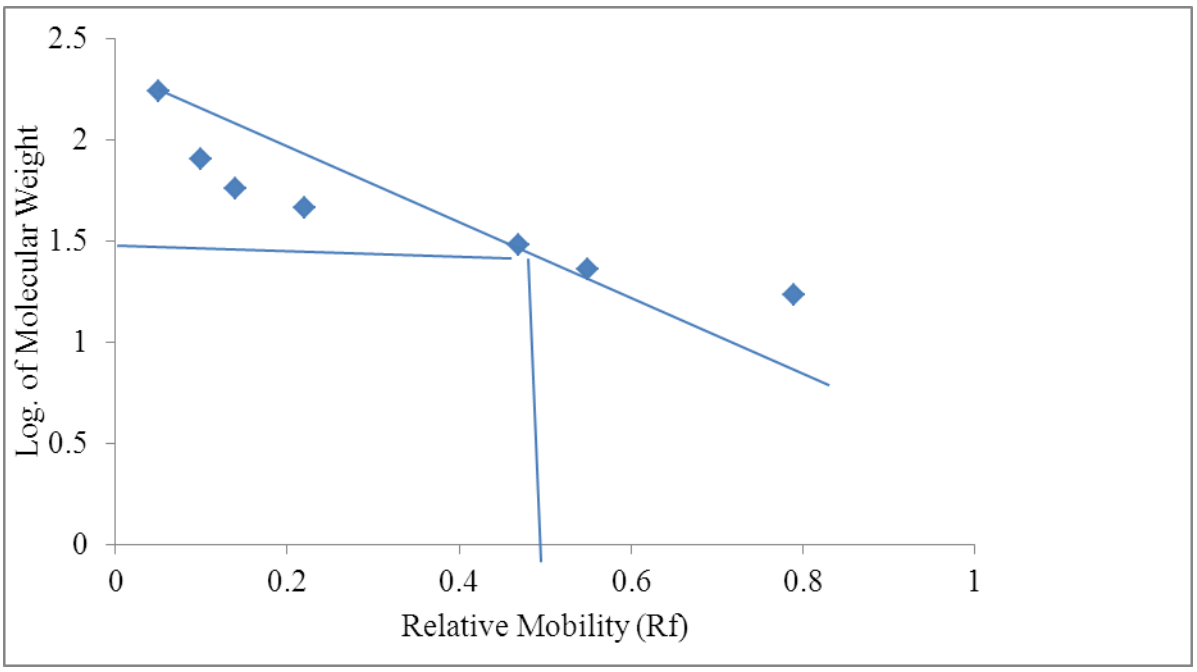


Figure 4.11 Plot of Log of Molecular Weight Against Relative Mobility (Rf) for Determination of Molecular Weight of Partially Purified Lipase from P. *aeruginosa*

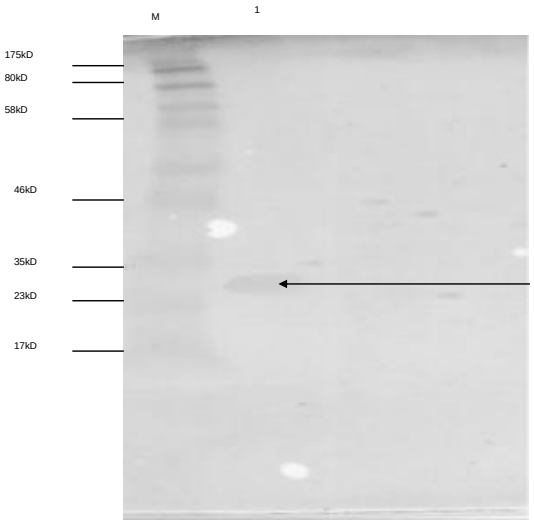


Plate 4.1 SDS-PAGE of Partially Purified Lipase from *P. aeruginosa*: Lane M Contains Molecular Weight Marker and Lane 1 Contains the Partially Purified Lipase

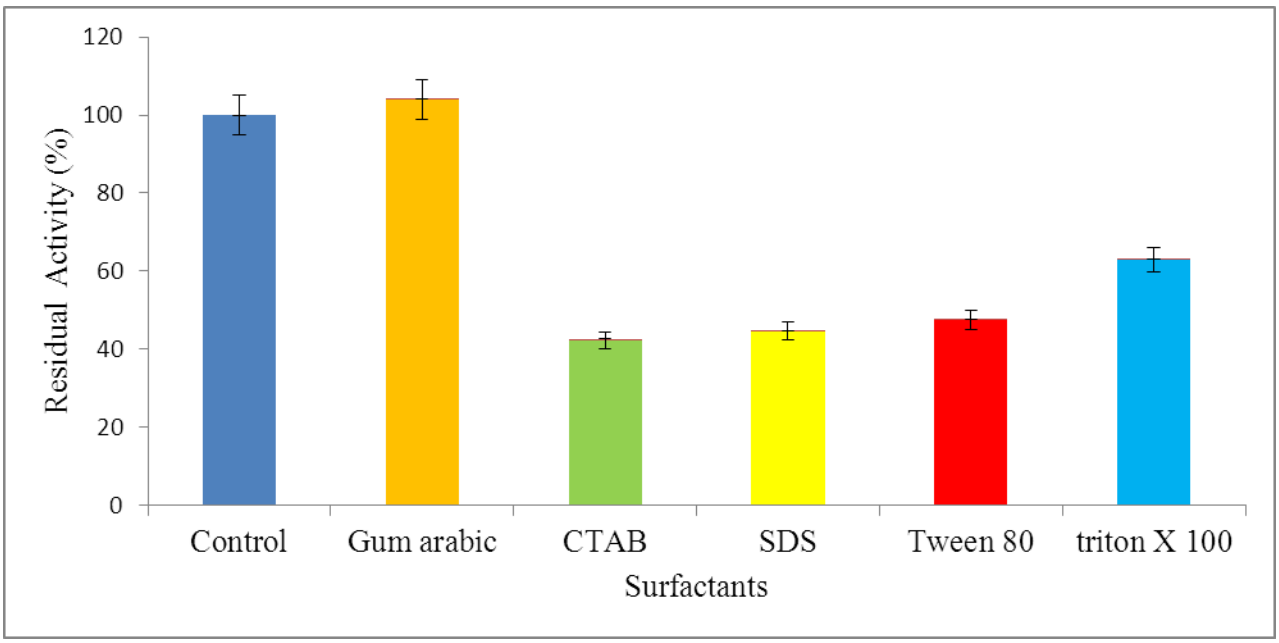


Figure 4.12.Effect of Some Surfactants on the Activity of Partially Purified Lipase from *P.aeruginosa*

CTAB= Cetyl trimethyl ammonium bromide

SDS= Sodium dodecyl sulphate

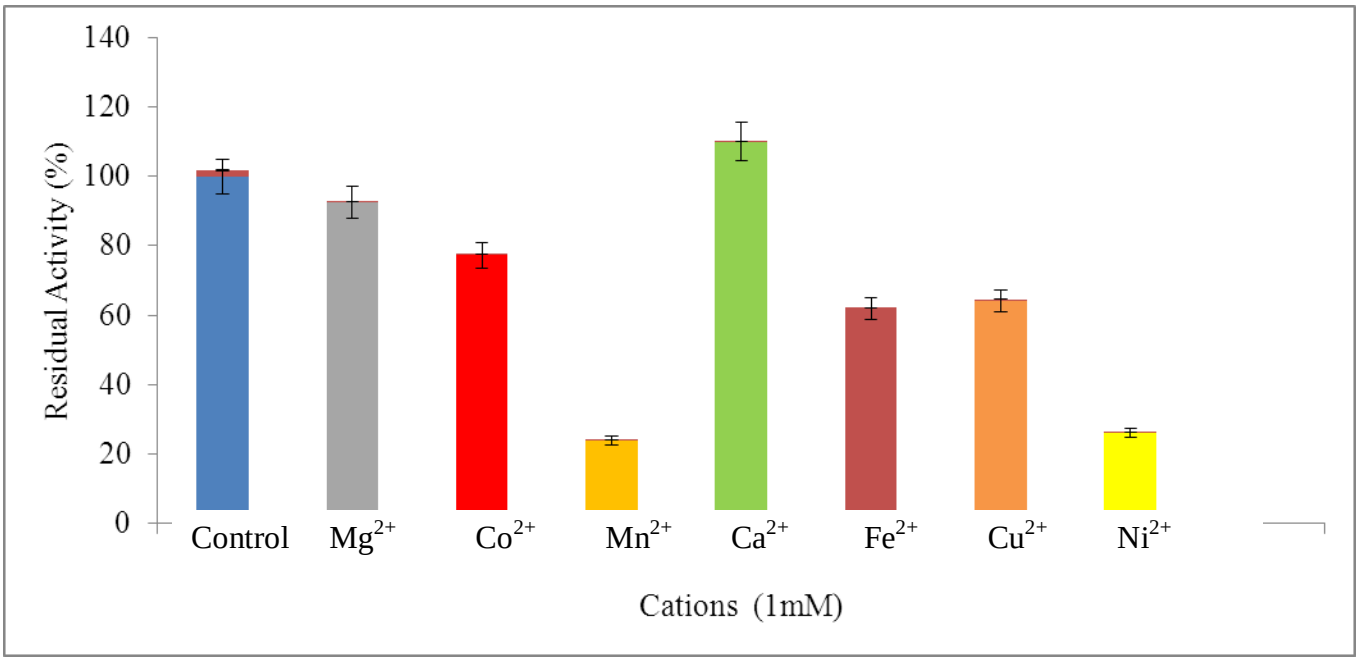


Figure 4.13 Effects of Some Cations at Concentration of 1 mM on the Activity of Partially Purified Lipase from *P. aeruginosa*

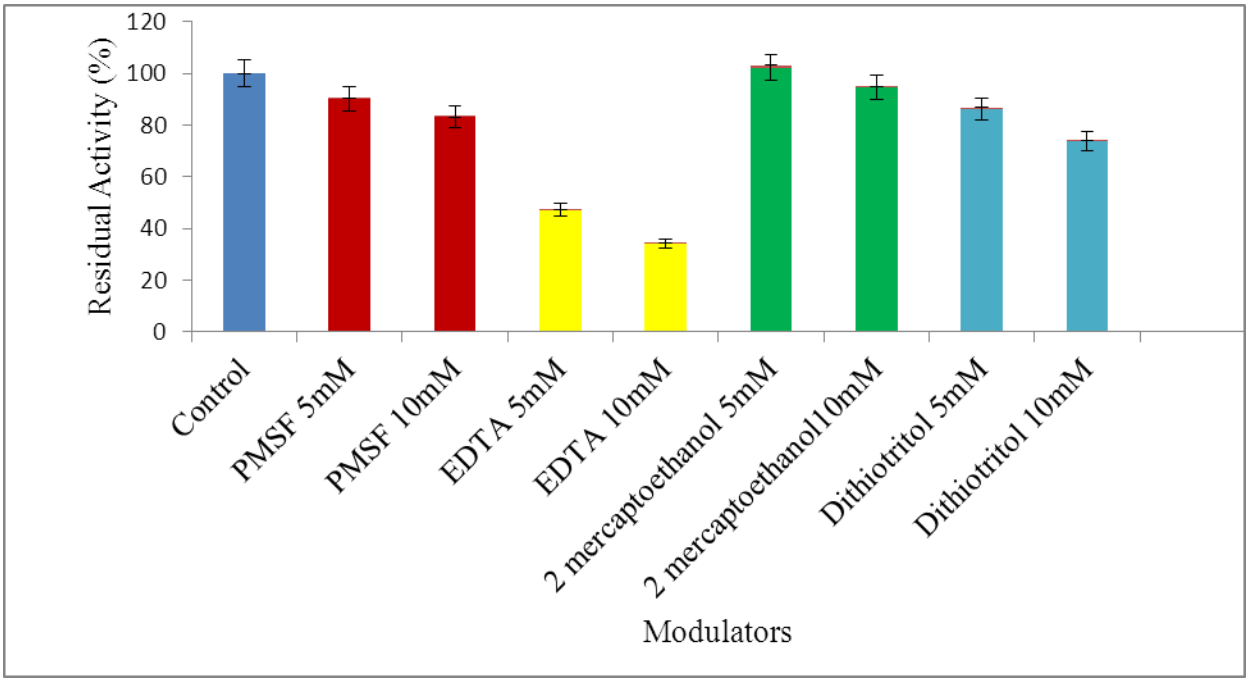


Figure 4.14 Effects of Some Modulators at Concentrations of 5 mM and 10 mM on the Activity of Partially Purified Lipase from *P. aeruginosa*

PMSF=Phenyl methane sulfonyl fluoride

EDTA=Ethylene diamine tetraacetic acid

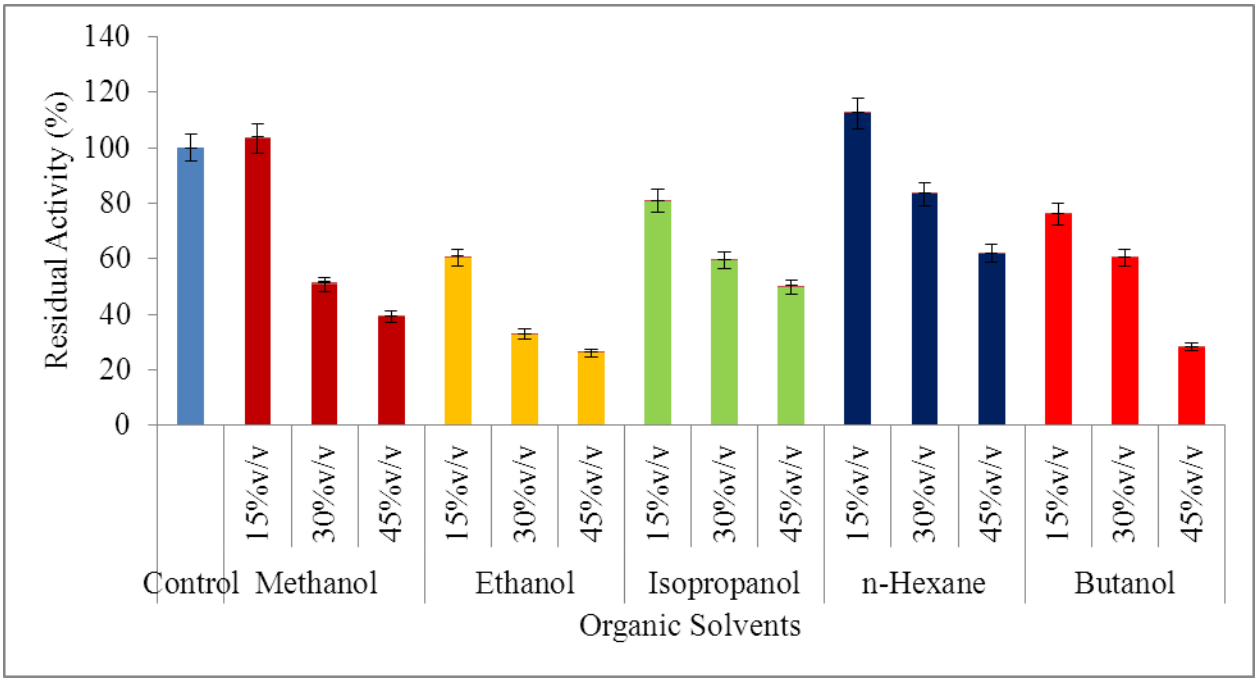


Figure 4.15 Effects of Some Organic Solvents at Concentrations of 15%, 30% and 45% (v/v) on the Activity of Partially Purified Lipase from *P. aeruginosa*

4.5 Characterization of Immobilized Lipase from *P. aeruginosa*

4.5.1 Effect of pH on the Activity and Stability of Immobilized Lipase

The effect of pH on the activity of immobilized lipase is shown in Figure 4.16. The enzyme had a broad pH of activity range with 19.0 U/ml at pH 7.5 and 8.0. No activity was detected at pH below 5.5 and above **9.**0. The pH stability of immobilized lipase is shown in Figure 4.17. At pH 6, (76.3%) of the activity was retained, but the enzyme retained 100% activity in the pH range of 7.5 to 8.5. However, there was a decrease in activity at pH 9.0, although 69.1% of activity retained.

4.5.2 Effect of Temperature on the Activity and Stability of Immobilized Lipase

The enzyme had maximum activity of 19.2 U/ml at 45°C, 50°C and 55°C but decreased to 18.4 U/ml and 17.2 U/ml at 60°C and 65°C respectively as shown in (Figure 4.18). The thermostability is shown in (Figure 4.19), there was a steady increase in activity of lipase at 30°C, 35°C and 40°C which was at its peak (100% activity) at 45°C, 50°C and 55°C. Subsequently a decrease was observed at 60°C and 65°C. The enzyme retained 65.4% at 70°C.

Figure 4.20 shows the initial velocity studies of immobilized lipase from *Pseudomonas aeruginosa*. The K_m and V_{max} value of 0.12 mM and 50.0 µmol/ min was obtained

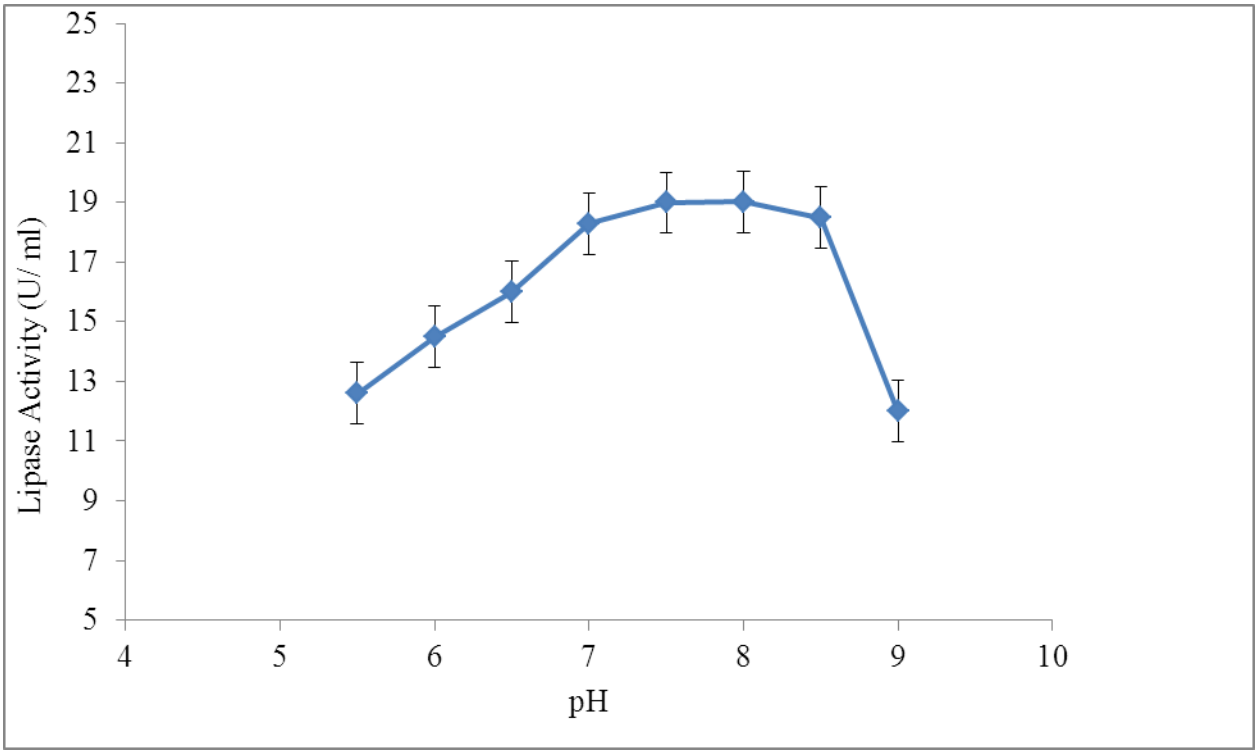


Figure 4.16. Effect of pH on the Activity of Immobilized Lipase from *P. aeruginosa*

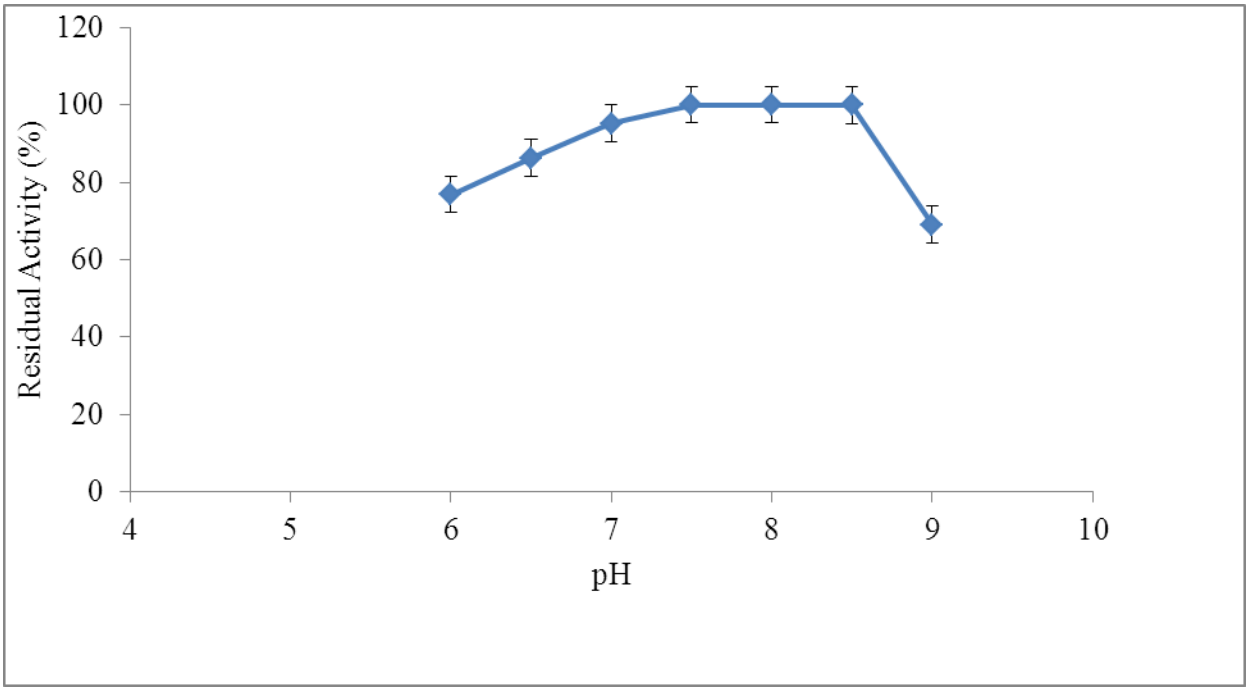


Figure 4.17 Effect of pH on the Stability of Immobilized Lipase from *P. aeruginosa*

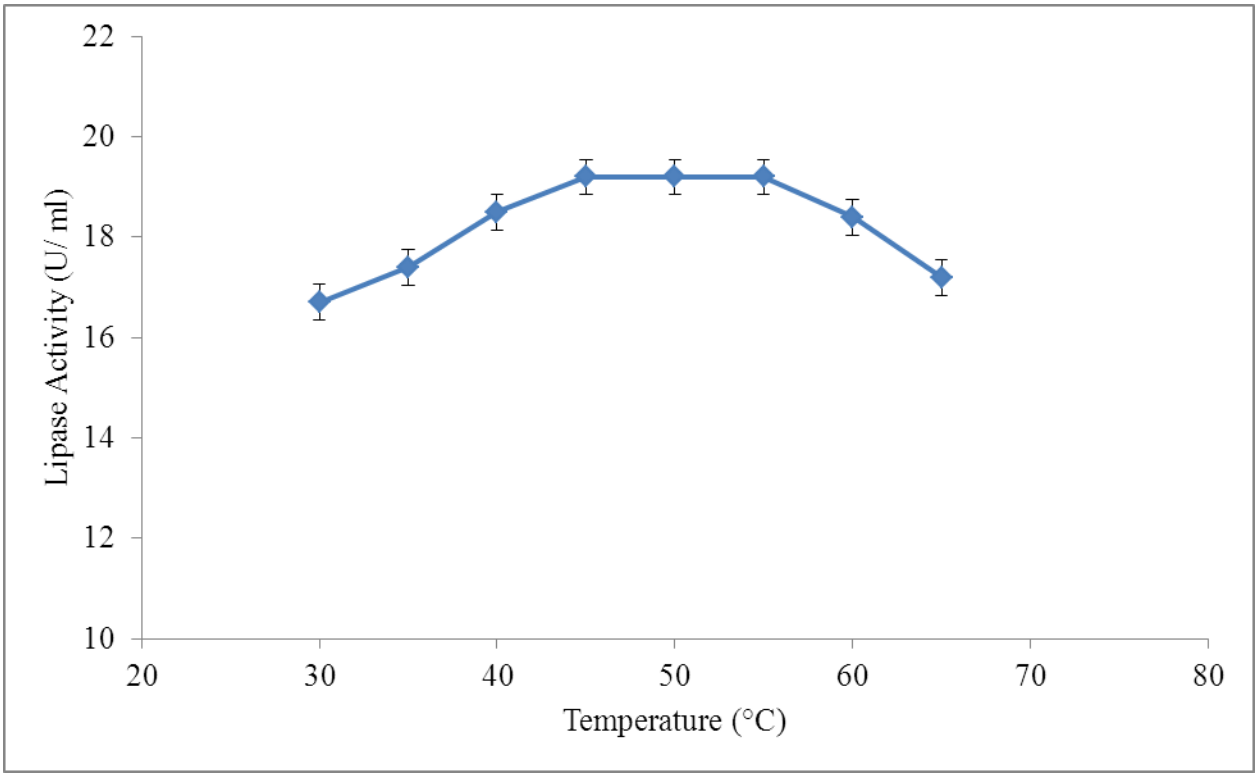


Figure 4.18 Effect of Temperature on the Activity of Immobilized Lipase from *P. aeruginosa*

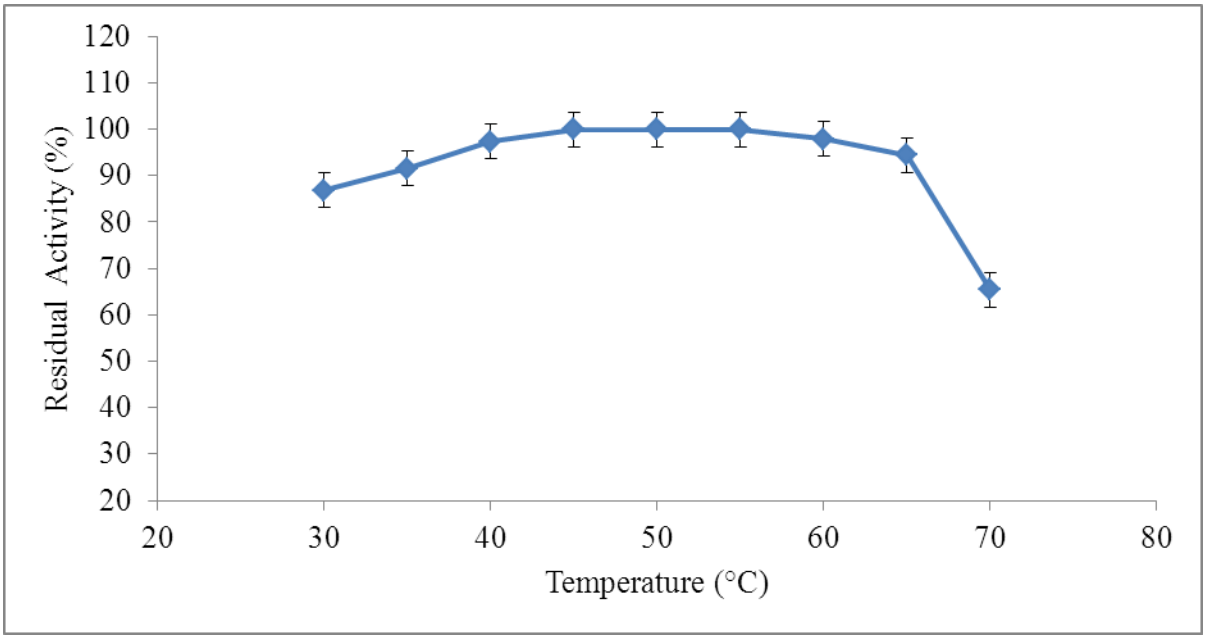


Figure 4.19: Profile of Stability of Immobilized Lipase from *P. aeruginosa* at Varying Temperatures (30^{o}C to 70^{o}C)

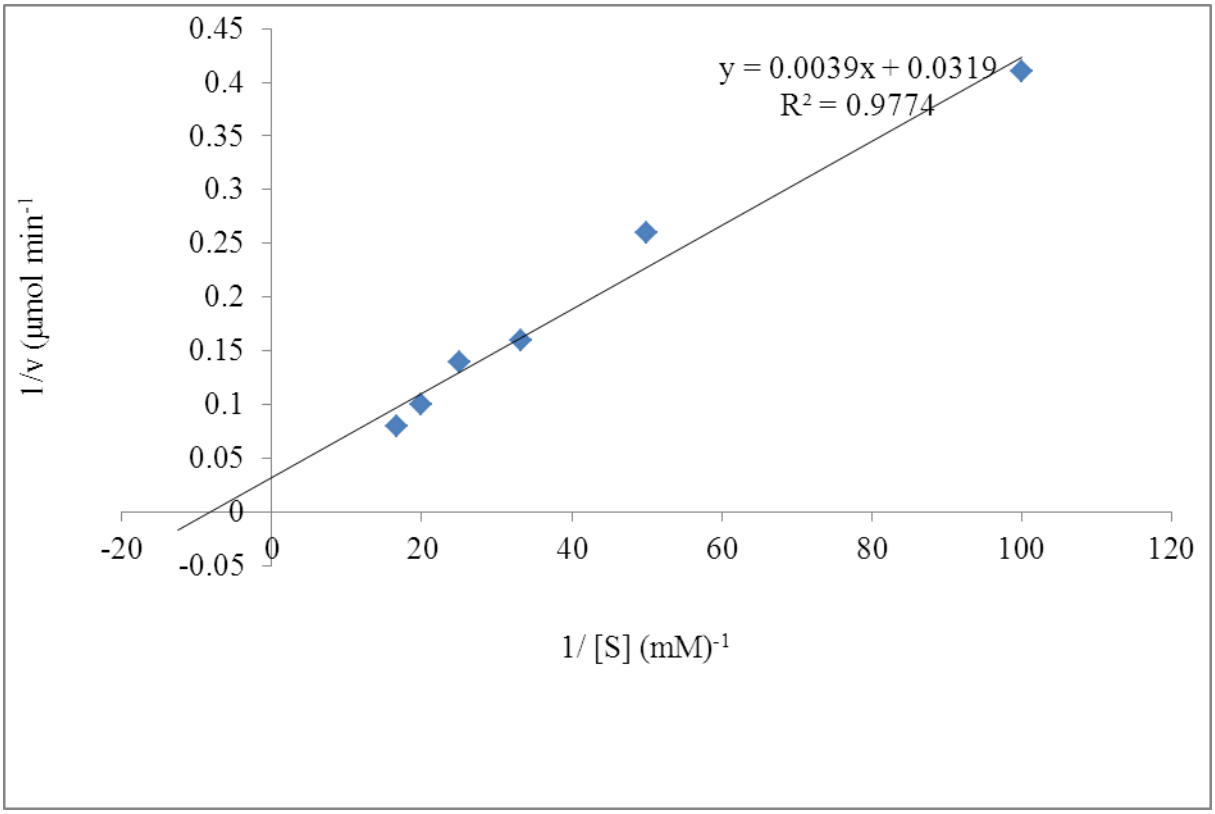


Figure 4.20 Lineweaver-Burk Plot for Immobilized Lipase from *P. aeruginosa*

4.5.3 Effect of Some Modulators on the Activity of Immobilized Lipase

Figure 4.21 shows the effect of some modulators on immobilized lipase activity. The residual activity of lipase in the presence of phenyl methane sulfonoyl fluoride (5 mM and 10 mM) was 91.3% and 83.1% respectively. In the presence of EDTA (5 mM and 10 mM), the residual activity of 51.0% and 36.7% was obtained. The activity of lipase was enhanced in the presence of 5mM and 10mM β mercaptoethanol with the residual activity of 103.4% and 78.6% respectively. In the case of 5 mM and 10 mM dithiotreitol, the residual activity was found to be 85.5% and 76.4% respectively.

4.5.4 Effect of Some Cations on the Activity on Immobilized Lipase

The effect of some cations on the activity of immobilized lipase is shown in Figure 4.22. The enzyme activity was enhanced in the presence of $Mg^{2+}$and Ca^{2} with the residual activities of 97.1% and 111.4%. The residual activities were decreased to 77.4% and 64.5% in the presence of Co^{2+},Fe^{2+} as well as 62.8% in the presence of Cu^{2+}. Similarly, a decrease in lipase activity was observed in the presence of $Mn^{2+}$and $Ni^{2+}$with the residual activities of 40.1% and 50.0% respectively.

4.5.5 Effect of Some Organic Solvents on the Activity of Immobilized Lipase

Figure 4.23 shows the effect of Some Organic Solvents on the activity of immobilized lipase from *P. aeruginosa*. The enzyme had residual activities of 104.1% in 15% methanol, 60.1% in 30% methanol and 49.3% in 45% methanol. The residual activities in the presence of 15% ethanol was (83.4%), in 30% ethanol it was 61.3% as well as 39.7% in 45% ethanol. While in the presence of 15%, 30% and 45% iso-propanol, the residual activities of 81.7%, 60.3% and 53.1%

respectively were obtained. The enzyme also had residual activities of 113.1%, 84.0% and 62.5% in the presence of 15%, 30% and 45% *n*-hexane respectively. Lipase residual activities retained in 15%, 30% and 45% butanol were 77.4%, 61.3% and 51.5% respectively.

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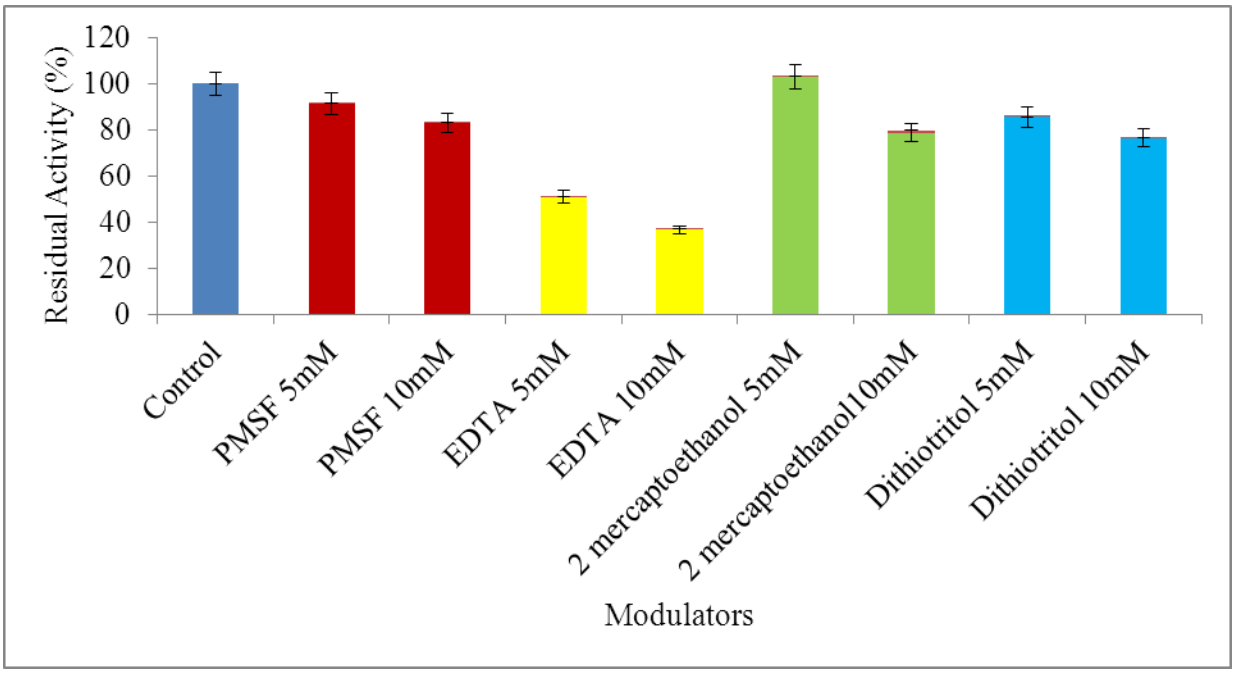


Figure 4.21 Effects of Some Modulators on the Activity of Immobilized Lipase from *P. aeruginosa*

PMSF=Phenyl methane sulfonyl fluoride

EDTA=Ethylene diamine tetraacetic acid

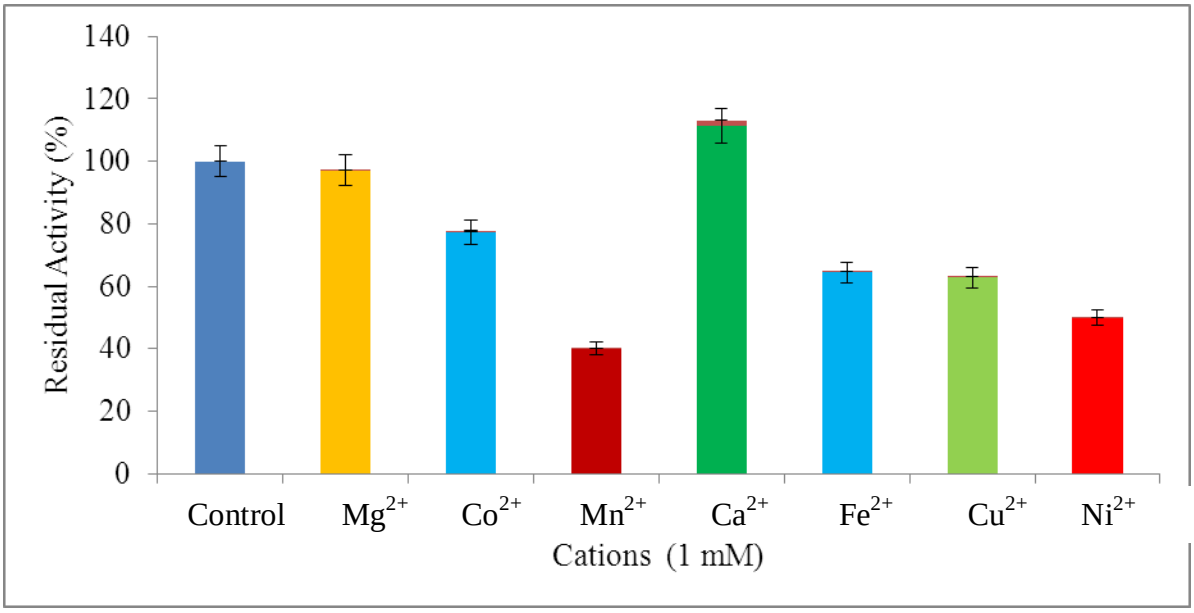


Figure 4.22 Effects of Some Cations on theActivity of Immobilized Lipase from *P. aeruginosa*

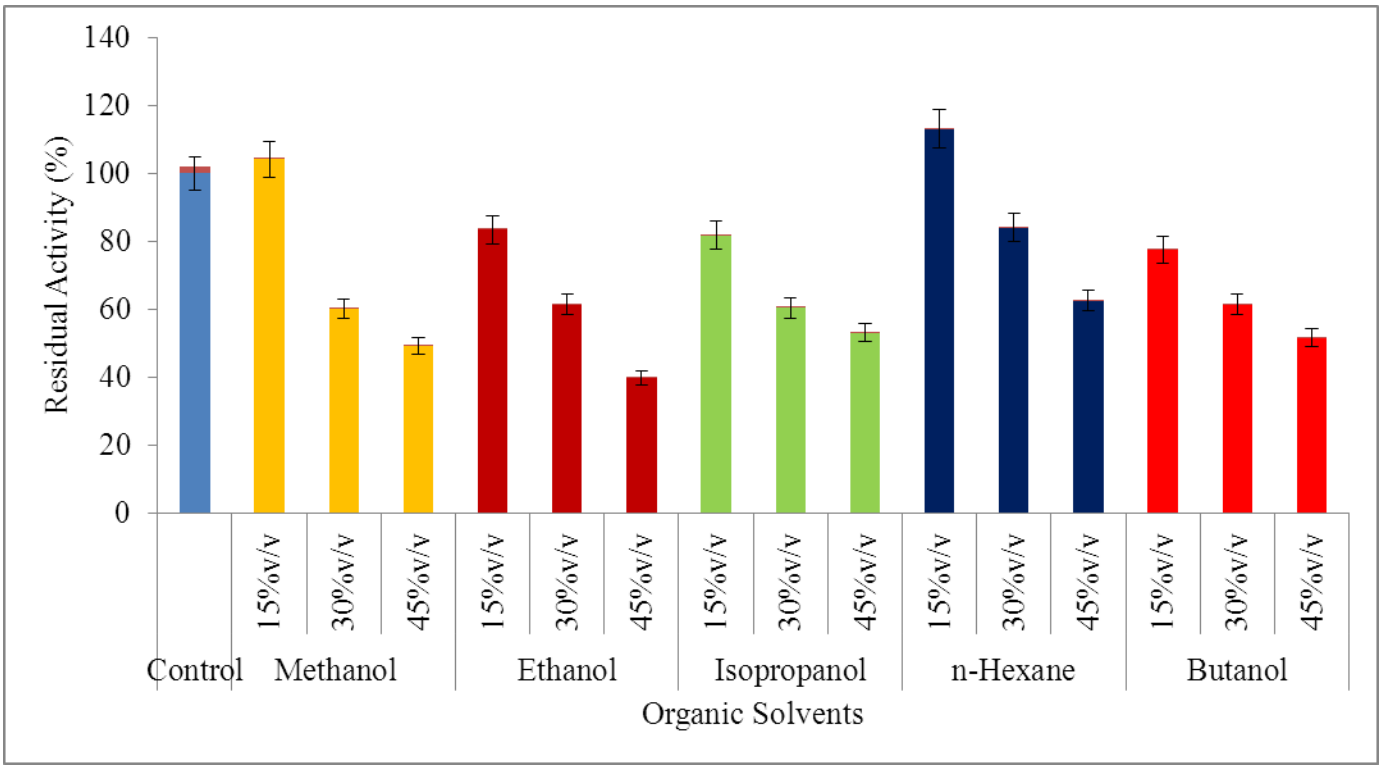


Figure 4.23 Effects of Some Organic Solvents at Concentrations of 15%, 30% and 45% (v/v) on the Activity of Immobilized Lipase from *P. aeruginosa*

4.5.6 Effect of Number of Transesterification Cycles on Immobilized Lipase Activity

The effect of number of cycles on transesterification mediated by immobilized lipase on methyl ester conversion is shown in Figure 4.24. Methyl ester conversion of 55.9% was found in cycles 1, 2 and 3. Cycles 4 and 5 had methyl ester conversion of 54.7% and 54.2% respectively. A percentage methyl ester conversion of 42.3% and 28.1 % were obtained in cycle 6 and 7 respectively. The lowest methyl ester conversion of 17.5% was recorded at cycle 8.

4.6 Response Values for Biodiesel Optimization Parameters

Table 4.5 shows the response values of the experimental and predicted biodiesel yield based on the response surface methodology (RSM) experimental design. The experimental response gave the highest yield of 62.4% while predicted response was 46.02% run1. The lowest response of 11.1% was recorded at run 28 while the predicted was 7.82%. For the pareto plot showing relationship between the predicted and actual biodiesel yield see appendix xxvi

Table 4.6 shows the analysis of variance of the quadratic model used for optimisation of biodiesel production from *A. nilotica* seed oil. The results of the analysis showed the model F-value of 2.78. The linear coefficient of incubation time (D), the quadratic coefficient of lipase (C^2) as well as the quadratic coefficient of incubation time (D^2) are the significant model terms for having "Prob > F" less than 0.050. While the linear coefficient of oil/methanol (A), *n*-hexane (B), lipase (C), the quadratic coefficient of oil/methanol (A^2), the quadratic coefficient of *n*-hexane (B^2), the interactive effect of oil/methanol and *n*-hexane (AB) , the interactive effect of oil/methanol and lipase (AC), the interactive effect of oil/methanol and iucubation time (AD),

the interactive effect of *n*-hexane and lipase (BC), the interactive effect of *n*-hexane and incubation time (BD) and the interactive effect of lipase and iucubation time (CD) are not significant model term for having p-value greater than 0.0500.

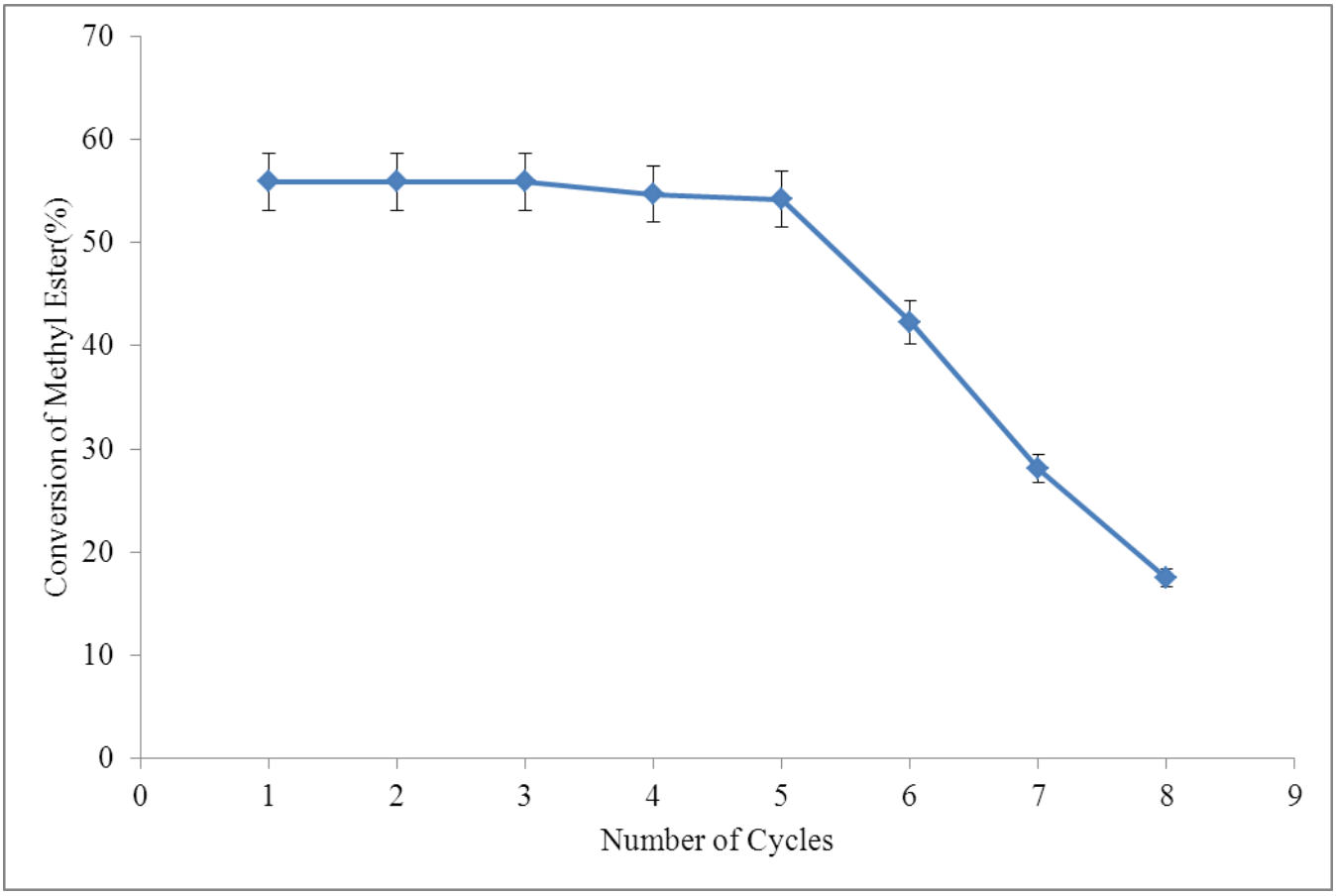


Figure 4.24 Effect of Number of Cycles on Transesterification Catalysed by Immobilized Lipase from *P. aeruginosa*

Table 4.5 Experimental and Predicted Surface Responses for Central Composite Second-Order Design

Run order	Oil/Methanol	n-hexane (ml)	Enzyme (U/ml)	Time (Minutes)	Actual yield (%)	Predicted yield (%)
1	0.14	0.40	64.90	195.00	62.4	46.02
2	0.02	0.10	111.20	30.00	20.6	25.50
3	0.10	0.10	111.20	30.00	34.9	39.42
4	0.10	0.70	111.20	30.00	22.2	33.49
5	0.06	0.40	111.20	360.00	19.02	18.85
6	0.06	0.40	64.90	195.00	53.90	52.62
7	0.02	0.70	18.60	360.00	26.6	30.72
8	0.06	0.40	64.90	-135.00	61.00	40.02
9	0.10	0.10	111.20	360.00	20.6	24.61
10	0.02	0.70	111.20	360.00	17.50	17.27
11	0.10	0.10	18.60	30.00	28.60	42.25
12	0.06	1.00	64.90	195.00	44.40	33.24
13	0.06	-0.20	64.90	195.00	42.90	31.99
14	0.02	0.70	111.20	30.00	17.70	21.24
15	0.02	0.07	18.60	30.00	27.00	36.42
16	0.06	0.40	-27.70	195.00	42.90	24.10
17	0.06	0.40	64.90	195.00	53.90	52.62
18	0.10	0.70	18.60	30.00	39.70	47.59
19	0.02	0.10	18.60	30.00	20.6	29.41
20	0.06	0.40	64.90	525.00	20.6	19.51
21	0.06	0.40	64.90	195.00	53.2	52.62
22	0.06	0.40	64.90	195.00	53.90	52.62
23	0.06	0.50	64.90	195.00	46.90	52.62
24	0.10	0.10	18.60	360.00	20.6	25.71
25	-0.02	0.40	64.90	195.00	37.30	31.60
26	0.06	0.40	64.90	195.00	53.90	52.62
27	0.02	0.10	111.20	360.00	20.60	21.36
28	0.06	0.40	157.50	195.00	11.10	7.82
29	0.10	0.70	18.60	360.00	22.70	31.22
30	0.02	0.10	18.60	360.00	21.40	23.54

Table 4.6. Analysis of Variance of the Response Surface Quadratic Model of *A, nilotica* Biodiesel

Parameters	Sum of squares	Mean squares	F- value	Prob>F
Model	36.51	2.61	2.78	0.0292
A	2.08	2.08	2.22	0.156
B	0.012	0.012	0.013	0.9118
C	4.10	4.10	4.38	0.0539
D	4.67	4.67	4.98	0.0413
A^2	2.23	2.23	2.38	0.1434
B^2	4.06	4.06	4.33	0.0550
C^2	17.60	17.60	18.78	0.0006
D^2	6.66	6.66	7.10	0.0176
AB	0.030	0.030	0.032	0.8596
AC	0.024	0.024	0.025	0.8753
AD	0.8753	0.8753	1.12	0.3076
BC	1.23	1.23	1.13	0.2707
BD	1.251×10^{-3}	1.251×10^{-3}	1.335×10^{-3}	0.9713
Lack of fit	13.86	1.39	35.20	0.0005

The Model F-value of 2.78 implies the model is significant. There is only a 2.92% chance that a "Model F-Value" this large could occur due to noise.
Values of "Prob > F" less than 0.0500 indicate model terms are significant

The "Lack of Fit F-value" of 35.20 implies the Lack of Fit is significant. There is only a 0.05% chance that a "Lack of Fit F-value" this large could occur due to noise. R^2 = 0.7220; Adjusted R^2 = 0.4626; Coefficient of Vartance=-0.5843and Adequate precision= 5.889

4.7 Effect of Tranesterification Variables on Biodiesel Yield

Figure 4.25 shows the interaction of *n*-hexane and oil methanol ratio on biodiesel yield. The lower and higher levels of *n*-hexane did not result in higher yield of biodiesel, but higher biodiesel yield was obtained at the centre of the plot. However, the biodiesel yield increased with increase in oil/methanol. Figure 4.26 shows the interaction of lipase and oil/methanol ratio on biodiesel yield. With respect to oil/methonol the biodiesel yield increased with its increase. There was no increase in biodiesel yield at lower and higher levels of lipase, but maximum biodiesel yield was obtained with 64.0U/ml (lipase). Figure 4.27 shows the interaction of incubation time and oil/methanol ratio on biodiesel yield. The yield decreased with increase in the incubation time but increased with increase in oil/methanol ratio. Figure 4.28 shows the interaction of lipase and *n*-hexane on biodiesel yield. At lower and higher levels of lipase, the biodiesel yield did not increase, but the yield increased with the increase in *n*-hexane.Figure 4.29 shows the interaction of incubation time and *n*-hexane on the biodiesel yield. The lower and higher levels of incubation time and *n*-hexane did not result in higher yield of the produced biodiesel. Figure 4.30 shows the interaction of incubation time and lipase on biodiesel yield. The interaction of incubation time and lipase at lower and higher levels has not resulted in maximum biodiesel yield.

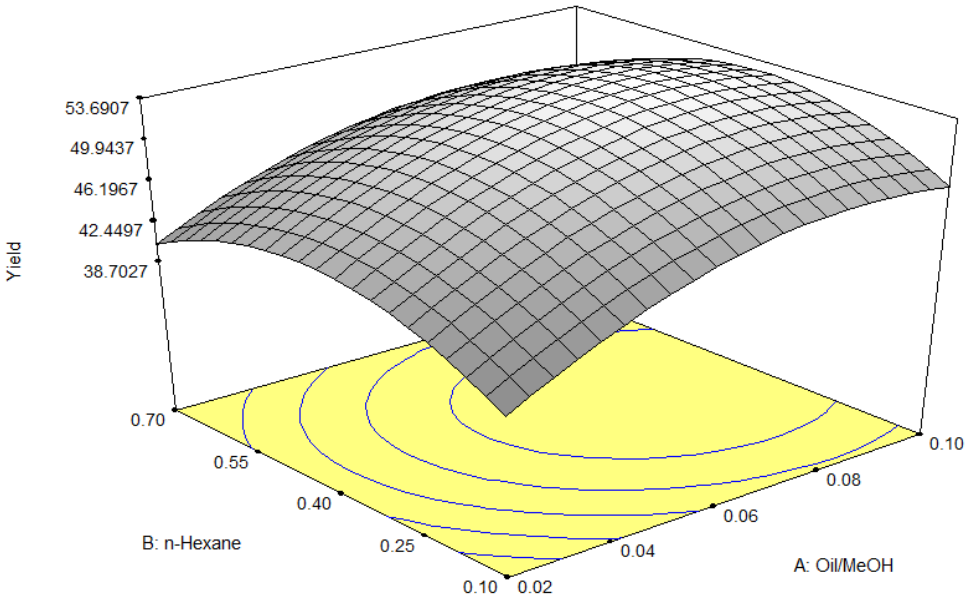


Figure 4.25 Three Dimensional Response Surface Plot Showing the Interaction of *n*-hexane and Oil/ Methanol Ratio on Biodiesel Yield

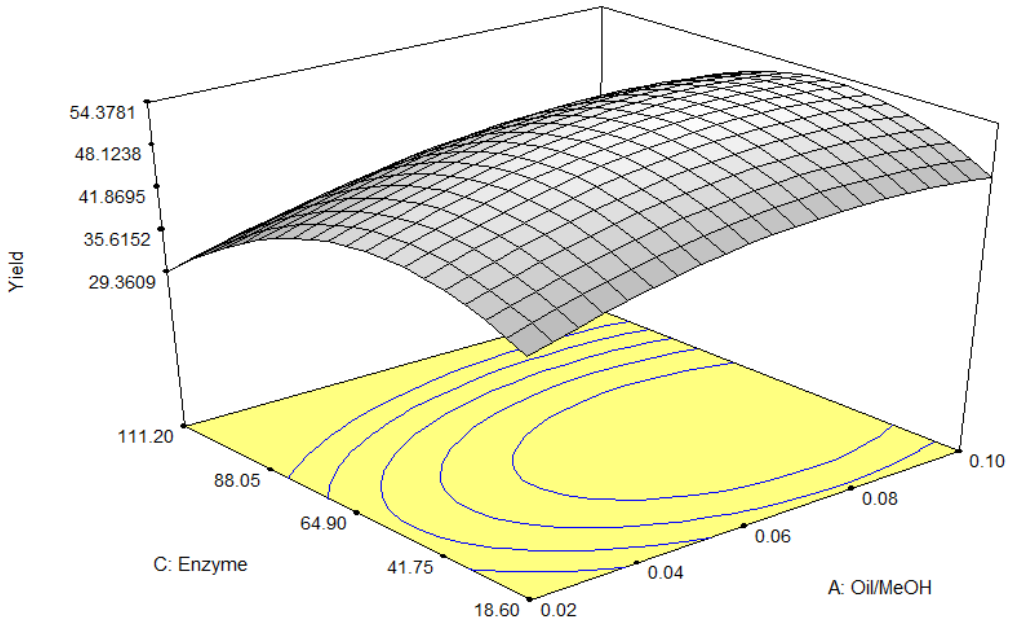


Figure 4.26 Three Dimensional Response Surface Plot Showing the Interaction of Lipase and Oil/ Methanol on Biodiesel Yield.

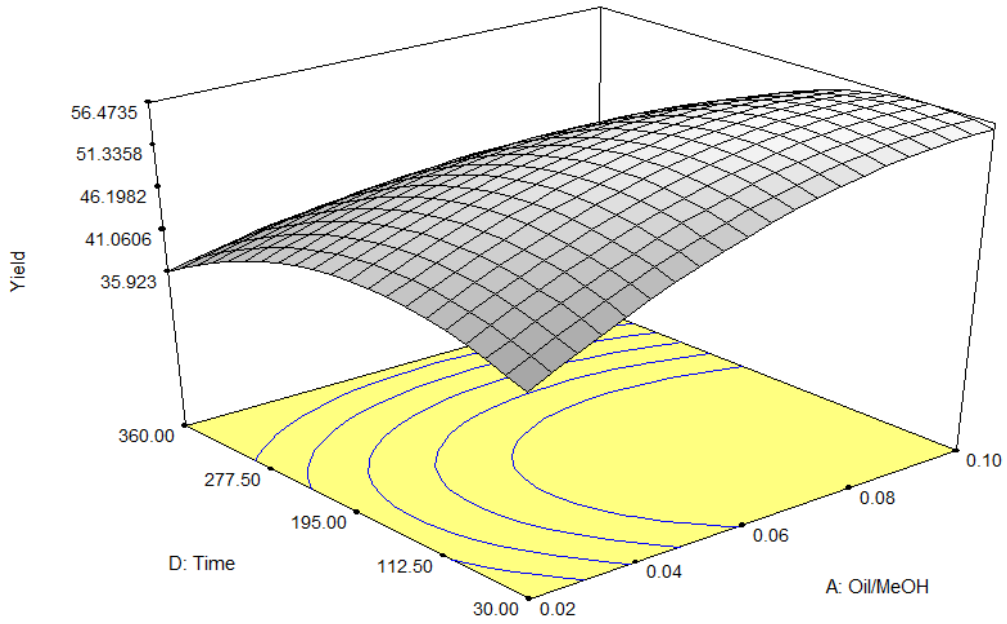


Figure.4.27 Three Dimensional Response Surface Plot Showing the Interaction of Incubation Time and Oil/ Methanol on Biodiesel Yield

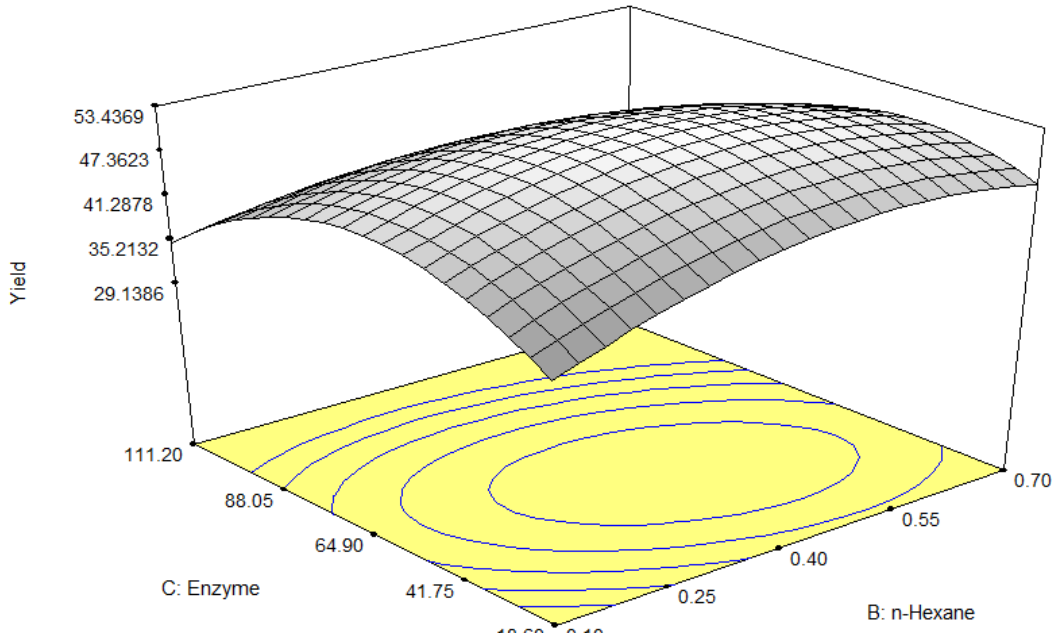


Figure 4.28 Three Dimensional Response Surface Plot Showing the Interaction of Lipase and *n*-Hexane on Biodiesel Yield

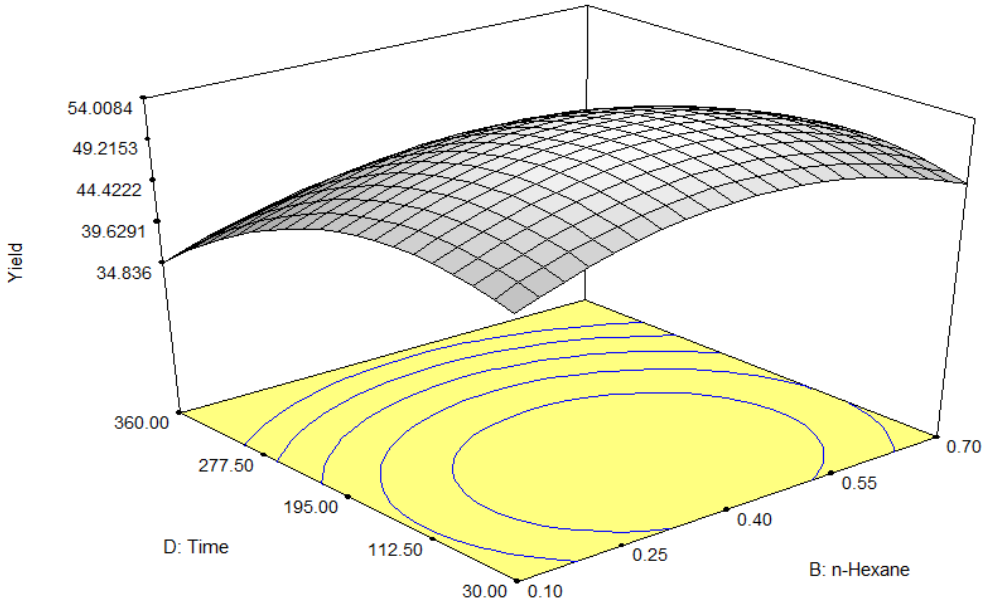


Figure.4.29 Three Dimensional Response Surface Plot Showing the Interaction of Incubation Time and *n*-Hexane on Biodiesel Yield.

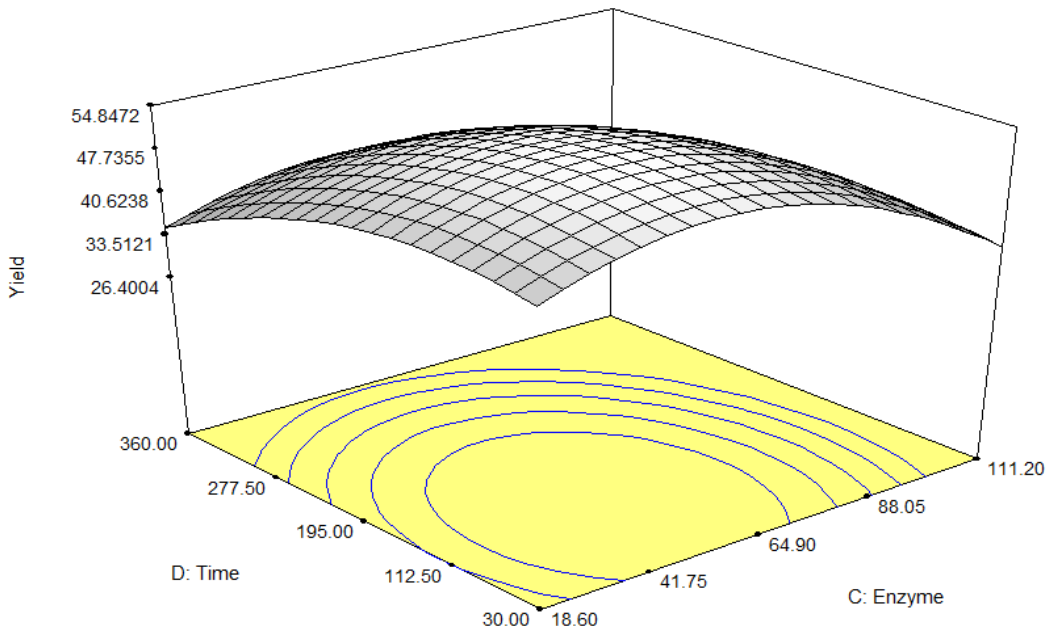


Figure 4.30 Three Dimensional Response Surface Plot Showing the Interaction of Incubation Time and Lipase on Biodiesel Yield

Table 4.7 Validation of the Developed Second Order Quadratic Model

Run order	Oil/Methanol	n-hexane (ml)	Lipase (U/ml)	Time (Minute)	Predicted yield (%)	Experimental yield (%)
1	0.04	0.25	64.9	195.0	55.5	54.8±0.2
2	0.06	0.70	64.9	195.0	50.8	49.7±0.2
3	0.1	0.4	64.9	195.0	55.8	55.9±0.1
4	0.02	0.4	111.2	195	47.6	46.6±0.2
5	0.04	0.4	41.75	195	51.4	50.7±0.3
6	0.08	0.4	88.05	195	52.5	51.8±0.3
7	0.02	0.4	64.9	30	44.3	40.4±0.1
8	0.04	0.4	64.9	112.5	53.6	55.2±0.3
9	0.1	0.4	64.9	360	38.5	34.9±0.4
10	0.06	0.25	41.75	195	51.6	51.9±0.2
11	0.06	0.55	88.05	195	49.2	48.9±0.2
12	0.06	0.10	18.60	195	38.2	39.1±0.1
13	0.06	0.25	64.9	112.5	56.2	54.9±0.1
14	0.06	0.55	64.9	30	54.8	55.1±0.05
15	0.06	0.7	64.9	360	37.4	36.8±0.2
16	0.06	0.4	41.75	112.5	56.5	55.4±0.1
17	0.06	0.4	88.05	272.5	49.7	48.3±0.1
18	0.06	0.4	64.9	30	56.1	53.8±0.3

Final model equation in terms of actual factors:

$$Sqrt(Yield) = +3.38 + 36.41\ (OMR) + 4.96\ (n - Hexane) + 0.045\ (E) + 6.34X\ 10n] - 3(T) - 178.37\ (OMR)^2 - 4.273\ (n - H)^2 - 3.73\ X\ 10 - 4\ (E)^2 - 1.809\ X\ 10 - 5\ \ (T)^2 - 3.62(OMR * n - H) + 0.0208(OMR * E) - 0.038\ (OMR * T) - 0.019\ (n - H\ *\ E) + 1.78\ X\ 10 - 4\ (n - H * T) + 4.53X\ 10 - 6\ (E *\ T)$$

----------(5.1)

4.8 Validation of the Developed Second Order Quadratic Model

Table 4.7 shows the values obtained from validation of the developed second order quadratic model for efficient biodiesel production. The predicted response gave the highest yield of 57.8±0.10% while experimental response was 55.9% run 3. The lowest predicted response of 38.5% was recorded at run 9 where the experimental response was 34.9±0.40%.

4.9. Fatty Acid Composition of *Acacia nilotica* Biodiesel

The percentage fatty acid methyl ester profile of *A. nilotica* seed oil is shown in Table 4.9. The fatty acid methyl esters composition indicated the presence of octadecenoic acid methyl ester in the highest proportion of (69.14%). Others included hexadecanoic and octadecanoic acid methyl ester at concentration of 15.47% and 7.92% respectively. While those present at low concentration included pentaflouropropionic acid methyl ester 1.90%, eicosanoic acid methyl ester (1.02%), docosanoic acid methyl ester (1.11%) and pentadecanoic acid methyl ester (0.64%) respectively. Others had 2.80%

4.10 Some Physical Properties of *Acacia nilotica* Biodiesel

Table 4.10 shows some physical properties of *Acacia nilotica* biodiesel. The specific gravity was 0.84 and refractive index was 1.34 ± 0.04. The biodiesel had the viscosity of 3.73 ± 0.15 (Pa. s) and the cetane number of 29.50±0.05. The calorific value and flash point were 34.2±0.10 (MJ/kg) and 113±0.10 respectively. The biodiesel had the cloud and pour point of 1.06±0.01 and 4.20±0.10^{o}C respectively.

4.11 Chemical Properties of *Acacia nilotica* Biodiesel

Table 4.10 shows some chemical properties of *Acacia nilotica* biodiesel. The iodine value was 187.6±0.40 gI_2/100 g, acid value was 5.61±0.01 mg KOH/g, saponification value was 189±1.00 mg KOH/g and peroxide value was 6.60±0.05 meq/kg

Table 4.8: Fatty Acids Methyl Ester Profile of Biodiesel Produced from *A. nilotica* Oil

Fatty acid methyl ester type	Proportion (%)	Similarity Index
Hexadecanoic acid methyl ester	15.47	93
Octadecenoic acid methyl ester	69.14	92
Octadecanoic acid methyl ester	7.90	92
Pentaflouropropionic acid methyl ester	1.90	83
Eicosanoic acid methyl ester	1.02	90
Docosanoic acid methyl ester	1.11	91
Pentadecanoic acid methyl ester	0.64	94
Others	2.80	-

Table 4.9 Some Physical Properties of *A. nilotica* Biodiesel

Fuel properties	*A.nilotica*	ASTM D 6751*	EN 14214 **
Specific gravity	0.84±0.005	0.85-0.90	-
Refractive index	1.34±0.04	1.20-1.80	-
Viscosity (P.as)	3.73±0.15	1.9–6.0	-
Cetane number	32.81±0.05	42.5	-
Calorific value(MJ/kg)	29.0±0.10	45–50	-
Flash point (^{o}C)	113±0.10	>130	>120
Cloud point(^{o}C)	1 .06±0.01	-	-
Pour point (^{o}C)	4.20±0.10	-	-

*The values were expressed as Mean ±SD.*Source: *Schinas, *et. al*,(2009) and ** Gerpen *et al* ., (2004)

Table 4.10 Some Chemical Properties of *A. nilotica* Biodiesel

Fuel properties	*A.nilotica*	ASTM D 6751*	EN 14214 **
Iodine value (g I_2/100 g)	187.6±0.40	125-150	<120
Acid value (mgKOH/g)	5.61±0.01	0.5	0.5
Saponification value (mg KOH/g	189.0.±1.00	185-196	-
Peroxide value (meq/kg)	6.60±0.05	4.0-7.5	-

The values were expressed as Mean ±SD .Source: *Schinas *et al*., (2009) and ** Gerpen *et al* ., (2004)

CHAPTER FIVE

5.0DISCUSSION

Oil has been extracted and characterized from different *A. nilotica* species. Attempt to produce biodiesel from *A.nilotica* seed oil by lipase catalysed transesterification has not been done. This work is first of its kind with respect to the lipase mediated transesterication of oil from aforementioned plant. Oil content of plant seeds is one of factors for assessing the economic viability of a feedstock for biodiesel production especially for the underutilized plant seeds. The oil yield of *A. nilotica* seed was found to be 13.5%. This result contradicts the earlier result reported by Kovo and Bawa (2007) who obtained the yield of 8.24% from *A. nilotica* oil extracted with acetone and 7.34% when extracted with petroleum ether-ethanol. Such variation in oil content across species might be attributed to the environmental and geological conditions of varied regions, age and time of harvest as well as the polarity of the solvent used in the extraction of oil.

The physicochemical properties of oil play a significant role in determining the quality of the biodieselproduced. The specific gravity of oil which is the ratio of the density of oil to the density of a reference substance is close to the standard range of 0.87–0.90 (Ejikeme *et al.*, 2008). The molecular weight, fatty acid chain length, degree of unsaturation and degree of conjugation determine the refractive index of oil which is a significant parameter to evaluate the state of biodiesel (Sadrolhosseini *et al*., 2011). The refractive index of *A. nilotica* seed oil in this study falls below the values reported for other seed oils 1.48 for *Teleferia occidentalis* seed oil, 1.47 for soybean and 1.47 for corn oil (Sodeke, 2005). The low refractive index is an indication that the oil is not viscous evidenced by high proportion of monounsaturated fatty acids. Viscosity which characterises the flow properties, the inherent friction of liquids and gases influences fuel

flow and atomization characteristics of oil. The value obtained was below ASTM reference range of 1.90–6.00. The calorific value of seed oil of *A. nilotica* was lower than petro-diesel as shown in (Table 4.1). The presence of chemically bound oxygen in the fatty acid chains is largely responsible for the low energy content of *A. nilotica* seedoil (Srivastava and Prasad, 2000). The cetane number of oil is determined by the distribution of fatty acids in the feedstock (Knothe *et al*., 2002). Higher cetane number is found in oils having more saturated and longer carbon chain of fatty acids. The low cetane number obtained could be due to high proportion of unsaturated fatty acids in *A.nilotica* seedoil evidenced by high iodine value and high proportion of octadeceinoic as shown in Tables 4.2 and 4.3 respectively.

The acid value obtained was lower than that of olive oil 17 (mg KOH/g) but higher than 1.20, the value reported for *Jatropha caucus* seed oil (ASTM, 2010). Tissouras *et al.,* (2013) reported acid values 10.93 mgKOH/g in *Acacia arabica* and 4 mg KOH/g in *Acacia raddiana* which signify purity of oil and makes it suitable for biodiesel production. The saponification value of *A. Nilotica* seed oil reported in this work as shown in (Table 4.2) agrees with the findings of Anchwagen *et al.,* (2004) who reported the saponification value of 184.26 mg KOH/g and 168.53 mg KOH/g in *Acacia arabica* and *Acacia raddiana* seed oil respectively. The result indicates the presence of median weight fatty acid in the oil. The iodine value obtained as shown in (Table 4.2) agrees with the findings of Kovo and Bawa (2007) who reported the iodine value of 187.54 gI_2/100g fat in *A. nilotica* seed oil. Oils having high iodine number cannot be preserved for a long period of time, they lower the pour point and are likely to polymerize in the heat engines if used as such (Sulistyo *et al.,* 2008). Iodine value of oil also indicates its degree of unsaturation and also serves as a standard for assessing its stability (Knothe (2002). The peroxide value of *A. nilotica* seed oil agrees with the findings of Abu Sayeed *et al*., (2004) who reported

peroxide values 5.85 to 6.03 meq/kg in *Mesua ferrea* seed oil. Mohammed and Hamza (2008) reported the peroxide value of 7.45–8 meq KOH/g in *Sesame indicum L*. Thus, the peroxide value obtained from the oil as shown in (Table 4.2) is an indication that the oil is less liable to rancidity at room temperature and can be used for biodiesel production as soon as it is extracted.

The percentage fatty acid profile of *Acacia nilotica* oil as shown in (Table 4.3) agrees with the the earlier findings of Rathee *et al.,* (1983) who reported oleic acid as the predominant fatty acids in *A. nilotica* seed oil. The difference in fatty acid composition in these oils could be due to variations in soil and climatic conditions (Egan *et al*., 1981). This result showed a potential for the industrial application of *A. nilotica* seed oil in biodiesel production owing to its high proportion of mono unsaturated fatty acid.

Extracellular lipases are known to be inducible enzymes and their efficient production depends on the appropriate choice of carbon sources (Lin and Ko, 2005). Induction of lipase production in microorganisms is enhanced when the growth medium contains lipidic compounds as carbon source such as olive oil, tributyrin and fatty acids (Lotti *et al.,* 1998). Prakasham *et al.,* (2006) reported that metabolism, growth and enzyme production pattern of each microbial strains differ with the fermentation condition. Ramesh *et al*., (2014) reported maximum activity (8.75 U/ml) of lipase in *Bacillus sp* produced by submerged fermentation at 36 hours in medium containing olive oil as carbon source. Faouzi *et al*., (2013) reported maximum lipase production (17 U/ml) in *Trichosporon coremiiforme* strain after 48 hours of incubation.

The elution of partially purified lipase from the anionic exchanger occured at low concentration of NaCl. The increased specific activity which is considered as a measure of enzyme purity and fold purification compared with the preceeding steps indicates that some level of purification

was achieved. Low yield can be attributed to activity loss during acetone precipitation (Schmidt-Dannert *et al.*, 1994). Benattouche and Aboun (2012) reported the purification fold of 3.97 and yield of 19.45% from *P.aeruginosa* lipase purified on DEAE cellulose ion exchanger. Ramani *et al.*, (2010) reported purification fold of 7.59 with 16.2 % from *Pseudomonas gessardii* lipase

Various molecular weights have been reported for lipase from different sources. The result of the findings agrees with Kok *et al.*, (1993) who reported the molecular weight of 32 kDa from *Aci.Calcoaceticus* BD 413 lipase on SDS-PAGE.

Two parameters, K_m and V_{max} define the Michaelis Menten kinetics. The $K_m$values of enzymes vary widely, but most industrially relevant enzymes have the K_m values in the range of 10^{-5} and 10^{-1}M (Fullbrook, 1996). Thus, the lower value of K_m obtained in this study signifies the higher affinity of the enzyme to *p*NPL. The differences in the affinities for lipase might be due to specie difference from which the lipases were obtained. Kambourova *et al.*, (2003) reported a lipase from *B. stearothermophilus* with K_m and V_{max} values of 0.33 mM and 188µM/min/mg respectively. An increase in K_m after immobilization as shown in (Figure 4.19) indicates that the immobilized enzymes have an apparent lower affinity for *p*NPL.This may be caused by the loss of enzyme flexibility necessary for substrate binding or by diffusional resistance to substrate transport (Şahin *et al.*, 2005).

Most lipases have optimal activity at neutral or slightly basic pH values (Bussamara *et al.*, 2010). Lipase produced by *Cunninghamella verticillata* was reported to be stable between pH range of 7.5–9.0 for 24 hours with pH optimum of 7.5 (Gopinath *et al.*, 2005). The loss of enzyme activity at low or high pH could result from formation of improper ionic form of enzyme or substrate or both. From the Dixon-Webb the asymptotes have been found to be histidine and

cysteine on the basis of their pKas of 6.5 and 7.0 respectively. The immobilization of lipase from *P. aeruginosa* has led to the shift in the optimum pH and temperature. The changes in the optimum pH for immobilized lipase could be due to the fact that the charged surface of beads and entrapped enzyme produces a charged micro environment, which ultimately affects the nature of the active enzyme protein and alters the pH of the entrapped enzyme (Orouzian, 2003). Greater optimum activity and temperature stability are attractive and desirable characteristics for industrially relavant enzymes (Asgher and Iqbal, 2011). The activity and stability of lipase from *P. aeruginosa* over a wide range of temperature suggests the stability of its three dimensional structure. The result of our finding as shown in (Figure 4.8) agrees with the findings of Saeed *et al*., (2005) who reported that lipase from *Pseudomonas pseudoalkaligenes* F-111 and *Pseudomonas* Ps-x retained stability up to 70ºC. The solubility of globular proteins increases with the temperatures 40 ^{o}C-65^{o}C. Above this temperature the thermal agitation tends to disrupt the tertiary structure leading to denaturation and sharp decrease in solubility which in turns affect the enzyme activity. Nawani and Kaur (2007) reported that lipase from thermophilic *Bacillus sp* was stable up to (60℃ to 70℃). As shown in (Figure 4.21) the optimum temperature of immobilized lipase contradicts the findings of Shuang *et al*., (2013) who reported the optimum temperature of 30°C and 35^{o}C for lipase immobilized on alginate hydrogel beads.

As shown in (Figure 4.22) the thermal stability of lipase entrapped in alginate matrices could be due to the fact that immobilization provided a more rigid external backbone for lipase molecules that reduces the effect of higher temperature in breaking the interactions that were responsible for the proper globular structure and catalytic activity of lipase (Dove and Madanwar, 2006). This shift of the enzyme's optimum temperature after immobilization could be an added

advantage in biodiesel production because temperature is a very important variable in lipase catalysed transesterification.

Different surfactants have different effects on lipase. The activation of lipase by gum arabic is in agreement with the report of Ali *et al*., (2001) who showed the enhancement in the activity of lipase by gum arabic. The stimulation of lipases by nonionic detergents could be due to their functions as stabilizers of interfacial area facilitating the access of the substrate to the enzyme, since the catalytic reactions of lipases occur at an oil-water interface (Karadzic *et al*., 2006). As shown in (Figure 4.12) the inhibition of lipase by CTAB agrees with the findings of Ren *et al.*, (2010) who reported the inhibiton of lipase activity by CTAB. Parvez *et al*., (2013) showed that SDS and CTAB led to inhibition of *Rhizopus niveus* lipase activity.The inhibitory effects of tween-80 could be due to competitive binding to lipase with *p*NPL. Xin *et al*., (2011) also demonstrated the inhibition of lipase activity by EDTA and activation by SDS and triton X-100 respectively. The inhibitory effect of SDS could be attributed to its effect in creating the imbalance of ions in the reaction mixture by absorption or release (Benjamin and Pandey, 1998). Our result contradicts the earlier report of You-Yan *et al*., (2000) who showed the activation of lipase by tween-80.

The inability of PMSF to inhibit lipase activity as shown in Figure 4.14 was reported in *Marcescens* Sr41 8000 by Matsumae and Shibatani (1994). The basis for the absence of inhibiton of lipase in the presence of PMSF could be that the serine residue is deeply located inside the molecule due to presence of a hydrophobic lid which imposes steric constraints thus making PMSF unable to contact with serine at the catalytic site (Schmidt-Dannert *et al*., 1994). The lack of inhibition of lipase by reducing agents indicates that the enzyme contains a few sulfhydryl groups or the enzyme is a monomeric enzyme as supported by the existence of a

single band from the electrophoregram shown in Plate 4.1. Vandana and Bera (2005) reported the inhibition of lipase from *Pseudomonas aeruginosa* MTCC2488 by EDTA. The decreased activity of lipase in the presence of EDTA indicates that the enzyme might be a metalloenzyme (Fox and Stepaniak, 1983).

The activation of lipase in the presence of calcium ions as shown in (Figure 4.13) is supported by Rashid *et al.,* (2001) who demonstrated maximum lipase activity in the presence of Ca^{2+}. Dong *et al.,* (1999) reported increased in the activity by 250% in lipase from *Pseudomonas spp* in the presence of Ca^{2+}.The inhibition of lipase activity by Co^{2+} and Mn^{2+} contradicts the earlier reports of Hiol *et al*., (2000) who observed that Mg^{2+},Co^{2+} and Mn^{2+} enhanced lipase activity but was inhibited by the Fe^{2+},Cu^{2+} and Ba^{2+}.Lotrakul and Dharmsthiti (1997) reported the inhibition of lipase by $Fe^{2+}$and Mg^{2+}**. The inhibition of lipase activity by Ni^{2+} contradicts the earlier report by** Mobarak *et al.,* (2011) who demonstrated that Ni^{2+}enhanced the activity of lipase from *Pseudomnas aeruginosa* KM110. Matsumae and Shibatani (1994) suggested that the effect of metals could be attributed to a change in the solubility and the behaviour of the ionized fatty acids at interfaces. The increase in activity of lipase due to certain ions can be attributed to the vital role played by a particular metal ion in building stable enzyme geometry as a result of its binding to the interior of enzyme (Ramani *et al.,* 2010).

The stability of lipase in organic solvents offers advantages for enzyme precipitation and synthetic applications. The sensitivity of lipases to solvents varies depending on their polarity with polar solvents being more destabilizing than non-polar solvents (Ahmed *et al.,* 2010). Our findings are in close agreement with those of Ramani *et al.,* (2010) in which lipase isolated from *Pseudomonas gessardii* showed good activity in non-polar solvents. Bancerz *et al*., (2005) showed that *P.simplicissimum* lipase has low stability in water-miscible solvents with good

stability in water-immiscible solvents such as *n*-hexane, heptane, benzene and isooctane. The result contradicts the earlier report by Davender *et al*., (2012) who observed the activation of lipase from *Bacillus sp*. strain DVL2 in the presence of ethanol, methanol and *n*-hexane. The inactivation of lipase by methanol and ethanol might be due to the fact that polar solvents removed the essential water molecules from active sites of enzyme required for conformational flexibility that caused denaturation of the enzyme. Rua *et a*l., (1993) reported that residues of the hydrophobic solvent, which may be transferred to the assay system with the enzyme after the pre-incubation step, keep it in the open conformation and therefore the lid of the enzyme does not block the active site crevice, keeping a flexible conformation and therefore increasing its stability.

One of the important characteristics of an immobilized enzyme is its stability and re-usability over an extended period of time.The repeated use of calcium alginate immobilized *P. aeruginosa* lipase in transesterification of *A. nilotica* oil was studied. Zorica *et al.*, (2002) reported the use of alginate-immobilized lipase for up to three reaction cycles with little loss of activity. Shefai and Alam (2010) reported that alginate immobilized lipase from *Penicillium chrysogenum* retained 72.09% activity after six cycles. Yi *et al.*, 2009) showed that lipase from *C. rugosa* immobilized on alanine chitosan beads retained 77% of the initial activity after 10 times of reuse. Won *et al.*, (2005) found that lipase from *C. rugosa* entrapped in calcium-alginate gel beads retained 72% after three cycles. The ability of lipase to be reused for a number of transesterification cycles might be due to its entrapment in calcium alginate beads. However, the decreased methyl ester conversion observed after five cycles could be due to the leakage of enzyme from the beads as a result of washing at the end of each cycle as no make-up quantities of enzyme was added

(Elnashar, 2010). However, Shimada *et al*., (1999) have reported more than 95% ester conversion even after 50 cycles (100 days) of the reaction.

Response surface statistical method was performed to determine factors contributing to the increase in biodiesel yield by lipase catalysed transesterification of *A. nilotica* seed oil. From the results of analysis of variance (Table 4.6) time has the lowest *p*-value and *F* value among the other variables indicating that time is an important variable in biodiesel production. According to the estimated coefficients exhibited in equation (1) time has a positive effect on the yield of the produced biodiesel. In statistical analysis probability value less than 0.05 means that the model is significant. This means that the probabilities D, C^2 and D^2 are the significant model terms .The F-value of 2.92% implies the significance of the model in predicting the biodiesel yield.

The variables affecting biodiesel yield could not be analyzed independently as a result of theirinteractive effects. In each of the three dimensional plots as shown in Figure (4.25 to 4.30) the interactive effect of the two variables was plotted while the third and fourth variables were fixed at the medium values. Non polar solvents such as *n*-hexane play a very important role in lipase catalysed trans-esterification in that they act as reaction medium and a binder between triacylglycerols and methanol. The increased biodiesel yield with respect to *n*-hexane and oil/methanol ratio interaction as shown in (Figure 4.25) could be due to the ability of nonpolar solvents to dissolve both methanol and glycerol there by elimin ating the inhibitory effects being caused on lipase (Watanabe *et al*., 2000). The decreased biodiesel yield in excess methanol might be due to the deactivation of lipase by exposure to methanol (Dossat *et al*., 2002). Li *et al*., (2006) reported maximum biodiesel synthesis from rapeseed oil at 1: 3.6 oil/ methanol ratio. Nie *et al*. (2006) obtained lowest biodiesel yield with polar solvents such as acetone. The activity of enzymes increases with increased substrate

concentration, a first order kinetic.The decrease in biodiesel yield at high lipase and oil/ methanol ratio could be that in the presence of a high amount of lipase the active site cannot be exposed to the substrates and many molecules of the enzyme aggregate together and thus results in low biodiesel yield (Liou *et al*., 1998). The elliptical form of the three dimensional response plots shows the significance of the interactions of variables on the biodiesel yield (Samukawa *et al*., 2000). As shown in (Figure 4.27) the reduction in biodiesel yield at 360 minutes could be because of reverse transesterification reaction which results in loss of methyl esters (Eevera *et al*., 2009). The result showed that the optimized conditions of biodiesel production resulted in 55.9% yield. Shah *et al.,* (2004) optimized the conditions for the activity of immobilized lipase from *Chromobacterium viscosum* for the transesterification of *Jatropha* oil with ethanol and obtained a yield of up to 92%. Zhao *et al*., (2007) reported ethanol molar ratio to oil of 3:1, temperature 70 °C, incubation time of 24 hours, impeller speed 180 rpm and iso-octane as the optimum conditions for obtaining 71% biodiesel yield from *Jatropha* by *Pseudomonas fluorescens* silica immobilized lipase. Minodora *et al.,* (2010) optimized with 50 U/g lipase, temperature 45^{o}C, molar ratio of methanol/ soybean oil 6:6, rate of stirring 400 rpm and incubation time (93.6 hours) and obtained 70% yield. Mehdi *et al.*, (2014) reported reaction temperature of 65°C, methanol to oil molar ratio of 9:1, NaOH concentration of 0.72% w/w, reaction time of 45 minutes as the optimum conditions for biodiesel production by alkali catalyzed transesterification of used frying oil with 93.6% methyl ester yield. Annapurna *et al.*, (2009) achieved 68% conversion with respect to methyl oleate with an oil/ methanol molar ratio of 1:4, 50 U of immobilized lipase/g of oil and a *tert*-butanol: oil volume ratio of 0.8:1 at 55°C after 48 hours of reaction. The low yield of biodiesel obtained from our study could be attributed to the following: (i) Internal and external mass transfer limitation for large molecules like

triacylglycerol and fatty acid alkyl ester that are caused by immobilization of lipase (Fjerbaek *et al.,* 2009) and (ii) Product inhibition due to the effect of glycerol on external film layer formation (Belafi-Bako *et al.*, 2002)

Transesterification process does not change fatty acid composition of the primary oil (Salvi and Panwar, 2012). The reason for the lack of change in the fatty acid composition is because, transesterification results in the reaction between fatty acids contained in a triacylglycerol molecule to form biodiesel in a reaction catalysed by an enzyme or a chemical catalyst without change in the composition, chain length or identity of fatty acids. As shown in (Table 4.8) the most abundant methyl ester in *A. nilotica* biodiesel is monounsaturated methyl octadecenoic acid. The implication of having monounsaturated fatty acids methyl ester predominantly improves the stability of the biodiesel as higher degree of unsaturation in the fatty acid methyl esters affects its suitability for use as fuel due to high polymerization tendency as result of peroxidation (Gaby and Peter, 1997). Peroxidation can be accelerated as a result elevated temperature experienced in combustion engines, which eventually causes the engine to be gummed with polymerized fatty methyl esters (Mohibbe *et al.,* 2005). The presence of saturated fatty methyl ester of hexadecanoic acid as the second most abundant in the produced biodiesel enhances its stability and reduces its susceptibility to peroxidation. Sokoto *et al*., (2013) reported fatty acid methyl ester of octadecenoate and hexadecanoate as the predominant esters in *L.vulgaris* biodiesel. Bamgboye and Hansen (2008) reported methyl octadecenoate and methyl hexadecanoate as the dominant esters in rape seed biodiesel.

Specific gravity is an important parameter for diesel fuel injection systems and for complete combustion, the values must be maintained within tolerable limits to allow optimal air to fuel ratios as high specific gravity of biodiesel can lead to incomplete combustion and particulate

matter emissions (Galadima *et al*., 2008). The viscosity of biodiesel has a significant impact on the operation of engine fuel injection and atomization systems and is known to affect engine wear and the rate of injector fouling. Viscosity of biodiesel depends on the amount and type of fatty acids of the feedstock used in biodiesel production. Fuels with low viscosity may not provide sufficient lubrication for fuel injection pumps resulting in leakage or increased wear. Biodiesel with high viscosity tend to form larger droplets on injection which causes poor combustion and increased smoke emission. As shown on (Table 4.9) the decrease in the viscosity of the produced biodiesel might be due to removal of glycerol from the reaction product.

The lower calorific value of *A. Nilotica* fatty acid alkyl esters were found to be in agreement with ASTM and EN standards. Aldo *et a*l., (2012) reported the calorific value of 30.4 $MJkg^{-1}$for castor oil biodiesel.

Cetane number indicates the ignition quality of the fuel. Meher *et al.*, (2006) reported that higher cetane number in biodiesel ensures good cold start and minimizes the formation of white smoke. Zahira *et al.*, (2013) reported cetane number of 54 for waste cooking oil biodiesel. The implication of low cetane number from our findings is the possibility of ignition delay of the biodiesel when used.

The flash point of *A. nilotica* oil methyl esters as shown in (Table 4.9) conforms to the ASTM and EN standards for biodiesel. This shows the less likelihood of the biodiesel produced to ignite accidentally and could be safely handled for storage. It is a parameter used to assess the overal flammability hazard of fuel (Knothe *et al.*, 2005).

The observed cloud point of *A. nilotica* biodiesel as shown in (Table 4.9) is lower than the values reported from lard oil methyl ester 13^0C, edible tallow oil methyl ester 16^0C and non edible tallow oil methyl ester 15^0C and higher than soybean oil methyl ester 1^0C and canola oil 0^0C (Vyas *et al*., 2009).

Pour point refers to the temperature at which the oil in solid form starts to melt or pour. In case where the temperature falls below the melt point, the entire fuel system including all fuel lines and fuel tank need to be heated. Hossain and Davies (2010) reported the pour point of -15^{o}C for sunflower and cotton seed oil biodiesel respectively. Rakibuddin *et al*., (2013) reported the pour point of 6^{o}C in biodiesel produced from waste cooking oil.

Iodine value determines the chemical stability of biodiesel fuels. The presence of air, heat, peroxides or the presence of double bonds influence the stability of fatty compounds such as biodiesel (Bajpai and Tyagi, 2006). Higher iodine value indicates less stability of biodiesel. The iodine value of the biodieselproduced from *A. nilotica* oil indicates its likelihood to undergo chemical oxidation that will affect its stability upon long time storage. As shown in (Table 4.10) the biodiesel produced from *A. nilotica* oil has iodine value indicating a moderate degree of unsaturation. The implication of the result is that the biodiesel produced will be stable owing to the absence of polyunsaturated fatty acids as the increased polyunsaturated fatty acid methyl esters content decreases its oxidation stability (Park *et al*., 2008). Ibeto *et al.,* (2011) reported the iodine value of 52.45 gI_2/100g fat in biodiesel produced from groundnut oil.Baboli and Kordi (2010) reported the iodine value of 156 gI_2/100 g fat in melon seed oil biodiesel.

Acid value of biodiesel indicates its quality as it determines the level of free fatty acids that may be present (Schinas *et al.,* 2009). The implication of the result is that the possibility of causing

wear in fuel systems and storage tanks is minimal. Zahira *et al.*, (2013) reported acid value of 3.6 mg KOH/g oil in biodiesel produced from waste cooking oil.

High peroxide value is associated with high rancidity rate. The low peroxide value obtained from of *A. nilotica* oil methyl ester is an indication that the oil is less liable to rancidity at room temperature.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

A. nilotica seed has a good oil yield with fatty acids mainly unsaturated. The refractive index and viscosity were suitable for biodiesel production. However, the high iodine value, acid value and peroxide value (187.0, 6.25 and 6.47) of the oil presents a problem of storage.

Lipase from *P. aeruginosa* was successfully isolated and partially purified with specific activity of 147.7 U/mg protein, purification fold of 4.63 and low K_m indicating high affinity for para nitrophenyl laurate. Its ability to retain activity over a wide range of temperature, pH and organic solvents makes it an ideal tool for biodiesel production.

The findings from this work have shown that the pH, temperature, organic solvent stability as well as the ability of lipase from *Pseudomonas aeruginosa* to be recycled improved with its immobilization on calcium alginate beads thus making the production of biodiesel from *Acacia nilotica* seed oil cost effective.

The analysis of variance for the quadratic model revealed that lipase and incubation time were the most significant factors in biodiesel production. The optimized condition for biodiesel production were oil/methanol ratio (1:10), *n*-hexane (1.2 ml), lipase concentration (64 U/ml) and incubation time of (195 minutes) .

The fuel properties of biodiesel produced under optimum conditions including viscosity, specific gravity, cloud point, pour point, flash point, calorific value, saponification value and iodine value were largely in conformity with ASTM 6751 and EN 14214 biodiesel standards.

6.2 Recommedation

Acacia nilotica seed oil should be considered for biodiesel production owing to its yield and desirable physicochemical characteristics.

Further studies should look into the use other bacterial species for isolation of lipase and its eventual usage for biodiesel production.

Other matrices should be considered for immobilization of lipase as well as whole cell immobilization.

The inclusion of other variables such as temperature, agitation speed in the optimization of biodiesel production should be considered for efficient yield.

The use of kits that are very specific for assaying lipase is also recommended..

Studies should also look at the possibility of genetic manipulation of *Pseudomonas aeruginosa* that will result in the expression of solvent tolerant lipase.

Contribution to Knowledge

i.From research I was able to come up with a model eq uation that fits my data by which any possible combination of transesterification variables can be used to obtain the desired biodiesel yield

ii. I was able to reducethe cost of biodiesel production from *A.niloyica* seed oil by immobilization of lipase which resulted in four transesterification cycles with out a significant loss of activity

iii. I was able to establish that oily environment can serve as a very importance natural source for isolation of lipase with desirable properties

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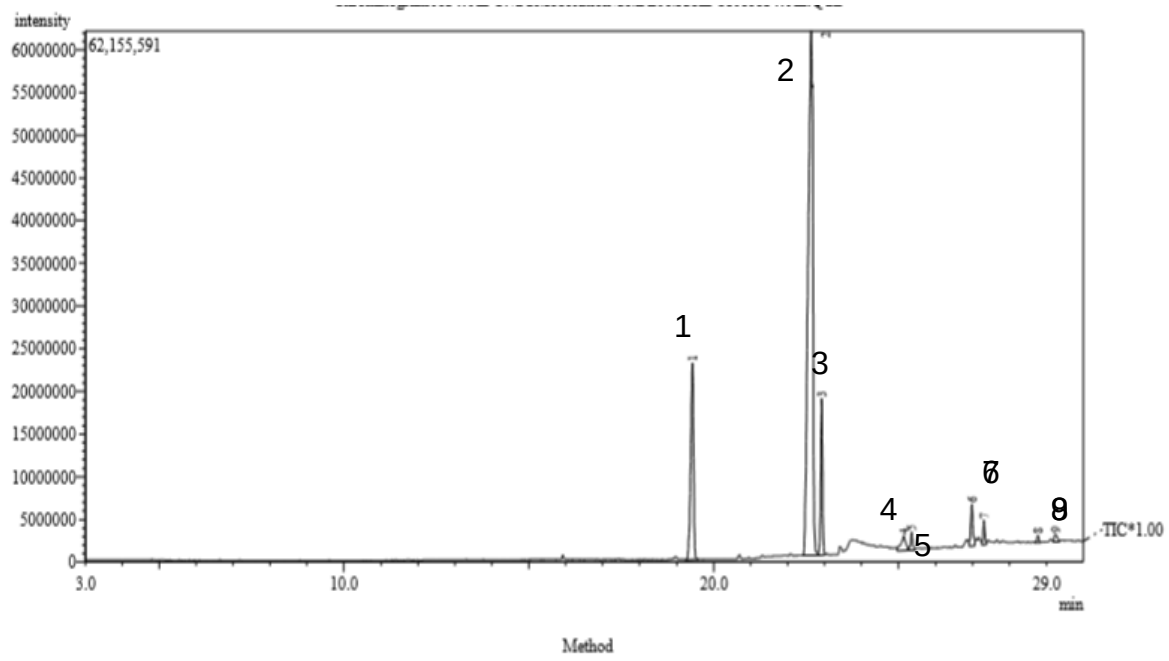
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APPENDICES



Appendix I: Gas Chromatogram Showing Peaks Corresponding to the Fatty Acid Content of *Acacia nilotica* Seed Oil

Appendix II:Time Course of Lipase Production by *P. aeruginosa*

S/No.	Incubation Time (Hours)	Lipase Activity (U/ml)
1	12	2.19
2	24	3.42
3	36	5.69
4	48	8.21
5	60	8.01
6	72	7.80
7	84	5.68
8	96	5.68

Appendix III: Elution Profile of Lipase from *Pseudomonas aeruginosa* (DEAE-Cellulose)

Fraction Number	Protein Concentration (mg/ml)	Lipase Activity (U/ml)
1	0.08	0
2	0.09	0
3	0.10	0
4	0.12	0
5	0.12	0
6	0.12	0
7	0.09	0
8	0.09	0
9	0.08	0
10	0.21	0
11	0.10	0
12	0.12	0
13	0.19	0
14	0.13	0
15	0.13	0
16	0.14	0
17	0.14	0
18	0.12	0
19	0.08	0
20	0.19	15.05
21	0.19	15.3
22	0.19	15.0
23	0.19	15.1
24	0.19	15.1

25	0.18	14.9
26	0.13	0
27	0.12	0
28	0.11	0
29	0.09	0
30	0.09	0
31	0.08	0
32	0.10	0
33	0.11	0
34	0.12	0
35	0.15	0
36	0.14	0
37	0.14	0
38	0.14	0
39	0.17	0
40	0.13	0
42	0.12	0
43	0.12	0
44	0.11	0
45		0
46		0
47		0

Appendix IV: Elution Profile of Lipase from *P. aeruginosa* (Sephadex G-75)

Fraction Number	Protein Concentration (mg/ml)	Lipase Activity (U/ml)
1	0.24	0
2	0.23	0
3	0.21	0
4	0.2	0
5	0.22	0
6	0.23	0
7	0.23	0
8	0.21	0
9	9.2	0
10	0.22	0
11	0.24	0
12	0.22	0
13	0.23	0
14	0.23	0
15	0.21	0
16	0.23	0
17	0.2	0
18	0.21	0
19	0.19	0
20	0.19	0
21	0.21	0
22	0.2	0
23	0.19	0
24	0.18	0

25	0.18	0
26	0.16	0
27	0.16	0
28	0.10	0
29	0.10	0
30	0.09	19.1
31	0.09	19.2
32	0.07	19.6
33	0.07	018.9
34	0.08	0
35	0.07	0
36	0.06	0
37	0.06	0
38	0.06	0
39	0.05	0
40	0.04	0
42	0.04	0
43		0
44		0
45		0
46		0
47		0
48		0

AppendixV: Lineweaver -Burk of Lipase from*P. aeruginosa.*

S/No.	1/[S]mmol	1/v (μmol/min
1	16.6	0.032
2	20	0.049
3	25	0.05
4	33.3	0.052
5	50	0.069
6	100	0.14

Appendix VI: Effect of pH on the Activity of Lipase from *P.aeruginosa*

S/No.	Ph	Lipase Activity (U/ml)
1	5.5	12.0
2	6.0	13.1
3	6.5	15.0
4	7.0	16.0
5	7.5	19.0
6	8.0	18.03
7	8.5	13.1
8	9.0	11,0

Appendix VII: pH Stability of Lipase from *P.aeruginosa*

S/No.	pH	Residual Activity (%)
1	5.5	68.83
2	6.0	79.18
3	6.5	83.96
4	7.0	100.1
5	7.5	100.1
6	8.0	100.1
7	8.5	78.88
8	9.0	78.17

Appendix VIII: Effect of pH on V_{max} and K_m of Lipase from *P.aeruginosa*

S/No.	pH	Log V_{max}/K_m
1	5.5	2.82
2	6.0	2.90
3	6.5	2.93
4	7.0	2.92
5	7.5	2.90
6	8.0	2.84
7	8.5	2.80

Appendix IX: Effect of Temperature on the Activity of Lipase from *P. aeruginosa*

S/No.	Temperature (^{o}C)	Lipase Activity (U/ml)
1	30	16.5
2	35	17.0
3	40	18.3
4	45	19.2
5	50	19.2
6	55	18.8
7	60	17.7
8	65	17.7

Appendix X:Thermostability Profile of Lipase from *P.aeruginosa*

S/No.	Temperature (^{o}C)	Residual Activity (%)
1	35	83.4
2	40	92.7
3	45	100
4	50	99.8
5	55	83.8
6	60	80.2
7	65	80.2

AppendixXI: Activation Energy Profile of Lipase from *P.aeruginosa*

S/No.	$1/T \times 10^{-3}$	Log v_o
1	3	1.283
2	3.05	1.235
3	3.1	1.262
4	3.15	
5	3.2	1.23
6	3.25	
7	3.3	1.217
8	3.35	

Appendix XII: Molecular Weight and Relative Mobility of the Molecular Weight Markers

S/No.	Relative Mobility (Rf)	Molecular Weight (kDa)
1	0.05	175
2	0.1	80
3	0.14	58
4	0.22	46
5	0.24	35
6	0.47	23
7	0.55	17

Appendix XIII: Effect of Some Surfactants on the Activity of Lipase from *P.aeruginosa*

S/No.	Surfactants	Residual Activity (%)
1	Gum Arabic	104.0
2	CTAB	42.2
3	SDS	44.6
4	Tween 80	47.5
5	Triton-X100	62.9

Appendix XIV: Effect of Some Cations on the Activity of Lipase from *P. aeruginosa*

S/No.	Cations	Residual Activity (%)
1	Mg^{2+}	92.4
2	Co^{2+}	72.2
3	Mn^{2+}	23.8
4	Ca^{2+}	110
5	Fe^{2+}	61.9
6	Cu^{2+}	64.1
7	Ni^{2+}	26.1

Appendix XV: Effect of Some Modulators on the Activity of Lipase from *P. aeruginosa*

Concentration	Modulators	Residual Activity (%)
5mm	PMSF	90.15
10mm	PMSF	83.0
5mm	EDTA	47.1
10mm	EDTA	34.2
5mm	β-Mercaptoethanol	102.2
10mm	β-Mercaptoethanol^{+}	94.5
5mm	Dithiotreitol	86.1
10mm	Dithiotreitol	73.7

Appendix XVI: Effect of Some Organic Solvents on the Activity of Lipase from *P. aeruginosa*

Concentration	Organic Solvents	Residual Activity (%)
15% v/v	Methanol	103.3
30% v/v	Methanol	50..8
45% v/v	Methanol	39.1
15% v/v	Ethanol	60.5
30% v/v	Ethanol	32.9
45% v/v	Ethanol	26.1
15% v/v	Isopropanol	80.8
30% v/v	Isopropanol	59.4
45% v/v	Isopropanol	49.9
15% v/v	n-hexane	112.3
30% v/v	n-hexane	83.3
45% v/v	n-hexane	61.9
15% v/v	Butanol	76.0
30% v/v	Butanol	60.5
45% v/v	Butanol	28.1

AppendixXVII: Lineweaver-Burk Plot of Immobilized Lipase from *P.aeruginosa*

S/No.	1/[S]mmol	1/v (μmmol/minute
1	16.6	0.08
2	20	0.10
3	25	0.14
4	33.3	0.16
5	50	0.26
6	100	0.41

Appendix XVIII: Effect of pH on the Activity of Immobilized Lipase from *P.aeruginosa*

S/N	pH	Lipase Activity (U/ml)
1	5.5	12.6
2	6.0	14.5
3	6.5	16.0
4	7.0	18.3
5	7.5	19.0
6	8.0	19.02
7	8.5	18.5
8	9.0	12.0

Appendix XIX:.Effect of pH on Stability of Immobilized Lipase from *P.aeruginosa*

S/No.	pH	Residual Activity (%)
1	6.0	76.9
2	6.5	86.3
3	7.0	85.2
4	7.5	100.1
5	8.0	100.1
6	8.5	100
7	9.0	69.1

Appendix XX: Effect of Temperature on the Activity of Immobilized Lipase from *P.aeruginosa*

S/No.	Temperature (^{o}C)	Lipase Activity (U/ml)
1	30	16.2
2	35	17.4
3	40	18.5
4	45	19.2
5	50	19.2
6	55	19.2
7	60	18.4
8	65	17.2

Appendix XXI: Profile of Stability of Immobilized Lipase from *P. aeruginosa* at Varying Temperatures

S/No.	Temperature (^{o}C)	Residual Activity (%)
1	30	86.9
2	35	91.6
3	40	97.4
4	45	100
5	50	100
6	55	100
7	60	97.9
8	65	94.4
9	70	65.4

Appendix XXII: Effect of Some Modulators on the Activity of Immobilized Lipase from *P.aeruginosa*

Concentration	Modulators	ResidualActivity (%)
5 mm	PMSF	91.3
10 mm	PMSF	83.1
5 mm	EDTA	51.0
10 mm	EDTA	36.7
5 mm	β-Mercaptoethanol	103.1
10 mm	β-Mercaptoethanol $^{+}$	78.6
5 mm	Dithiotreitol	85.5
10 mm	Dithiotreitol	76.4

Appendix XXIII: Effect of Some Cations on the Activity of Immobilized Lipase from *P. aeruginosa*

S/No.	Cations	Residual Activity (%)
1	Mg^{2+}	97.1
2	Co^{2+}	77.4
3	Mn^{2+}	40.1
4	Ca^{2+}	111.4
5	Fe^{2+}	64.5
6	Cu^{2+}	62.8
7	Ni^{2+}	50.0

Appendix XXIV: Effect of SomeOrganic Solvents of the Activity of Immobilized Lipase from *P. aeruginosa*

Concentration	Organic Solvents	Residual Activity (%)
15% v/v	Methanol	104.1
30% v/v	Methanol	60.1
45% v/v	Methanol	49.3
15% v/v	Ethanol	83.4
30% v/v	Ethanol	61.3
45% v/v	Ethanol	39.7
15% v/v	Isopropanol	81.7
30% v/v	Isopropanol	60.3
45% v/v	Isopropanol	53.1
15% v/v	n-hexane	113.4
30% v/v	n-hexane	84.0
45% v/v	n-hexane	62.5
15% v/v	Butanol	77.4
30% v/v	Butanol	61.3
45% v/v	Butanol	51.5

AppendixXXV: Effect of Number of Cycles on Transesterification Mediatedby Immobilized Lipase

Number of Cycles	Conversion of Methyl Ester (%)
1	55.9
2	55.9
3	55.9
4	54.7
5	54.2
6	42.3
7	28.1
8	18.5

Appendix XXVI: Relationship Between Predicted and Actual Biodiesel Yield

