

OPTIMIZATION OF BIODIESEL PRODUCTION FROM *JATROPHA* SEED-OIL  
USING MICROBIAL LIPASE-CATALYZED TRANSESTERIFICATION REACTIONS

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## DECLARATION

I hereby declare that this thesis entitled ‘OPTIMIZATION OF BIODIESEL PRODUCTION FROM *JATROPHA* OIL USING MICROBIAL LIPASE-CATALYZED TRANSESTERIFICATION REACTIONS’ has been performed by me in the Department of Biochemistry under the supervision of Prof. H.C. Nzelibe and Dr A.S. Agbaji. The information derived from the literature has been duly acknowledged in the text and a list of references provided. To the best of my knowledge and belief, no part of this project report was previously presented for another degree or diploma at any University.

Abbas, Mary

.....

Signature

.....

Date

## CERTIFICATION

This project titled: OPTIMIZATION OF BIODIESEL PRODUCTION FROM *JATROPHA* OIL USING MICROBIAL LIPASE-CATALYZED TRANSESTERIFICATION REACTIONS, by Abbas Mary meets the regulation governing the award of Masters Degree in Biochemistry of Ahmadu Bello University Zaria, and is approved for its contribution to knowledge and literary presentation.

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## DEDICATION

This work is dedicated to my husband and my lovely three little angels; Maltibweh, Deborah and Esther Mankilik whose patience, endurance and sacrifices has led to realization of this dream.

## ACKNOWLEDGEMENT

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## ABSTRACT

The environmental concern and diminishing reserves of fossil fuels has increased the demand for the study of biodiesel production. This study was undertaken to optimize biodiesel production from *Jatropha* seed-oil using microbial lipase-catalyzed transesterification reactions. Oils were extracted using Soxhlet extractor and the free fatty acids (%) were evaluated using Gas chromatography (GC). The highest oil yield (>60%) was of *Jatropha* seed provenances from Kebbi (*Wasugu Maga Danko*), Zamfara (*Dandotodaji Dandotodaji*) and Kastina (*Chanranchi Kuki*) states. While the least oil yield (<30%) was from Sokoto (*Shagari Kajiji*), Jigawa (*Birnin Kudu*) and Zamfara (*Magazu 2 Tsafe*) states. The fatty acids found in both non-transesterified and the transesterified *Jatropha* seed-oil provenances were linoleic, oleic, palmitic methyl ester, and stearic acids as the dominant fatty acids in variable compositions. Also, ecosanoic, palmitic and palmitoleic acids were the least fatty acids present. Fatty acid methyl esters in better composition were found in the transesterified *Jatropha* seed oil compared to those in the non-transesterified *Jatropha curcas* seed oil. The study revealed fatty acids profile which could be useful as feedstock for biodiesel and other industrial applications. It could also be concluded that transesterification of *Jatropha* seed-oil by enzymatic catalysis improved the quality of biodiesel production.

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# CHAPTER 1

## INTRODUCTION

### 1.1 GENERAL INTRODUCTION

Alternative fuel sources like bioethanol and biodiesel are being produced to combat the threats of global warming and high cost of fossil fuel. Emission of green house gases is on the increase everyday with rapid depletion of oil resources. Thus, biofuel like bioethanol provides numerous benefits in terms of environmental protection, economic development and national security (Arthe *et al.*, 2008). Biodiesel is a variety of ester-based oxygenated fuels derived from natural renewable biological sources such as vegetable oil. Through a process termed transesterification, in most developed countries, biodiesel is produced from rape seed, sun flower, soybeans, peanut which are essentially edible. More research is being geared towards finding an alternative renewable fuel through biological ways because of its positive environmental benefits (Bridgewater, 2006 and Arthe *et al.*, 2008).

Two approaches for transesterification of vegetable oils for the production of biodiesel have been suggested (Haas *et al.*, 2002). The first is a chemical one in which alcoholysis of oil by methyl or ethyl alcohol in the presence of a strong acid or base produces biodiesel and glycerol (Fukuda *et al.*, 2001). Chemical transesterification is efficient in terms of reaction time; however, the chemical approach to synthesize biodiesel from triglycerides has drawbacks, such as difficulty in the recovery of glycerol and the energy-intensive nature of the process. The second approach is the enzymatic one, in which lipase-catalyzed transesterification is carried out in non aqueous environments.

A number of plant species including *Jatropha curcas L.* are being established as feedstocks, but in the case of *Jatropha* the seed cake is toxic and can only be used as a less valuable fertilizer or combusted as a fuel (Jongschaap, *et al.*, 2007). Seed oil of *Jatropha* has been used as fuel substitute during world war 11. Enzyme-catalysed

transesterification has more advantages over chemical method because of the reduced feedstock limitations, downstream processing and environmental impact (Rosenthal *et al.*, 1996 and Jegannathan *et al.*, 2008). A lot of research work has been reported on the benefits of oil transesterification using enzymatic methods (Nelson *et al.*, 1996; Wu *et al.*, 1999; Abigor *et al.*, 2000; Kaide *et al.*, 2001; Belafi *et al.*, 2002; Oznur *et al.*, 2002 and Shieh *et al.*, 2003).

Today, enzymatic oil transesterification using commercially available enzymes like Lipzyme RMIM<sup>®</sup> and Lipozyme TLIM<sup>®</sup> which are lipases from microorganism have emerged as a promising technique for transesterification of oil from plant materials (Rosenthal *et al.*, 2001 and Sharma *et al.*, 2002). In this present work, crude lipase from microorganism (*Enterobacter aerogenes*, *Aspergillus niger* and *Aspergillus flavus*) was screened for their ability to transesterify *Jatropha* seed oil, the most promising enzyme from the microorganism was characterised.

## **1.2 STATEMENT OF THE PROBLEM**

The consumption and demand for petroleum products are increasing every year due to increase in population, standard of living and urbanization. In addition, limited and diminishing resources of fossil fuels, increasing prices of crude oil and environmental concerns have been the reasons for exploring the use of vegetable oil as an alternative to fossil fuel. Combustion of fuels such as coal, oil and natural gas has increased the concentration of carbon dioxide in the atmosphere thus increasing green house effects.

## **1.3 JUSTIFICATION OF THE STUDY**

Since chemical approach to transesterification of biodiesel from triglycerides has drawbacks, such as difficulty in the recovery of glycerol and the energy-intensive nature of the process, there is therefore the need to source alternative methods of transesterification of vegetable oil. Therefore, transesterification of *Jatropha curcas* oil to

biodiesel would be of great advantage such as reduction of global warming by reducing sulfur/carbondioxide (CO<sub>2</sub>) emmision.

#### **1. 4 AIMS AND OBJECTIVES OF THE STUDY**

The objective of the work is to optimize biodiesel production from *Jatropha curcas* seed oil using lipases from microogamisms (*Enterobacter aerogenes*, *Aspergillus niger*, and *Aspergillus flavus*).

##### **1. 4. 1 Specific Objective**

- i. To determine the percentage oil in some *Jatropha* seeds provenances.
- ii. To Screen some microorganism lipase activity for use in transesterification of *Jatropha* oil.
- iii. To characterise the crude lipase from organism with high activity in transesterification of the seed oil.

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 BIOFUELS

Biofuel can be broadly defined as any sort of fuel that is made from organic matter (Biomass). The most common biofuels are biodiesel and bioalcohols, including bioethanol and biobutanol also called biogasoline (Rafael, 2008). The concept of biofuel is not new. Rudolph Diesel was the first to use a vegetable oil (peanut oil) in a diesel engine in 1911 (Akoh *et al.*, 2007 and Antczak *et al.*, 2009).

The use of biofuels in place of conventional fuels would slow the progression of global warming by reducing sulfur and carbon oxides and hydrocarbon emissions (Fjerbaek *et al.*, 2009). Because of economic benefits and more power output, biodiesel is often blended with diesel fuel in ratios of 2:5 or 20% (Vasudevan and Briggs, 2008). The higher the ratio of biodiesel to diesel the lower the carbon dioxide emission (Fukuda *et al.*, 2001 and Harding *et al.*, 2007). Fukuda *et al.* (2001) reports that a mixture of diesel containing 20% biodiesel reduces carbon dioxide net emissions by 15.66% while using pure biodiesel significantly reduce the net emission of carbon dioxide (Vasudevan and Briggs, 2008).

Biodiesel has shown its ability to meet the energy demand of the world in the transportation, agriculture, commercial and industrial sectors of the economy (Akoh *et al.*, 2007; Basha *et al.*, 2009; Shafiee and Topal, 2009 and Robles *et al.*, 2009). The annual world consumption of diesel is approximately 934 million tons, of which Canada and the United States consume 2.14 and 19.06%, respectively (Marchetti *et al.*, 2008). As a green renewable and potentially unlimited, biodiesel has recently come out as the superlative alternative fuel which can be used in compression ignition engines with minor or no modifications (Xu and Wu, 2003; Vasudevan and Briggs, 2008; Robles *et al.*, 2009 and Leung *et al.*, 2010).

Biodiesel is a mixture of Fatty Acid Methyl Esters (FAMES) which is produced from renewable sources (Srivastava and Prasad, 2000). However, fats and oils are often used interchangeably referring to the feedstock employed in biodiesel production. The raw materials used for production of biodiesel can be crude, refined or waste such as frying oils/fats (Marchetti *et al.*, 2008). The feedstock can also be classified as plant derived, animal derived, microbial or waste materials (Akoh *et al.*, 2007). Subramanian *et al* (2005) identified more than 300 oil-bearing plants/trees that can be utilized to make biodiesel.

The most popular plant derived oils used for biodiesel production are: canola, coconut, cottonseed, groundnut, jatropha, karanj, olive, palm, peanut, rapeseed, safflower, soybean and sunflower oils (Demirbas, 2003; Akoh *et al.*, 2007 and Robles *et al.*, 2009). Waste oils and fats (beef tallow, lard and yellow grease), hemp oil, waste cooking oil, the greasy by-product from omega-3-fatty acids production from fish oils and microalgae oil are also considered as potential alternative feedstocks for biodiesel production (Demirbas, 2003; Marchetti *et al.*, 2008; Ranganathan *et al.*, 2008 and Antczak *et al.*, 2009).

However, there is a concern about using plant derived oils and fats since the crops used for biodiesel production are also needed for food, feed and oleochemical industries (Li *et al.*, 2007 and Jegannathan *et al.*, 2008). Biodiesel factories must compete with food, cosmetic, chemical and livestock feed demands for the crops (McNeff *et al.*, 2008). There is, also, an environmental concern because an increased demand for vegetable oils requires an increase in the use of fertilizers which contribute to greenhouse gas emissions. In fact, biodiesel production from heavy fertilized crops could result in a 70% increase in greenhouse gas emission

(Jegannathan *et al.*, 2008).

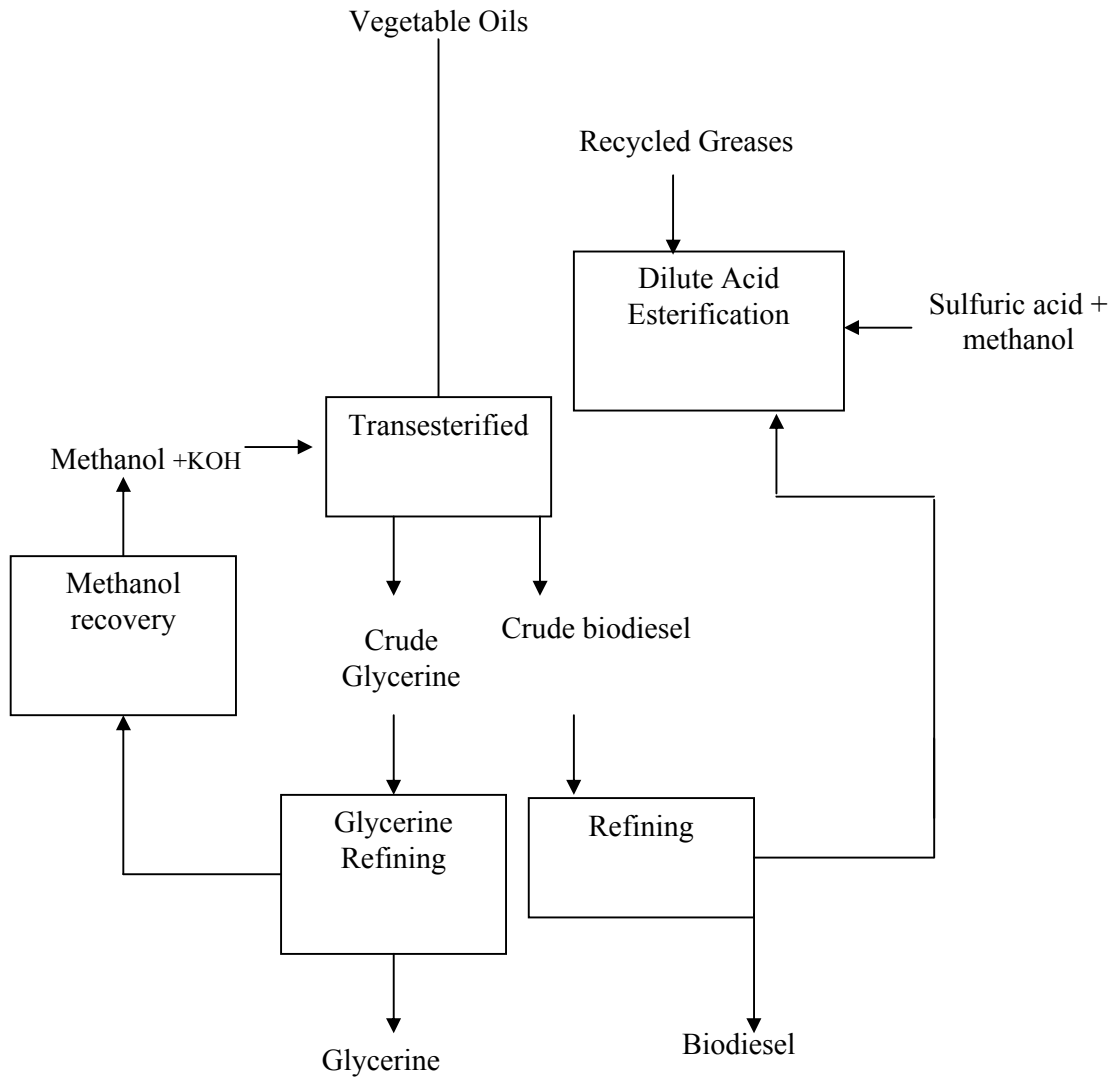
The choice of feedstock depends on where the biodiesel is being produced and used which could meet norms of internationally accepted ASTM standards. Parameters such as saponification number, iodine value and cetane number of fatty acid methyl esters of the oil, also, play an important role in selection of feedstock for biodiesel production (Sharma and Singh, 2010). Today, the United States largely uses soybean oil, Europe uses rapeseed and sunflower oils, Southeast Asia uses palm oil and the Philippines uses coconut oil (Bhatti *et al.*, 2008 and Murugesan *et al.*, 2009).

Another important factor in biodiesel production is the fatty acid composition of the source of oil or fat. Oils containing higher levels of saturated fatty acids than unsaturated fatty acids (have one or more double bonds) may solidify and clog the fuel lines during the winter condition (Pinto *et al.*, 2005; Akoh *et al.*, 2007 and Demirbas, 2008). Biodiesel which contains high levels of unsaturated fatty acids are less viscous and show higher pour and cloud points properties which make biodiesel suitable for warm and cold weather conditions (Robles *et al.*, 2009).

It can be said that the choice of feedstock is a balance between the unsaturation and the chain length of fatty acid (Robles *et al.*, 2009). It has been predicted that feedstocks with a high level of oleic acid (an unsaturated fatty acid that is 18 carbons long with a single double bond) are the best suited for biodiesel production. Biodiesel produced from feedstocks containing oleic acid has characteristics that are the most similar to conventional diesel (Knothe, 2005 and Robles *et al.*, 2009). Table 2.1 below shows the approximate fatty acid profile by percentage of many fats and oils used for biodiesel production.

**Table 2.1. Fatty acid profile of oils and fats used for biodiesel production (Akoh *et al.*, 2007; Marchetti *et al.*, 2007)**

<b>Oil/fats</b>	<b>Arachidic (20:0)</b>	<b>Linoleic (18:2)</b>	<b>Linolenic (18:3)</b>	<b>Oleic (18:1)</b>	<b>Palmitic (16:0)</b>	<b>almitoleic (16:1)</b>	<b>Stearic (18:0)</b>	<b>Other</b>
Canola		22.3	8.2	64.4	3.5		0.9	0.7
Coconut				6.0	5.0		3.0	86.0
Cotton seed		57.5		13.3	28.3		0.9	
Groundnut		26.0		51.6	8.5		6.0	7.9
Jatropha	0.2	36.2		37.0	16.4	1.0	6.2	3.0
Karanj	1.6	17.7	3.6	51.8	10.2		7.0	8.1
Microalgae		2.2	0.9	1.3	15.5	17.3	0.3	62.5
Olive	0.4	8.5	0.7	74.2	11.8	1.5	2.6	0.3
Palm Oil		10.1	0.2	40.5	42.6	0.3	4.4	1.9
Peanut	1.3	32.0	0.9	48.3	11.4		2.4	3.7
Rapeseed		22.3	8.2	64.4	3.5		0.9	0.7
Safflower seed		77.0		13.5	7.3	0.1	1.9	0.2
Soybean	0.3	53.8	9.3	20.8	11.4		4.4	
Sunflower	0.3	62.4		25.5	7.1		4.7	
Tallow				44.5	29.0		24.5	2.0



Adapted from <http://www.cogeneration.net/transesterification.htm>

Figure 2.1. Flow chart of Basic Technology for biofuel production from vegetable oil

### **2.1.1 Advantages of biodiesel**

The higher oxygenated state compared to conventional diesel leads to lower carbon-monoxide (CO) production and reduced emission of particulate matter (Graboski, and McCormick, 1998).

Biodiesel also contains little or no sulphur or aromatic compounds; in addition to the reduced CO and particulate emissions, the use of biodiesels confers additional advantages, including a higher flash point, faster biodegradation and greater lubricity than conventional diesel and blending biodiesel with low sulphur fuel restores lubricity (Knothe *et al.*, 2005).

In addition to reduced carbon dioxide and particulated emissions, the use of biodiesel confers advantages, among the benefits include;

- A higher flash point, the higher flash point of biodiesel allows safe handling and storage.
- Faster biodegradation; the biodegradability of biodiesel is particularly advantageous in environmentally sensitive areas where fuel leakages pose large hazards.
- Lubricity: the lubricity issue has become increasingly important with the widespread mandated adoption of low sulphur diesel fuels. The elimination of sulphur containing compound from conventional diesel also removes the fuel constituents that contribute to the inherent lubricity of the fuel. Biodiesel has greater lubricity than conventional diesel and blending biodiesel with low sulphur fuel restores lubricity (Knothe *et al.*, 2005).

The biggest advantage of using biodiesel, especially given today's environmental and political concern is that biodiesel in principle is a sustainable source of liquid transportation fuels and is essentially neutral with respect to the production of carbon dioxide. Taking into consideration other inputs such as fertilizer and energy for transportation and conversion, biodiesel returns almost double the energy used for its

production; its subsequent combustion in place of conventional diesel reduces green house gas emission by 40% (Hill *et al.*, 2006).

### **2.1. 2 Biofuel from *Jatropha curcas* plant**

*Jatropha* is a particularly good example of non-food crop for biofuel production since it thrives on poor soil and land unsuitable for food crops, actually creating topsoil and gives high oil yield (Gubitz *et al.*, 1999). Recently, high yield of oil content of *Jatropha* seed (66.4%), attracted global attention for biofuel (Openshaw 2000, Chent *et al.*, 2006; Adebowale and Adedire, 2006, and Li *et al.*, 2007). The fact that *Jatropha* oil can not be used for nutritional purposes without detoxification makes it use as energy or fuel source very attractive as biodiesel. In Madagascar, Cape Verde and Benin, *Jatropha* oil was used as mineral diesel substitute during the Second World War (Agarwal, 2007).

According to Emil *et al* (2009), the fatty acid compositions of the *Jatropha* seed oil were; oleic, linoleic, palmitic and stearic fatty acid. Oleic acid showed the highest percentage composition of 42.8% followed by linoleic acid with 32.8%. Thus, *Jatropha* seed oil can be classified as oleic – linoleic oil compared to other vegetable oil. *Jatropha* oil seed has highest oleic content than palm oil, palm kernel, sunflower, coconut and soybean oil. *Jatropha* has been reported to produce large amounts of oil, as much as 1300/ha<sup>-1</sup> (Fairless, 2007), but large scale production may require the development of mechanized harvest and processing technologies.

## **2. 2 THE PLANT “*JATROPHA CURCAS L*”.**

*Jatropha curcas L* is a multipurpose drought resistant perennial plant, and belongs to the family *Euphobiaceae*. The genus *Jatropha* comprises of about 70 species growing in tropical and sub-tropical countries like Brazil, India and Nigeria among others. The seed of the *Jatropha curcas* offers potential for biodiesel production. The percentage oil content of *Jatropha* seed ranges between 40-60% (Makkar *et al.*, 1997).The compositions of the

oil is similar to those found in vegetable oil which is used for edible purposes. However, presence of some antinutritional compounds in *Jatropha* seed oil such as phorbol esters renders it unsuitable for cooking (Gubitz *et al.*, 1998).

The young leaves of *Jatropha* plant may be safely eaten, steamed or stewed; they are flavoured for cooking with goat meat to counteract the smell. Though purgative, the nuts are sometimes roasted and eaten. In India, the pounded leaves are applied near horses' eyes to repel flies. The leaves are used for topical application for treatment of piles and inflammation of the tongue in babies, scabies, eczema and ringworm (Rajore and Batra, 2005). The latex has proven medical benefits, it exhibits tendency to oppose cancerous growth in human body (Kosasi *et al.*, 1989). The seeds contain semi-dried oil that can be used as an efficient substitute for diesel engines (Bhasasabutra and Sutiponepeibum, 1982). *Jatropha curcas* is also efficacious in treatment of dropsy and paralysis, and has antihelminthic properties (Kirtikar and Basu, 1933). Besides its medicinal properties, it is also an excellent species for agro forestry programme. It can be used in the control of flood, nutrient leaching, soil erosion and shifting of sand dunes (Saika, *et al.*, 2008).

### **2. 2. 1 Cultivation of *Jatropha curcas* plant**

*Jatropha* plants can be cultivated in places where enough rainfall and warm climates exists. It grows on almost any soil, such as sandy-saline. It is resistant to long periods of drought and can withstand short spells of light frost. The trees may produce for over 35 years. Thus, it is known that *Jatropha* can be established from seed, seedling and vegetative from cuttings. Plant from seeds develops a typical and lateral roots and cutting do not develop a taproot (Heller, 1996). *Jatropha* is a fast growing plant which can achieve a height of three meters within three years under a variety of growing conditions (plate 1). Use of branch clustering for propagation is easy and results in rapid growth, the plant can be expected to start bearing fruit within one year of planting (Jones and Miller, 1992).

*Jatropha* grows well in low rainfall condition and it can also respond to higher rainfall particularly in hot climatic condition. Considerable plantation of *Jatropha* has been undertaken by a number of active organizations in its promotion including the Agricultural Research Trust, Biomass Users' Network and Plant Oil Producers Association. In equatorial region, where moisture is not a limiting factor (i.e. continuously wet tropics or under irrigation), *Jatropha* can bloom and produce fruit all year. A drier climate has been found to improve the oil yields of the seeds (Jones and Miller, 1992). *Jatropha* products from the fruits – the flesh, seed coat and seed cake are rich in nitrogen, phosphorous and potassium (NPK) and providing humus and fertilizers to the soil. This can further enhance the productivity of other agricultural crops.



Source: (<http://www.jatrophacurcasplantations.com>).

Plate I. A view of *Jatropha curcas L* plantation

### **2. 2. 2 The seed of *Jatropha curcas* plant**

The seed pod contains 2 or 3 large black oily seed (plate IIa and IIb). This seeds about 2.2cm long becomes mature when the fruit changes from green to yellow. The seed contain high percentage (37%) of clean oil used for candles, soaps and biofuel production. The seed have been used in the production of biodiesel. Estimate of *Jatropha* seed yield vary widely due to lack of research data, the genetic diversity of the crop and the range of environment in which it is grown. Seed yield ranges from 1500-2,000kg/hectare (Dar, 2006). The seed germinates easily (7-14 days) in organic mix soil with good moisture. The high quality viable seeds have germination rate of 90% or higher (<http://www.africanjatropha.com>).



Source: (<http://www.africanjatropha.com>).

Plate IIa. A view of *Jatropha curcas* matured fruits



## *Jatropha curcas* Nuts

Source: (<http://www.africanjatropha.com>).

Plate IIb. Dried matured *Jatropha curcas* nuts

### **2. 2. 3 Toxicity of *Jatropha curcas* plant**

Characteristics of many members of the family *Euphorbiaceae*, *Jatropha* plants contain several toxic compounds, including lectin, saponin, carcinogenic phorbol etc. The black shelled seeds are very toxic. They contain toxalbumin, curcin, phytate, trypsin inhibitor, amylase inhibitor etc (Rakshit *et al.*, 2006). Despite this, the seeds are occasionally eaten after roasting, which reduces some of the toxicity. In 2005, Western Australia banned *Jatropha gossypifolia* as invasive and highly toxic to people and animals (<http://www.timesonline.co.uk>).

## **2. 3 TRANSESTERIFICATION OF VEGETABLE OIL**

Transesterification is a process for the conversion of vegetable oil to its corresponding fatty esters and thus biodiesel. For several years, the transesterification of vegetable oils to form esters, especially methyl esters, has received considerable attention. Transesterification of vegetable oils by chemical catalysis have been extensively investigated and processes were also patented by many workers (Cvengros and Povazanec, 1996; Haas *et al.*, 2002; Ergun *et al.*, 2002).

### **2. 3. 1 Transesterification catalysts**

The transesterification process is catalyzed by alkalis, acids and enzymes. However, the use of alkali catalysts is 100% in commercial sector. The most common alkaline catalysts are sodium hydroxide (NaOH) and potassium hydroxide (KOH) (Schuchardt *et al.*, 1998; Marchetti *et al.*, 2008; Robles *et al.*, 2009). Other alkaline catalysts include carbonates, methoxide, sodium ethoxide, sodium propoxide and sodium butoxide (Fukuda *et al.*, 2001). These chemicals proved to be the most economic because of higher conversion rate of esters under a low temperature and pressure environment and short reaction time (Bacovsky *et al.*, 2007; Leung *et al.* 2010).

The main drawback of the technology is the sensitivity of alkaline catalysts with respects to Feedstock purity. The presence of free fatty acids and water in the feedstock has a significant impact on the transesterification reaction (Leung and Guo, 2006; Marchetti *et al.*, 2008). Besides the multi step purification of end products, alkaline transesterification requires treatment. The amount of waste water produced is approximately 0.2 ton per ton biodiesel produced. The need for extensive downstream processing makes alkaline transesterification expensive and not environmentally friendly (Fjerbaek *et al.*, 2009).

The second commercially used catalysts are acid-catalysts. The most commonly employed acids are: Sulphuric acid, hydrochloric acid and sulphonic acid. Despite the fact that yield is very high and no soap formations, the corrosive nature of acid, very slow reaction rate and higher temperature conditions limit the use of the technology for esterification reactions (Freedman *et al.*, 1984 and Bacovsky *et al.*, 2007). The acid and alkali Transesterification processes are energy intensive and require extensive downstream processing (Xu and Wu, 2003). Post treatments are required after the completion of transesterification reaction as the end products are a mixture of esters and glycerol. These post treatments include a multi-step purification of end products which include:

- i. Separation of glycerol by gravitational settling or centrifugation,
- ii. Neutralization of the catalyst,
- iii. Deodorization
- iv. Removal of pigments (Antczak *et al.*, 2009; Banerjee and Chakraborty, 2009).

Enzymatic transesterification is, therefore, an attractive method for biodiesel production over chemical methods because of the reduced feedstock limitations, downstream processing and environmental impact (Jegannathan *et al.*, 2008). The use

of enzyme catalysts eliminates these problems associated with acid and alkali catalysts as well as presents other production benefits. Unlike the alkaline catalysts, enzymes do not form soaps so there is no restriction on free fatty acid content (Harding *et al.*, 2007; Fjerbaek *et al.*, 2009). Unlike the acid catalysts, enzymes are not severely inhibited by water, so there is little concern about water production (Dizge and Keskinler, 2008). Since the enzymes are capable of completely converting free fatty acids to Fatty acids acyl esters with low cost feedstocks such as waste oils and lard can be used (Fukuda *et al.*, 2001).

### **2. 3. 2 Chemical transesterification:**

#### *2. 3. 2. 1 Base catalyzed transesterification*

Several conventional and non-conventional base-catalyzed transesterification processes have been reported in a review by Knothe *et al* (1997). Boiler ashes, potassium hydroxide (KOH) amongst other catalysts were successfully used in the ethanolysis and methanolysis of palm and coconut oils with yields as high as 90% (Encinar *et al.*, 2002, and Ejikeme *et al.*, 2007). It has also been reported that methyl and ethyl esters with 90% yield can be obtained from palm and coconut oil from the press cake and oil mill and refinery waste with the ashes of the wastes (fibers, shell, and husks) of these two oil seeds, and with lime, clay, zeolites, *etc* (Grailler *et al.*, 1982).

Methanolysis has been reported to yield 96-98% esters when palm oil is refluxed for 2 hours. Using coconut-shell ash and other ashes from the combustion of plant wastes such as fibers of palm tree that contain potassium and sodium carbonate (Grailler *et al.*, 1982). Calcium oxide or magnesium oxide has been shown, at 60-63<sup>0</sup>C, to be the best catalyst system amongst potassium carbonate, sodium carbonate, iron (III) oxide, sodium methoxide, sodium

aluminate, zinc, copper, tin, lead and zinc oxide in the methanol transesterification of low-erucic rapeseed oil (Peterson and Scarrah, 1984).

Generally, the mechanism of the base-catalysed transesterification of vegetable oils involves four steps. The first step is the reaction of the base with the alcohol, producing an alkoxide and the protonated catalyst. The second step is the nucleophilic attack of the alkoxide at the carbonyl group of the triglyceride generating a tetrahedral intermediate (Meher *et al.*, 2006). The third step involves the formation of the alkyl ester and the corresponding anion of diglyceride. The final step involves deprotonating the catalyst, thus regenerating the active species, which is now able to react with a second molecule of the alcohol, starting another catalytic cycle.

#### *2. 3. 2. 2 Acid-catalyzed transesterification*

The acid-catalyzed transesterification process does not enjoy the same popularity in commercial applications as its counterpart, the base-catalyzed process. The fact that the homogeneous acid-catalyzed reaction is about 4000 times slower than the homogeneous base-catalyzed reaction has been one of the main reasons (Srivatava and Prasad, 2000). However, acid-catalyzed trans- esterification holds an important advantage with respect to base-catalyzed ones; the performance of the acid catalyst is not strongly affected by the presence of free fatty acids in the feedstock. Thus, a great advantage with acid catalysts is that they can directly produce biodiesel from low-cost feedstocks, generally associated with high free fatty acid concentrations (Srivatava and Prasad, 2000).

In the transesterification of triglyceride feedstock using acid catalysts, Mittelbach *et al* (1995) compared the activities of a series of layered aluminosilicates with sulphuric acid for the transesterification of rapeseed oil. These researchers used an initial molar ratio of 30:1 alcohol-to-oil and 5% catalysts. Among the catalysts tested,

sulphuric acid showed the highest activity. The solid catalysts showed varied activities depending on reaction conditions. Kaita *et al* (2002) designed aluminum phosphate catalysts with various metal-to-phosphoric acid molar ratios and used these materials for the transesterification of kernel oil with methanol. According to the authors, durable and thermostable catalysts were obtained with good reactivity and selectivity to methyl esters. However, the use of these materials still needed high temperatures (200°C) and high methanol to-oil molar ratios (60:1) in order to be effective.

The transesterification process in biodiesel production is catalyzed by Bronsted acids like HCl, BF<sub>3</sub>, H<sub>3</sub>PO<sub>4</sub>, H<sub>2</sub>SO<sub>4</sub> and sulphonic acids (Formo, 1954). Preferably, sulphuric and sulphonic acids are mostly used. These catalysts give very high yields in alkyl esters, but the reactions are slow, requiring typically, temperatures above 100°C and from 3-48hours to reach complete conversion (Zheng *et al.*, 2006). Freedman *et al* (1984) showed that the methanolysis of soybean oil, in the presence of 1mol of H<sub>2</sub>SO<sub>4</sub>, with an alcohol/oil molar ratio of 30:1 at 65°C, takes 50h to reach complete conversion of the vegetable oil (>99%), while the butanolysis (at 117°C) and ethanolysis (at 78°C) using the same quantities of catalyst and alcohol take 3hours and 18hours, respectively.

Peter *et al* (2002) studied the methanolysis of palm oil in a 6:1 molar ratio of methanol to oil using the following metal salts of amino acids; cadmium, cobalt, copper, iron, lanthanum, nickel and zinc. Arginate of zinc was shown to result in the highest yield and the reasonable rate of reaction estimated to be obtained at temperatures higher than 130°C. Report also indicated that soybean oil can be transesterified in methanol using sulphated zirconia-alumina and tin oxide as well as tungstated zirconia-

alumina acid catalysts, though the latter was adjudged most effective as it gave 90% conversion in 20h at 250°C (Furuta *et al.*, 2004). Other sulphated compounds of zirconium have also been studied with varying results. New solid acid/base catalysts as well as metal oxides have also been used in the transesterification process (Karmee and Chadba, 2005).

Reaction rates in acid-catalyzed processes may be increased by the use of larger amounts of catalyst. A rate enhancement was observed with the increased amounts of catalyst and ester yield went from 72.7 to 95.0% as the catalyst concentration was increased from 1 to 5%. The dependence of reaction rate on catalyst concentration has been further verified by the same authors and other groups (Crabba *et al.*, 2001). A further complication of working with high acid catalyst concentration becomes apparent during the catalyst neutralization process, which precedes product separation. Since CaO addition during neutralization is proportional to the concentration of acid needed in the reactor, high acid concentration leads to increased CaO cost and higher production cost (Crabba *et al.*, 2001).

### **2. 3. 3 Enzymatic transesterification**

Enzymes are biological catalysts which allow many chemical reactions to occur within the homeostasis constraints of a living system. Enzymes have enormous potential for reducing energy requirements and environmental problems in the chemicals and pharmaceutical industries. Over the last two decades, substantial research has been performed on the use of enzymes in the synthesis of various organics (Roberts, 1989; Arnold, 1998).

Large scale applications of enzymes have been reported in the production of detergents, drinks, textiles, leather, pulp, drugs and cosmetics (Kudli-Shrinivas, 2007).

Enzyme catalyzed transesterification reactions have been extensively used in production

of drug intermediates, biosurfactants and designer fats (Shah *et al.*, 2003). Enzymatic approach for production of biodiesel has been extensively reported, although this technology has not received much commercial attention except in china where the first industrial scale for biodiesel production in the world (with lipase as the catalyst at a capacity of 20,000 tons year<sup>-1</sup>) is in operation (Du *et al.*, 2008). The benefits of using enzymes as catalyst over the acid and alkali catalysts are:

- i. No soap formation.
- ii. Have ability to esterify both Free Fatty Acids (FFA's) and triglycerides in one step without the need of a washing step.
- iii. Capitate a higher quality glycerol.
- iv. Ability to handle large variation in raw material quality.
- v. A second generation raw materials like waste cooking oils, animal fat and similar waste fractions, with high Free Fatty acids and water content, can be catalyzed with complete conversion to alkyl esters with significantly condensed amount of waste water.
- vi. Reaction conditions are milder and there is less energy consumption with lower alcohol to oil ratio than chemical catalysts (Narasimharao *et al.*, 2007; Tamalampudi *et al.*, 2008 and Fjerbaek *et al.*, 2009).

#### 2. 3. 3. 1 Lipase-catalyzed transesterification

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) constitute a diverse and ubiquitous family of enzymes which are produced by animals, plants and microorganisms. The animal lipase most commonly used is the pancreatic lipase. Plant lipases include papaya latex, oat seed lipase and castor seed lipase (Akoh *et al.*, 2007). Microbes have been found to produce high yields of lipases in compares to the animals and plants. The commercialization of microbial lipases and their involment in enzymatic biodiesel production are more common than animal and plant due to their ease in

bulk production (Hasan *et al.*, 2006; Akoh *et al.*, 2007 and Antczak *et al.*, 2009). Lipases from microorganisms (bacterial and fungal) are the most used as biocatalysts in biotechnological applications and organic chemistry.

The physical and biochemical properties vary among lipases. As such, each industrial application requires lipases with specific properties. Therefore, there is always interest in new lipases that could be used in new applications (Aires-Barros *et al.*, 1994; Abramic *et al.*, 1999). Lipases have been successfully used in novel biotechnological applications for the synthesis of biopolymers and the production of enantiopure pharmaceuticals, flavor compounds, agrochemicals and biodiesel (Jaeger and Eggert, 2002).

Lipases are considered hydrolases which naturally hydrolyse triacylglycerols (Salis *et al.*, 2005) and are capable of catalyzing other unnatural reactions such as the alcoholysis of 15 triglycerides (Jaeger and Reetz, 1998; Joseph *et al.*, 2008). They act on the ester bonds of carboxylic acids (Fig. 2.2) allowing them to carry out their primary reaction of hydrolyzing fats (Joseph *et al.*, 2008). Many lipases are limited because their fatty acid chain is length specific, substrate specific and regioselective. However, the majorities of lipases are capable of converting triglycerides, diglycerides, monoglycerides and Free Fatty acids to Fatty Acid Acyl Esters (FAAEs) in addition to fat hydrolysis (Akoh *et al.*, 2007; Joseph *et al.*, 2008). It is the stability of lipases that allows them to catalyze the unnatural reaction of transesterification (Jegannathan *et al.*, 2008). The advantages of using lipases in biodiesel production are:

- i. Ability to work in very different media which include biphasic systems, monophasic system (in the presence of hydrophilic or hydrophobic solvents).
- ii. They are robust and versatile enzymes that can be produce in bulk because of their extracellular nature in most producing system.

- iii. Many lipases show considerable activity to catalyze transesterification with long or branched chain alcohols, which can hardly be converted to fatty acid esters in the presence of conventional chemical catalysts.
- iv. Products and byproduct separation in downstream process are extremely easier, the immobilization of lipases on a carrier has facilitated the repeated use of enzymes after removal from the reaction mixture and when the lipase is in a packed bed reactor, no separation is necessary after transesterification.
- v. Higher thermostability and short-chain alcohol-tolerant capabilities of lipase make it very convenient for use in biodiesel production (Bacovsky *et al.*, 2007 and Kato *et al.*, 2007).

The limitations of using lipases in biodiesel production include:

- i. Significant cost.
- ii. The risk that glycerol inhibits the lipase by covering it, due to its accumulation in the reaction mixture.
- iii. Initial activity may be lost because of volume of the oil molecule (Marchetti *et al.*, 2008 and Robles *et al.*, 2009). However, more research is needed in order to be able to use modified lipase on a large scale.

### 2. 3. 3. 2 Microbial lipase

Microbial lipases come from a variety of sources, Gupta *et al* (2004). The microbes that have been suggested as a sources from which common lipase are derived for biodiesel production include: *Aspergillus niger*, *Bacillus thermoleovorans*, *Burkholderi cepacia*, *Candida antarctica*, *Candida cylindracea*, *Candida rugosa*, *Chromobacteriu viscosum*, *Fusarium heterosporum*, *Fusarium oxysporum*, *Getrichum candidum* *Humicola lanuginose*, *Oospora lactis*, *Penicillium cyclopium*, *Penicillium roqueforti*, *Pseudomonas aeruginosa*, *Pseudomonas cepacia*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Rhizomucor miehei*, *Rhizopus arrhizus*, *Rhizopus chinensis*

*Rhizopus circinans*, *Rhizopus delemr*, *Rhizopus fusiformis*, *Rhizopus oryzae*, *Saccharomyces cerevisiae*, *Staphylococcus hyicus*, *Thermomyces lanuginose* (Akoh *et al.*,2007 and Fjerbaek *et al.*,2009).

Of these microorganisms, *Candida antarctica*, *Candida rugosa*, *Pseudomonas cepacia*, *Pseudomonas fluorescens*, *Rhizomucor miehei*, *Rhizopus chinensis*, *Rhizopus oryzae* and *Thermomyces lanuginosa* have produced the most effective lipases for transesterification (Vasudevan and Briggs, 2008). *Candida Antarctica* displayed high activity in methanolysis and ethanolysis but showed a lower conversion yield for other alcohols. Methanolysis using *Candida antarctica* in solvent free environment resulted in a 90% conversion in majority of studies.

Ethanolysis using *Candida antarctica* in a solvent free medium resulted in 82% conversion (Mittelbach, 1990). Rodrigues *et al* (2008) reports that the conversion yield decreases proportionally to the increase in carbon length of the alcohol. The combination of two or more lipases has also been suggested in order to lower cost and optimize conversion. Li *et al* (2006) used a combination of *Candida antarctica* and *Thermomyces lanuginosa* and obtained a 95% conversion in methanolysis using a *tert*-butanol solvent. Lee *et al* (2002) was successful in using a combination of *Rhizopus oryzae* and *Candida rugosa*.

The lipases produced by organisms can be used in various application sectors in different forms: extracellular or intracellular. Extracellular lipase refers to the use of the enzyme that has been previously extracted from the producing organism and purified. Intracellular lipase refers to the use of the enzyme while it is still contained in the producing organism (Robles *et al.*, 2009). Both extracellular and intracellular lipase can be immobilized on a solid support (Jegannathan *et al.*, 2008). Lipases can also be regiospecific which means they only act on specific bonds of the triglyceride

molecule (Robles *et al.*, 2009).

#### *Extracellular lipase:*

Microbial lipases are mostly intracellular which can be produced by submerged fermentation or solid state fermentation. The fermentation process is followed by purification step as a certain degree of purity simplifies their successful usage as biocatalysts (Balaji and Ebenezer, 2008 and Barberis *et al.*, 2008). The important purification step for producing extracellular lipase is a complex process and it depends on the origin and structure of the lipase (Palekar *et al.*, 2000 and Saxena *et al.*, 2003).

The large scale production of extracellular lipases should be economical, fast, easy and efficient. Unfortunately, the cost of novel purification technologies is higher (Bandmann *et al.*, 2000; Joseph *et al.*, 2008). The majority of immobilized lipases that are commercially available are extracellular (Robles *et al.*, 2009). The most commonly used ones are: Novozym 435 which is lipase from *Candida antarctica*, Lipzyme RMIM<sup>®</sup> which is lipase from *Rhizomucor miehei* and Lipozyme TLIM<sup>®</sup> which is lipase from *Thermomyces lanuginosus* (Robles *et al.*, 2009).

#### *Intracellular lipase:*

The biggest issue with enzymatic biodiesel production is the cost of enzymes. Thus, eliminating the costly step (the purification needed for extracellular lipases) has led to using whole cells as biocatalysts. Direct use of compact cells for intracellular production of lipases or fungal cells immobilized within porous biomass support particles as a whole biocatalyst represents an attractive process for bulk production of biodiesel and polyesters (Iftikhar *et al.*, 2008). The utilization of lipase while still contained in the cells is referred to as intracellular lipase (Robles *et al.*, 2009).

Some microorganisms can be spontaneously immobilized on certain supports. This

eliminates the costly purification step and the need for an extended immobilization process, which is necessary with extracellular lipase (Fukuda *et al.*, 2001). Using intracellular lipases as opposed to extracellular lipases slows down the transesterification process (Robles *et al.*, 2009), although their use increases the conversion efficiency since the lipase is relatively stable (Klibanov, 1983; Ranganathan *et al.*, 2008). Only a handful of microorganisms have been used as whole cell biocatalysts: *Candida antarctica*, *Rhizopus chinensis*, *Rhizopus oryzae* and *Saccharomyces cerevisiae*, with the latter being the least popular option (Fukuda *et al.*, 2008; 2009 and Robles *et al.*, 2009).

It has been shown that *Rhizopus oryzae* whole cells can efficiently catalyze the methanolysis of vegetable oils and *Rhizopus chinensis* whole cells are efficient in transesterification of short chain fatty acids (Qin *et al.*, 2008). In comparison with *Candida antarctica*, *Candida rugosa*, porcine pancreas and *Pseudomonas cepacia*, *Rhizopus chinensis* showed the highest catalytic ability in the transesterification of soybean oil in a solvent free system (Qin *et al.*, 2008).

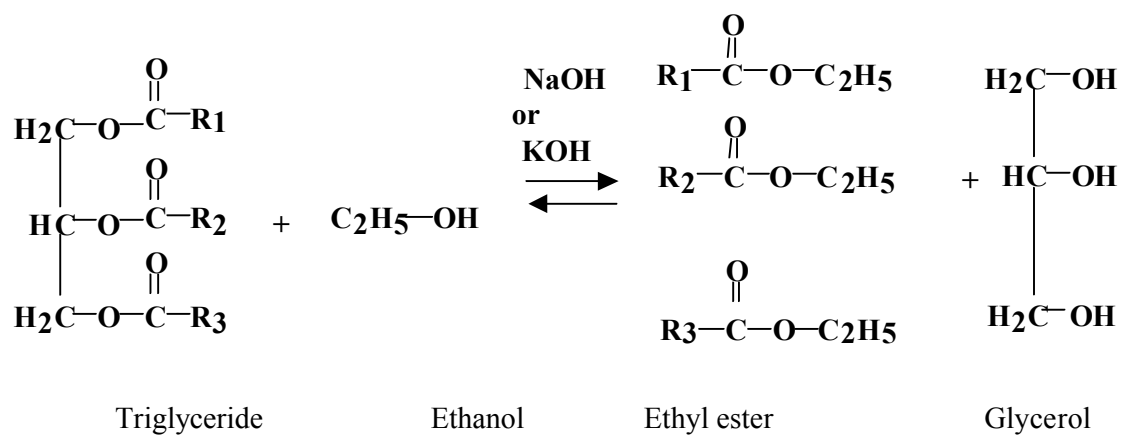


Figure 2.2 Transesterification of Vegetable Oil

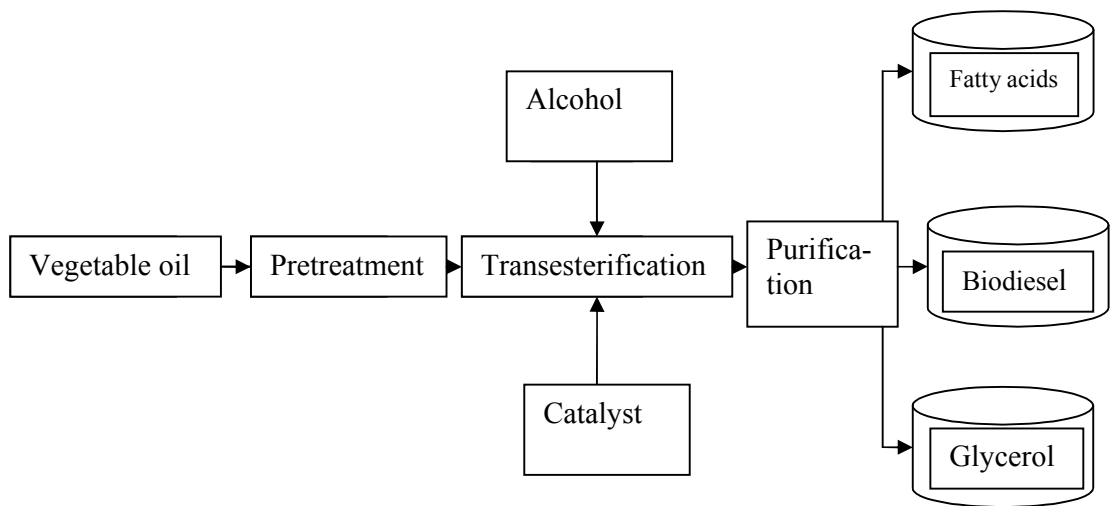


Figure 2.3. Sequence of Transesterification process (Pierre, 2008)

Table 2.2 Comparison of chemical catalyst and biocatalyst transesterification  
(Shah *et al.*, 2003 and Fukuda *et al.*, 2001).

<b>Major factors</b>	<b>Alkali catalyst transesterification</b>	<b>Biocatalyst transesterification</b>
Temperature	60-80°C	20-60°C
Presence of FFA's in Feed stock	Soap formation	Complete conversion into the methyl ester
Presence of water	Towards more soap formation as hydrolysis of the oil may take place	No effect on final product
Yield of biodiesel Production	High, nearly 99%	Comparatively lower than alkali catalyst, around 90%
Down stream Processing	Multi-step purification of end products	None
Biodiesel production Cost	Cheap, as catalysts are comparatively cost less	Really expensive as biocatalysts are expensive
Commercialization	100% commercialized	Not exactly
Waste water generation	Saline and alkaline effluents need treatment before discharge	No waste water generation

### 2.3.3.3 Lipase-catalyzed transesterification of *Jatropha curcas* Oil

Three different lipases such as *Chromobacterium viscosum*, *Candida rugosa*, and *Porcine pancreas*, were screened for a transesterification reaction of physic nut (*Jatropha curcas* L.) oil (that contained about 44.8% oleic acid and 34% linoleic acid) in a solvent-free system to produce alkyl ester. Other report also found that only lipase from *Chromobacterium viscosum* was found to give appreciable yields. A maximum yields (92%) was obtained in 10 hours using 10 % (w/w) of immobilized enzyme preparation (Shah *et al.*, 2004). Lipases ability to catalyze reactions other than hydrolysis, such as esterification and interestification can be exploited in the production of some specific compounds, such as tailor made lipids and bioemulsifiers (Arcos *et al.*, 1998).

### 2. 3. 3. 4 Properties of lipases

#### *Lipase specificity:*

The specificity of a lipase refers to its regioselectivity for specific positions on the triglyceride molecule. Lipases can be classified according to their selectivity for the acyl position (regioselectivity) on the glycerol backbone (Chandler, 2001). Each lipase has been deemed one of three types: 1,3 specific, 2 specific, or non specific (Koskinen and Klibanov, 1996; Rahman *et al.*, 2005). 1,3 specific lipases act primarily on the ester bonds on the extreme positions of the triglyceride molecule and rarely attack the middle ester bond. 2 specific lipases primarily attack the middle ester bond on the triglyceride molecule. Non specific lipases show no preference to the ester bonds they attack (Macrae, 1983).

The most common 1,3 specific lipases are: *Rhizopus oryzae*, *Thermomyces lanuginosus*, *Aspergillus niger*, *Rhizopus delemar* and *Rhizomucor miehei* (Shimada *et al.*, 1997; Fukuda *et al.*, 2001; Lanser *et al.*, 2002 and Robles *et al.*, 2009). The only 2 specific lipase that has been mentioned in the literature is *Geotrichum candidum* which is not

Commonly used for transesterification (Macrae, 1983). The most commonly used non specific lipases are; *Candida antarctica*, *Candidacylindracea*, *Candida rugosa*, *Pseudomonas cepacia* and *Pseudomonas fluorescens* (Fukuda *et al.*, 2001).

Regioselective lipases were not believed to be applicable to biodiesel production since they do not act on all ester bonds of the triglyceride molecules. It was later discovered that they efficiently catalyze transesterification with yields often greater than 90%, exceeding the estimated 66% yield (Antczak *et al.*, 2009). It was suggested that the reason for the unexpected high yield is spontaneous acyl migration (Fukuda *et al.*, 2001). It was later verified by thin layer chromatography, that acyl moieties migrate from the 2 position to either the 1 or 3 positions on the partial triglyceride in aqueous environments (Fukuda *et al.*, 2009). In order to promote acyl migration and, therefore, reaction productivity, it has been suggested to use polar immobilization supports and to add silica gel to the reaction mixture (Akoh *et al.*, 2007 and Robles *et al.*, 2009).

#### *Lipase stability:*

The stability of the lipase without losing its catalytic activity is the most important enzymatic characteristics when used in biodiesel synthesis (Moreira *et al.*, 2007 and Zheng *et al.*, 2009). The environment in a reactor is often more harsh for the enzyme than when *in vivo* since enzymes are known to be more stable in their natural cell environment. Therefore, many enzymes do not remain stable when used industrially. The higher temperature, inactivating impurities and aggressive surfaces of the reactors assist in enzyme deactivation and inhibition (Klibanov, 1983).

In addition to mechanical forces, lower chain alcohols, the by-product glycerol, water content and high alcohol to oil ratios can also cause destabilization and deactivation of the enzyme (Malcata *et al.*, 1990; Marchetti *et al.*, 2007 and Robles *et al.*, 2009). The loss of enzyme activity over time is often a result of thermal degradation and alcohol

inhibitions (Torres *et al.*, 2008). Methods that have been suggested to improve lipase stability include: Genetic engineering, molecular biology, chemical modification, physical treatments, immobilization techniques and reaction and reactor engineering (Malcata *et al.*, 1990; Reetz, 2002; Mateo *et al.*, 2007 and Illanes *et al.*, 2008).

### **2. 3. 4 Factors affecting enzymatic transesterification**

There are several factors which affect the rate at which transesterification proceeds and the ultimate yield of biodiesel. These include:

- i. Selection of alcohol.
- ii. Use of solvents.
- iii. Alcohol to oil molar ratio.
- iv. Reaction temperature.

#### *2. 3. 4. 1 Selection of Alcohol:*

There are a number of different compounds that have been deemed acceptable acyl acceptors for transesterification. Methyl acetate and ethyl acetate have both been seen as appropriate acyl acceptors (Xu and Wu, 2003, and Modi *et al.*, 2007), but have also been found to be much more expensive than the more commonly used alcohols (Vasudevan and Briggs, 2008, and Robles *et al.*, 2009). Primary, secondary, straight chained and branched alcohols can all be employed in the transesterification reaction (Fukuda *et al.*, 2001).

The most commonly used alcohols are: methanol, ethanol, propanol, iso-propanol, 2-propanol, n-butanol and iso- butanol (Iso *et al.*, 2001; Antczak *et al.*, 2009, and Varma and Madras, 2010). Alcoholysis of triolein using *Pseudomonas cepacia* was carried out in a solvent free medium with a multitude of alcohols. Methanol showed 40% conversion, ethanol showed 93% conversion, propanol showed 99% conversion, 1-butanol showed 99% conversion, 2-butanol showed 83% conversion, 2-methyl-1-propanol showed

99% conversion and a mixture of pentanol isomers resulted in 99% conversion (Salis *et al.*, 2005). However, deactivation of the enzymes with alcohol have been estimated to be inversely proportional to the number of carbon atoms in the alcohol which means that methanol is the most deactivating alcohol (Chen and Wu, 2003; Ranganathan *et al.*, 2008).

It is also thought that the rate of the transesterification reaction using lipase increases with the length of carbon chain of the alcohol, implying that the use of ethanol over the use of methanol increases the rate of the transesterification reaction (Antczak *et al.*, 2009). Two solutions have been suggested to overcome the inhibiting effects of lower chained alcohols: (a) stepwise addition of the alcohol or the sequential addition of alcohol aliquots (Shimada *et al.*, 1997; 2002; Watanabe *et al.*, 2002; Soumanou and Bornscheuer, 2003 and Matassoli *et al.*, 2009) and (b) the use of solvents (Nelson *et al.*, 1996; Mittelbach, 1990 and Modi *et al.*, 2007).

#### 2.3.4.2 Use of solvents

Inhibition by lower chained alcohols is often due to alcohol insolubility. Solvents are used to protect the enzyme from denaturation by alcohol by increasing alcohol solubility (Kumari *et al.*, 2009). The solvent can also increase the solubility of glycerol which is beneficial since the byproduct can coat the enzyme and inhibit its performance (Royon *et al.*, 2007). The most common solvents used in transesterification are hydrophobic organic ones: hexane, isooctane, n-heptane, petroleum ether, cyclohexane, 2-butanol and tert-butanol (Holmberg and Hult, 1990; Nelson *et al.*, 1996; Soumanou and Bornscheuer, 2003; Ghamguia *et al.*, 2004; Lara and Park., 2004 and Coggon *et al.*, 2007). The use of solvents has become a recognized solution for reducing inhibitory effects of lower chained alcohols. However, several disadvantages of the use of solvents have been identified (Ranganathan *et al.*, 2008). These include:

- i. Solvent must be separated from the final desired product (biodiesel) which requires additional processing (Vasudevan and Briggs, 2008).

- ii. The use of organic solvents can compromise safety since they are generally volatile and hazardous.
- iii. Reactor volumes must also increase to compensate for the additional volume of solvent added to the reaction mixture. All of these disadvantages of using solvents could ultimately lead to increased capital and running costs of biodiesel production (Fjerbaek *et al.*, 2009).

#### 2.3.4.3. Alcohol to substrate molar ratio

A molar excess of alcohol to oil is needed for the transesterification reaction to proceed at a reasonable rate. Generally, the greater the molar ratio of alcohol to oil the faster the reaction rate, long as the alcohol is soluble in the reaction mixture (Antczak *et al.*, 2009). When a portion of the alcohol remains insoluble (in excess) it forms droplets which coat the enzyme causing deactivation. Many authors stressed that the alcohol employed in transesterification must be completely dissolved (especially methanol) implying that there is an optimum alcohol to oil molar ratio which allows for the fastest reaction rate (Jeong and Park, 2008).

As a guideline, if the alcohol has less than three carbons it is likely to inhibit the lipase enzyme since its solubility is less than the stoichiometric ratio. Methanol and ethanol typically are soluble at 1/2 and 2/3 of their stoichiometric amounts respectively. Alcohols with greater than three carbons typically do not cause any inhibition since they often dissolve in the feedstock in their stoichiometric ratios (Shimada *et al.*, 2002; Robles *et al.*, 2009). In an organic solvent reaction, an excess amount of alcohol is needed in order to achieve a satisfactory reaction rate and a Fatty Acid Acyl esters (FAAE) yield. Typically, in a solvent system, methanol to oil molar ratios should be in the range of 3:1 - 6:1 (Matassoli *et al.*, 2009).

Salis *et al.* (2005) experimented with ratios of 3:1, 6:1, 9:1 and 12:1 in the butanolysis of

triolein with *Pseudomonas cepacia*. The best ratios were found to be 3:1 and 6:1, both reached 100% conversion after 4 hours. Ratios of 9:1 and 12:1 resulted in 100% conversion after 5 hours and 6 hours. Jeong and Park (2008) evaluated the effect of methanol to rapeseed oil between 1:1 and 6:1 using *Candida antarctica*. It was determined that any ratio between 2:1 and 5:1 resulted in a high conversion and any ratio above 6:1 reduced conversion. However, it is important to realize that the optimum alcohol to oil molar ratio is vastly dependent on the individual system employed and the alcohol, feedstock and enzyme used.

#### 2. 3. 4. 4 Reactive temperature

Lipase is known to have a fairly large thermal stability (Marchetti *et al.*, 2008). The conversion of transesterification is rarely influenced by temperature fluctuations so long as the temperature remains between 20 and 70°C. However, most lipases have optimal temperatures between 30 and 60°C. It is important to note that the optimum temperature for a given lipase increases when the enzyme is immobilized (Fjerbaek *et al.*, 2009). Overall, the optimum temperature is dependent on enzyme stability, alcohol to oil molar ratio and the type of organic solvent used (Antczak *et al.*, 2009).

Methanolysis using *Candida antarctica* was performed in a temperature range of 25-55°C (Jeong and Park, 2008). The optimum temperature was found to be 40°C, anything above this causes a decrease in overall conversion. This was again verified by Lu *et al.* (2009) using *Candida* sp. 99-125<sup>®</sup>. Salis *et al.* (2005) evaluated butanolysis using *Pseudomonas cepacia* within a temperature range of 20-70°C. Methanolysis using *Rhizopus chinensis* was found to have an optimal temperature of 30°C in the range of 20-60°C (Qin *et al.*, 2008).

## CHAPTER 3

### MATERIALS AND METHODS

#### 3.1 MATERIALS

##### 3.1.1 Plant Sample Collection and Identification

*Jatropha* seeds were collected from the Institute of Agricultural Research (IAR) Samaru, Zaria and authenticated at the same Institute.

##### 3.1.2 Seed Sample

Healthy *Jatropha* seed were selected, cleaned and de-shelled. Seeds were pulverized prior to analysis and extraction of oil.

##### 3.1.3 Chemical and Solvents

All chemicals and solvents used were of analytical grade obtained From BDH, England and Fluka, USA. The chemicals include; n-Hexane, Ethanol, Tris Base (Trishydroxymethyl), Amino methane, Hydrochloric acid, Sodium Hydroxide, Glycine (Aminoacetic acid), Calcium Chloride, Sodium Chloride, Disodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ ), Sodium dihydrogen phosphate ( $\text{NaH}_2\text{PO}_4$ ), Olive oil, Sodium Acetate, Citric Acid, Ethyldiamine tetracetic acid (EDTA), Barium chloride, Lauric acid, Thymolphthalien indicator, Lipase (commercially available), Potassium Chloride, and Cupper Oxide among others.

##### 3.1.4 Equipment

The equipment used included; pH meter (Hanna pH 210), Oven, Metler Balance (Meter H.80), Incubator Memmert GMB (KG 8540), Autoclave, Gas chromatography Mass Spectrometer (Qp2010) Plus SHIMADZU-Japan.

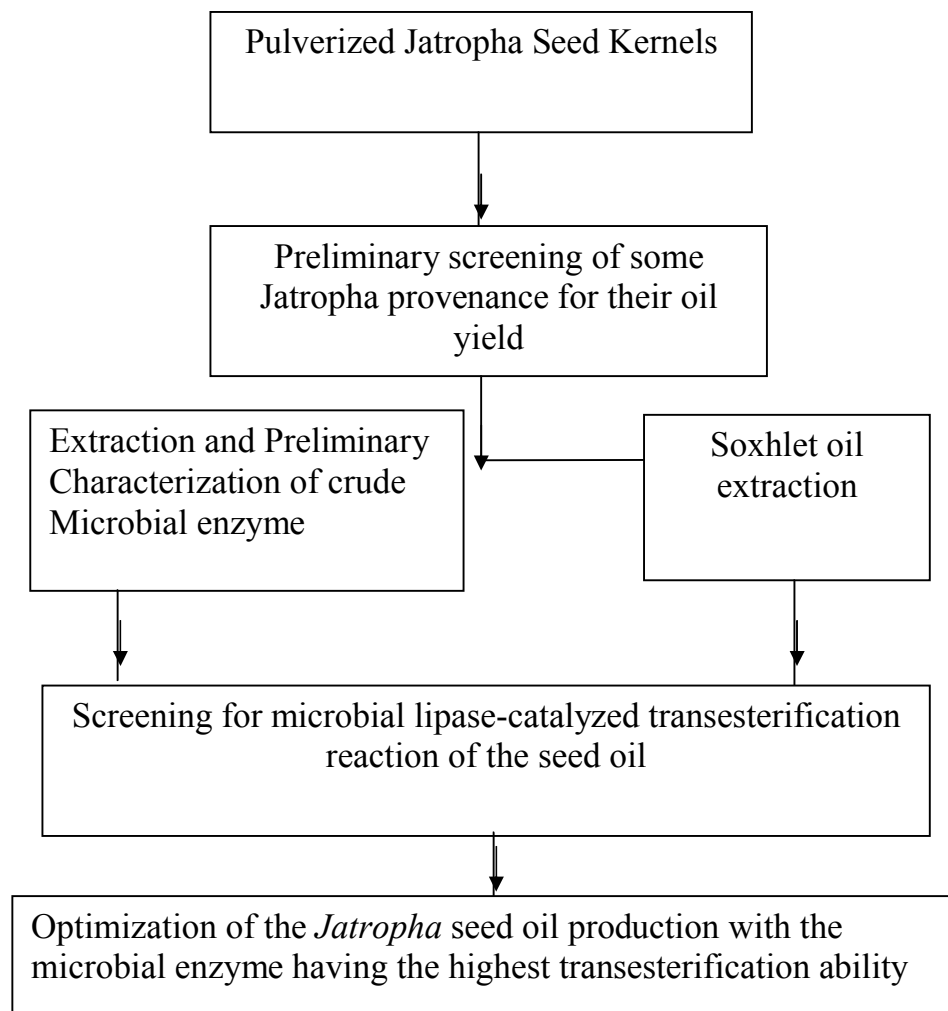


Figure 3.1 Experimental design

### 3. 2 MICROORGANISMS

Pure cultured microorganism isolates: *Enterobacter aerogenes*, *Aspergillus niger* and *Aspergillus flavus* were obtained from the Department of Microbiology, Ahmadu Bello University, Zaria.

### 3. 3 SAMPLE PREPARATION AND OIL EXTRACTION

*Jatropha* seeds were cracked, the shell carefully removed and the kernels thus obtained were pulverized and used for oil extraction.

### 3. 4 EXTRACTION OF OIL FROM POWDERED KERNEL

Soxhlet extraction method for oil extraction from *Jatropha* seed kernels was carried out.

#### 3. 4. 1 Soxhlet oil extraction

Oil from *Jatropha* powdered kernel was extracted by soxhlet extraction using n-Hexane as solvent at 60°C (Horowitz, 1984; AOAC, 1990 and Makkar *et al.*, 1997).

**Principle:** This involves the continuous extraction with non-polar organic solvent such as petroleum ether for about an hour or more.

**Procedure:** 250ml clean boiling flask was dried in an oven at 105-110°C for about 30minutes. This was then transferred into desiccator and allowed to cool, 2g of the sample (pulverized kernel) was carefully poured into a labelled thimble. The boiling flask was filled with about 300ml of n-Hexane. The extraction thimble was plugged lightly with cotton wool. The soxhlet apparatus was assembled and refluxing was carried out for about 6 hours. The thimble was carefully removed and the collected n-hexane in the top container of the set up was drained into another container for re-use. When the flask was almost free of n-Hexane, it was removed and dried at 105°C – 110°C for an hour. It was transferred from the oven into a desiccator and allowed to cool then weighed.

Calculation:

$$\% \text{ Oil output} = [\text{Weight of Oil obtained (gms)}/\text{Weght of Sample}] \times 100$$

### **3. 5 LIPASE PRODUCTION**

#### **3. 5. 1 Nutrient Agar for Bacterial Growth**

Nutrient Agar was used as the growth medium for the bacteria. Media (28g) was dissolved in 1.0 liter of distilled water in a conical flask capped with a cotton wool and boiled for a few minutes over a Bunsen burner for complete dissolution to occur. This was then sterilized in an autoclave at 121°C for 15 minutes. The system was allowed to cool to 45°C and 15-20mls aliquots were poured into sterilized Petri-dishes, covered and allowed to solidify. The media were then inoculated with the test bacteria by spread plate technique. It was then incubated at 38°C in an incubator for 24-48 hours (Lennette et al., 1974).

#### **3. 5. 2 Saboround Dextrose Agar (SDA) for Fungal Growth**

Exactly, 65g of SDA was dissolved in 1.0 liter of distilled water and sterilized at 121°C for 15 minutes in an autoclave. The suspension was poured into sterilized Petri dishes, allowed to solidify with the microorganism inoculated (Lennette et al., 1974).

### **3. 6 EXTRACTION OF ENZYME**

After the incubation period, the microorganism cultured was harvested using a sterilized loop under a sterilized condition. Microbial colonies (1g) were extracted with 100ml phosphate buffer (pH 7.0, 100mM) and then filtered through four layers of cheese cloth. The filtrate was used directly as the crude preparation of lipases (Chakraborty *et al.*, 1998).

### **3. 7 TRANSESTERIFICATION REACTIONS**

Lipase-catalyzed transesterification reactions were carried out in a screw-capped vials that were magnetically agitated (200rpm) by using the magnetic stirrer hot plate and held at the desired constant temperature (35-40°C) (Kumari *et al.*, 2009)

**Procedure:** *Jatropha* seed oil (0.5ml) was mixed with ethanol in the ratio of 1:4 (mol mol<sup>-1</sup>) in a screw-capped vial. To this mixture, 50mg of enzyme preparation (crude-lipases) was added and incubated at 40°C for fungal lipase and 50°C for bacterial lipase with constant

shaking at 200rpm. The reaction was monitored for 40 minutes and was appropriately diluted with 2ml hexane. To the diluted sample, 0.2ml lauric acid was added as an internal standard before analysis by Gas chromatography (GC) (Shweta *et al.*, 2003).

### **3. 8 GC- ANALYSIS**

Lipid composition was analyzed by Gas Chromatography instrument (QP2010 plus) equipped with a FID Detector and a high temperature HT5 AQ (SGE), 12 m x 0.22 mm column. The Column flow was 1.80 ml/min, purge flow was 3.0ml/min and total flow was 40.8 ml/min. The injector temperature was 250°C; the oven temperature was programmed from 70°C to 280°C at 10°C/minute and was held at 250°C for 5 minute.

#### **3. 8. 1 Methylation Process for the Gas Chromatography (GC) Analysis**

i. Preparation of Methanolic Sodium Hydroxide:

Exactly 2.00g sample of sodium hydroxide (NaOH) was weighed and dissolved in 100cm<sup>3</sup> methanol solution. The mixture was stirred for 2 minutes till a clear solution was obtained forming methanolic sodium hydroxide solution.

ii. Methylation of the oil sample:

Exactly 0.2g of the oil was weighed into quick fit conical flask and 6ml of methanolic sodium hydroxide solution was added. It was refluxed for 10 minutes on a steam bath. Then, 10ml of a solution consisting of 30ml Concentrated HCl and 20ml methanol was added and refluxed for another 10 minutes on the steam bath. Then, 10ml of hexane was added and refluxed for 2 minutes on the steam bath and allowed to cool. Finally, 10ml of distilled water was added and the mixture poured into a separating funnel. The organic layer (upper layer) was collected and dried with calcium chloride. This was then used for GC analysis (The Analyst 108 (1287)).

### 3. 9 CHARACTERISATION OF CRUDE LIPASE

#### 3. 9. 1 Lipase Characterization

Lipase activity was characterised by titration method (Benzonana and Desnuelle, 1968; Jensen, 1983). In this procedure; native substrate (triacylglycerols) are hydrolyzed to yield fatty acids. Samples at predetermined time are withdrawn and reactivity was quenched by the addition of ethanol (95%). The amount of fatty acid released during the reaction was determined by direct titration with 0.05M NaOH to a thymolphthalein end point .The titration was done thrice and the average titer value was calculated for each.

**Procedure:** Into each of eight 25ml Erlenmeyer flask, 2ml of 95% (v/v) ethanol and 2-3 drops of 1% (w/v) thymolphthalein indicator was poured. Into a 50ml Erlenmeyer flask with stopper, place 20ml of 5% (w/v) olive oil/gum Arabic emulsion substrate and preincubated for 15 minutes in a 37°C water bath with magnetic stirring. Exactly, 5ml of enzyme was added to initiate lipolysis on the emulsion substrate for 15 minutes after which 5ml reaction mixture was removed and transferred into a separate flask containing 2ml titrate cocktail prepared to quench reaction. The content of each flask was titrated with 0.05M NaOH using a burette until light blue colours appear. Into the last 25ml Erlenmeyer flask containing titration cocktail, 5ml phosphate buffered, olive oil/gum Arabic emulsion substrate was added and mixed well. Using a burette, the contents of this flask was also titrated with 0.05M NaOH which served as a reagent blank.

Calculation:

Lipase activity was determined using the formula:

$$\mu\text{mol of fatty acid/ml subsample} = \frac{[(\text{ml } N_s - \text{ml } N_b) \times N \times 1000]}{5\text{ml}}$$

and is expressed as  $\mu\text{mol}$  of fatty acid/ml under the test condition.

Where,  $N$ = Normality of the NaOH titrant used

$N_s$ = ml of NaOH of sample

$N_b$ = ml of NaOH of blank

### *3. 9. 1. 1 Effects of temperature*

The optimum temperature for enzyme activity was determined at various temperatures ranging from 30°C-80°C (at 10°C interval). Briefly, 1ml of crude enzyme was added to 5ml of the substrate emulsion (5% each of Olive oil and gum Arabic homogenized in 100ml of 0.05M phosphate buffer, pH 8) and allowed to react for 40 minutes at various temperatures (30°C-80°C) and the reactivity was quenched by the addition of ethanol (95%). The enzyme activity was assayed as described above.

### *3. 9. 1. 2 Thermostability of the enzyme*

Thermostability of the enzyme was determined by incubating 1ml of crude enzyme in phosphate buffer (pH 7.0, 100mM) at different temperatures between 30°C-80°C (at 10°C interval) for 1hr. Then, 5ml of the substrate emulsion (5% each of Olive oil and gum Arabic homogenized in 100ml of 0.05M phosphate buffer, pH 8) was added and allowed to react for 40 minutes and the reactivity was quenched by the addition of ethanol (95%). The enzyme activity was assayed as described above.

### *3. 9. 1. 3 Effects of pH on lipase activity*

The effects of pH on lipase activity were determined at various pH ranging from pH<sub>4</sub>-pH<sub>10</sub>. Acetate buffer (0.2M) pH 4-5, Phosphate buffer (0.2M) pH 6-8, and Tris-HCl buffer (0.2M) pH 9-10 were used. Briefly, 1ml of crude enzyme was added to 5ml of the substrate emulsion (5% each of Olive oil and gum Arabic homogenized in 100ml of appropriate buffers, pH 4-10) and allowed to react for 40 minutes at 37°C, the reactivity was quenched by the addition of ethanol (95%). The enzyme activity was assayed as described above

### *3. 9. 1. 4 pH stability of the enzyme*

pH stability of the enzyme was determined by incubating 1ml of crude enzyme in appropriate buffers above at different pH from pH 4-10 for 1hr at 30°C. Then, 5ml of the substrate emulsion (5% each of Olive oil and gum Arabic was homogenized in 100ml of

0.05M phosphate buffer, pH 8) was added and allowed to react for 40 minutes and the reactivity was quenched by the addition of ethanol (95%). The enzyme activity was assayed as described above.

#### *3. 9. 1. 5 Effects of organic solvents on lipase*

The effects of organic solvents were determined by preincubating 1ml of crude lipase solution for 1 hour at 30°C (pH 7) and 0.1M Tris-HCl buffer with methanol, ethanol, butanol, acetone and hexane one at a time. Then, 5ml of the substrate emulsion (5% each of Olive oil and gum Arabic was homogenized in 100ml of 0.05M phosphate buffer, pH 8) was added and allowed to react for 40 minutes and the reactivity was quenched by the addition of ethanol (95%). The enzyme activity was assayed as described above.

### **3. 10 STATISTICAL ANALYSIS**

The data obtained were analysed statistically and presented as Mean  $\pm$  Standard deviation. The standard deviation (or standard error) is a measure of dispersion that measures the absolute deviation of a set of values from the mean or other measures of central tendencies (mode and median). It is generally defined as the root mean square of the deviation from the common mean of a set of values (Francis, 1988).

## CHAPTER 4

### RESULTS

#### 4. 1 Oil Yield of *Jatropha* Seed-Kernels

Table 4.1 shows the percentage oil out-put of *Jatropha* seed-kernel Provenances. *Jatropha* seed obtained from *Wasugu Maga Danko* (KBWD) in Kebbi State, *Dandatodaji dandatodaji* (ZMDD) from Zamfara State and Katsina Chanranchi Kuki (KTCK) from Katsina state gave the highest Percentage oil out-put as; 60.62%, 58.97% and 58.68% respectively while the least percentage oil out-put was obtained from *Shagari Kajiji* (SKSK) in Sokoto State (8.32%), *Magazu II Tsafe* (ZMMT) from Zamfara State (15.84%) and *Birnin Kudu* (JGBK) in Jigawa State (17.08%).

Table 4.1. Percentage of Oil Out-put for *Jatropha* seed-kernel Provenances.

<b>Jatropha seed provenances</b>	<b>Mean Percentage oil yield (%)</b>
JGBK	17.05 ± 0.07
JGBM	30.53 ± 0.74
KBDW	60.30 ± 0.42
KBJB	52.50 ± 0.71
KDBJ	38.33 ± 0.25
KDDJ	39.08 ± 1.31
KDGI	51.85 ± 1.20
KDSS	53.80 ± 0.28
KNDT	36.50 ± 0.71
KNGG	33.25 ± 0.35
KNRK	32.75 ± 1.06
KNWN	45.13 ± 0.18
KTCK	58.85 ± 0.21
KTMK1	48.68 ± 0.46
KTSD1	45.08 ± 0.11
KTSD2	35.38 ± 0.53
SKKT	25.48 ± 0.67
SKPB	44.28 ± 0.32
SKSK	8.90 ± 0.85
SKTB	36.83 ± 0.25
ZMBZ	52.72 ± 0.39
ZMDD	59.48 ± 0.74
ZMDT	22.25 ± 1.06
ZMMT	16.18 ± 0.46

NB:

**JGBK**=Jigawa BirinKudu , **JGBM**= Jigawa Birin kudu Masaya, **KBDW**=Kebbi Wasugu Maga danko, **KBJB**=Kebbi Aleiro J Birnin, **KDGJ**=Kaduna Birnin Gwari Jiba, **KDDJ**=Kaduna Dan Jawei, **KDGI**= Kaduna Giwa Iyatawa, **KDSS**= Kaduna soba, **KNDT**=Kano Dirpindai Takai, **KNGG**=Kano Gwarzo, **KNRK**=Kano Ruwan Kanyan, **KNWM**=Kano Welare Makoda, **KTCK**=Katsina Chanranchi Kuki, **KTMK1**=Katsina Dutsen Karohi 1, **KTSD1**=Katsina Kankia Sagawa Doka1, **KTSD2**= Katsina Kankia Sagawa Doka2, **SKKT**=Sokoto Kwara Takai, **SKPB**=Sokoto Pompo Bodinga, **SKSK**=sokoto shagari kajiji, **SKTB**=Sokoto tanbulwal Barkeji, **ZMBZ**=Zamfara Bukuyan Zugu, **ZMDD**=Zamfara Dandotodaji Dandotodaji, **ZMDT**=Zamfara Daki Takwas, **ZMMT**=Zamfara Magazu 2 Tsafe

## **4. 2 EFFECTS OF TEMPERATURE AND THERMOSTABILITY ON CRUDE MICROBIAL LIPASE ACTIVITY**

The crude lipase from *Aspergillus niger* and *Aspergillus flavus* showed their lipolytic activity in the temperature range of 40-50°C with maximum activity at 40°C (Figure 4.1). Thermostability for both the crude lipases was observed at 40°C (Figure 4.2). On the other hand, crude lipase from *Enterobacter aerogenes* was most active between 40°C-50°C with maximum at 50°C and thermostability was observed at 50°C (Figures 4.1 and 4.2).

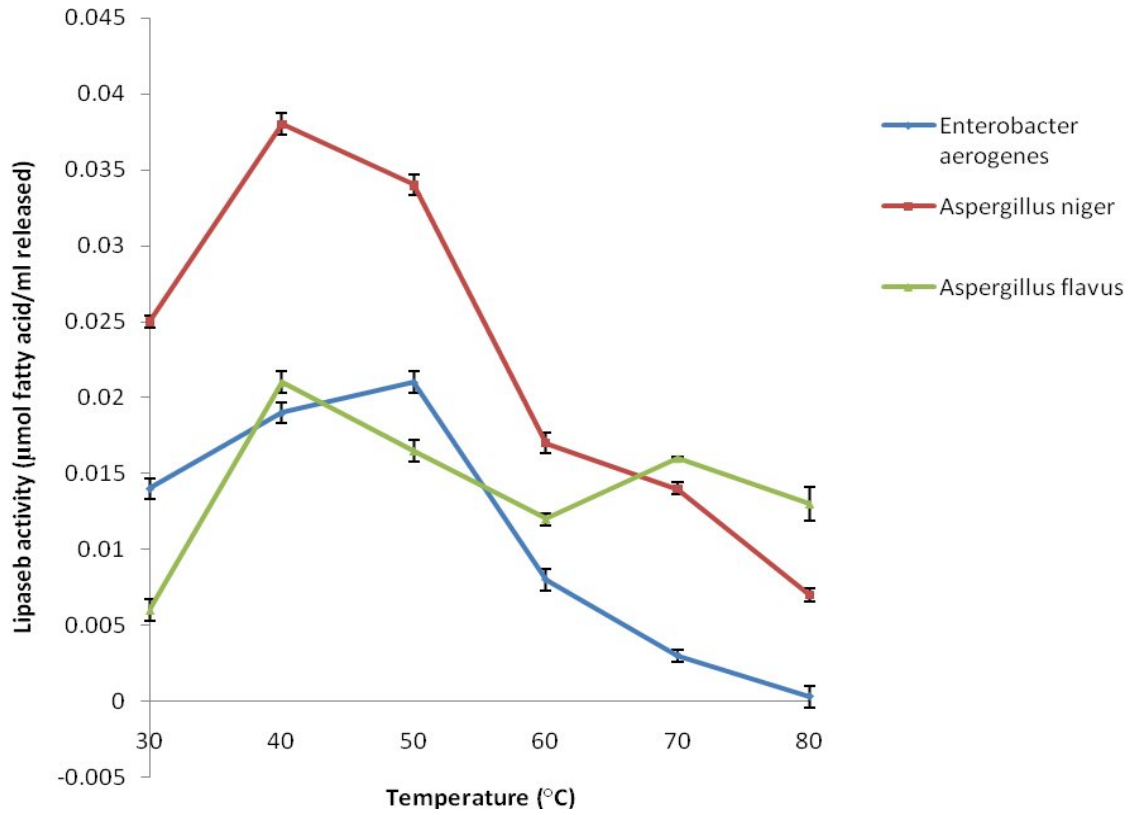


Figure 4.1. Effects of Temperature on Crude Microbial Lipase Activity (*Enterobacter aerogenes*, *Aspergillus niger* and *Aspergillus flavus*).

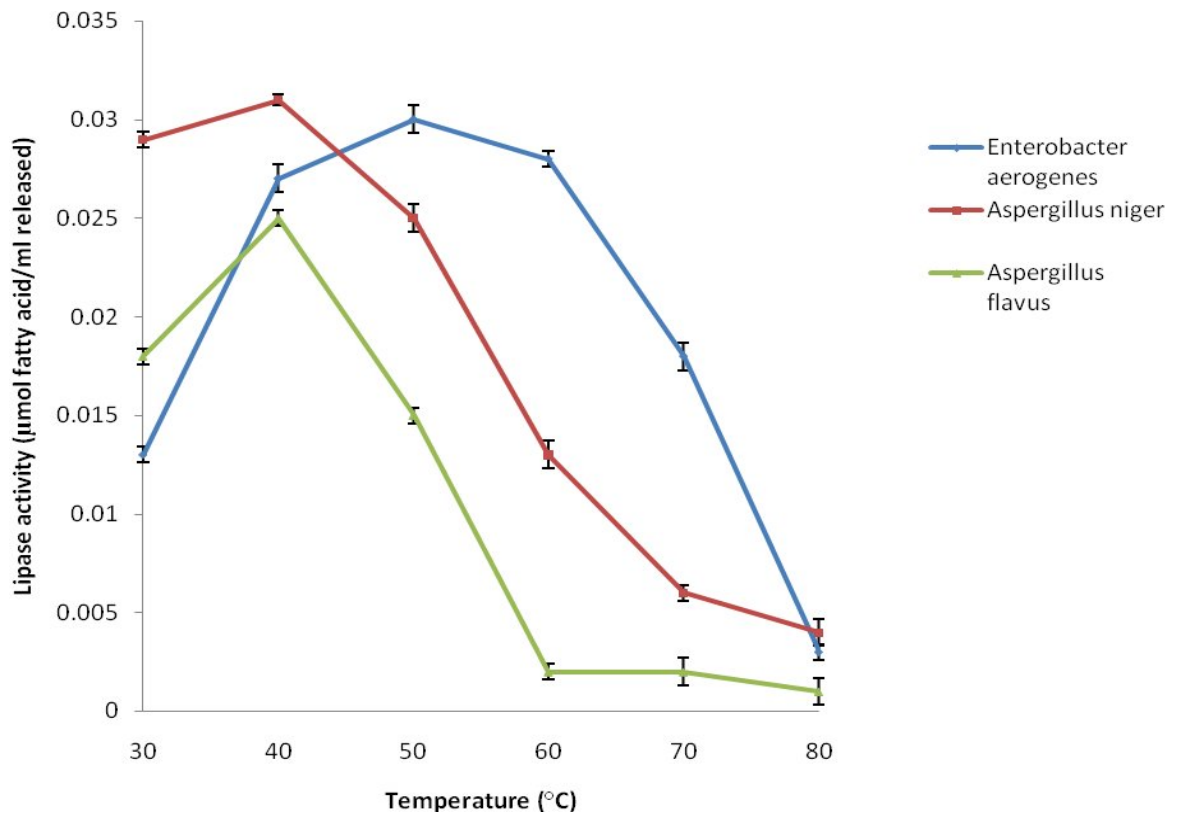


Figure 4.2. Thermostability of Crude Microbial Lipase Activity (*Enterobacter aerogenes*, *Aspergillus niger* and *Aspergillus flavus*).

### **4. 3 EFFECTS OF pH ON CRUDE MICROBIAL LIPASE ACTIVITY**

The optimal pH and stability for the microbial lipase are presented in Figures 4.3 and 4.4. The pH activity of *Aspergillus niger* and *Aspergillus flavus* was observed in the range of pH 6-8 with pH optimal at pH 6. Their pH stability was found at pH 6. While, optimal pH for crude lipase activity from *Enterobacter aerogenes* was between pH 7-8. The enzyme was most stabled at pH 8.

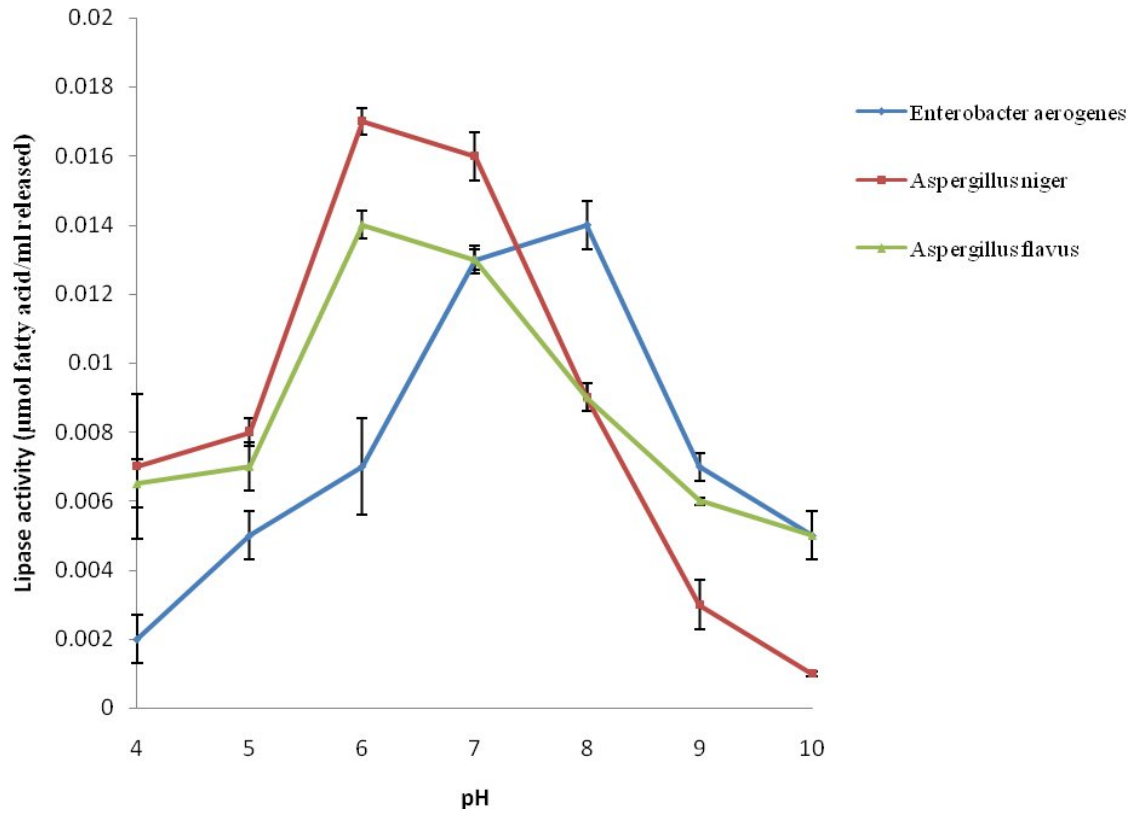


Figure 4.3. Effects of pH on Crude Microbial Lipase Activity (*Enterobacter aerogenes*, *Aspergillus niger* and *Aspergillus flavus*).

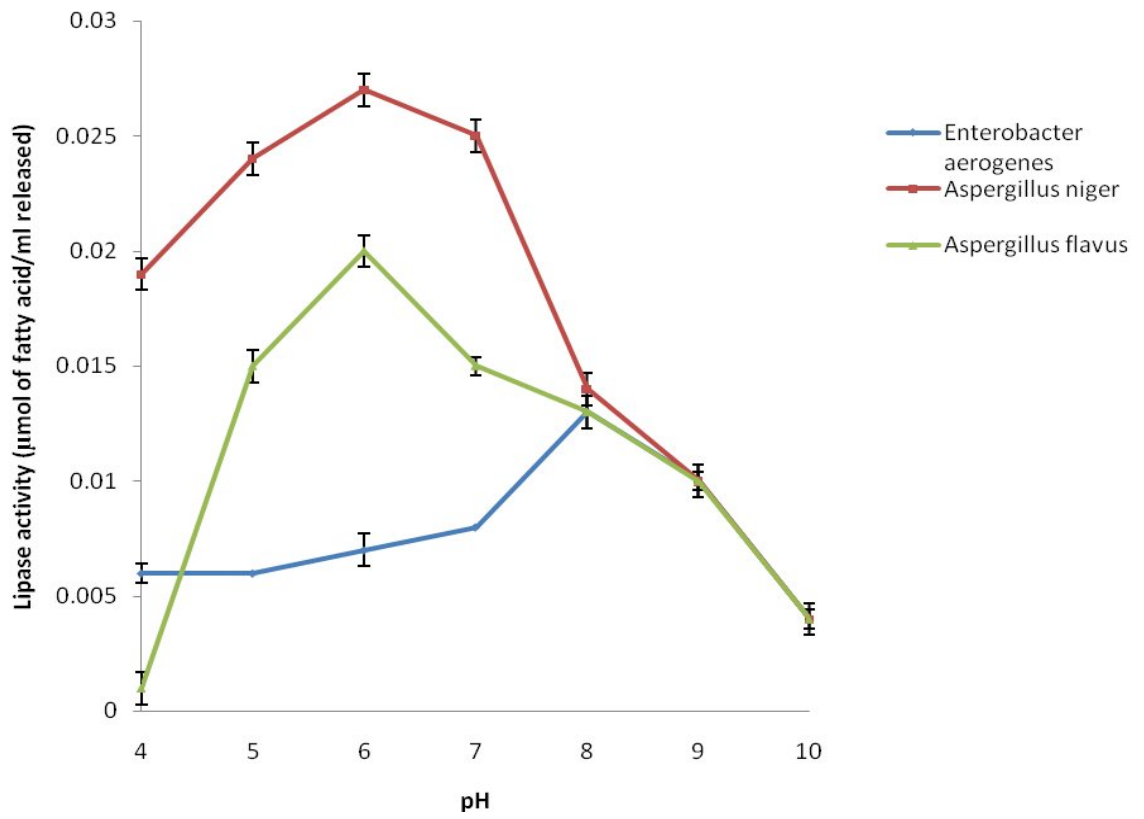


Figure 4.4. pH Stability on Crude Microbial Lipase Activity (*Enterobacter aerogenes*, *Aspergillus niger* and *Aspergillus flavus*).

#### **4. 4 EFFECTS OF ORGANIC SOLVENTS ON MICROBIAL LIPASE ACTIVITY**

Lipase catalyzed reaction involving transesterification often occurs in organic solvents or organic-aqueous solution media. In this study, incubation of crude lipase of *Aspergillus niger*, *Aspergillus flavus* and *Enterobacter aerogens* in 30% ethanol and methanol did not cause much loss of their activities while a sharp decrease in their activities were observed in the presence of 30% butanol and acetone (Table 4.2).

Table 4. 2. Effects of Some Organic Solvents on Crude Microbial Lipase Activity (Mean±SD)

Organic Solvents	Relative Activity (%)		
	<i>Aspergillus flavus</i>	<i>Aspergillus niger</i>	<i>Enterobacter Aerogenes</i>
Positive Control (Phosphate buffer, pH 7.0 + Lipase)	100.00±0.00	100. 00±0.00	100.00±0.00
Acetone	31.58±0.52	32.97±1.87	24.09±0.43
Methanol	55.90±0.49	50.17±1.77	32.92±1.59
Ethanol	45.14±0.98	53.04±1.76	36.28±0.43
Butanol	31.87±0.10	32.26±1.78	21.65±0.43
Hexane	40.97±0.98	42.65±0.30	30.49±1.72

\* Standard Deviation ( $\pm$ ) is mean values of duplicate determination.

#### **4.5 FATTY ACIDS PROFILE OF JATROPHA SEED OIL PROVENANCES**

The percentage fatty acids profile for *Jatropha* seed oil provenance obtained from *Birnin kudu* (JGBK) in Jigawa State and *Wasugu-maga Danko* (KBWD) in Kebbi state before and after transesterification with crude lipases are presented in Tables 4.3 and 4.4. Linoleic methyl esters, oleic methyl ester, palmitic methyl ester, and Stearic acids methyl ester were detected as the dominant fatty acids in both seed oils provenance. While, ecosanoic, palmitic and palmitoleic acids were the least fatty acids present. Stearic and palmitic acid methyl esters were the the major saturated fatty acids observed in both seed oils.

Table 4.3. Fatty Acids Composition for *Jatropha curcas* Seed Oil Provenance from *Birnin Kudu* (JGBK) Following Transesterification with Lipases (Industrial lipase, *Enterobacter aerogenes*, *Aspergillus niger* and *Aspergillus flavus*)

<b>Indices</b>	<b>Palmitoleic acid methyl ester</b>	<b>Palmitic acid methyl ester</b>	<b>Linoleic acid methyl ester</b>	<b>Oleic acid methyl ester</b>	<b>Stearic acid methyl ester</b>	<b>Others</b>
Fatty acids profile before transesterification (%)	1.07 ±0.01	16.44 ±0.02	30.10 ±0.01	36.10 ±0.00	8.57 ±0.02	6.91 ±0.01
Fatty acid profile after Lipase catalyzed Trans-esterification (%)						
- <b>Industrial lipase</b>	----	6.42 ±0.03	6.96 ±0.02	9.86 ±0.03	2.50 ±0.01	74.26 ±0.01
% difference	----	(-10.02)	(-23.14)	(-26.2)	(-6.07)	(+67.35)
- <b><i>Enterobacter aerogenes</i></b>	----	23.83 ±0.00	8.53 ±0.03	33.15 ±0.00	----	34.48 ±0.02
% difference	----	(+7.39)	(-21.57)	(-3.77)	----	(+27.57)
- <b><i>Aspergillus niger</i></b>	----	4.50 ±0.03	6.58 ±0.01	----	2.40 ±0.01	86.51 ±0.01
% difference	----	(-11.94)	(-23.32)	(-33.80)	(+6.06)	(+79.60)
- <b><i>Aspergillus flavus</i></b>	----	17.98 ±0.03	25.52 ±0.02	34.77 ±0.00	9.91 ±0.01	11.74 ±0.01
% difference	----	(+1.54)	(+4.58)	(+2.15)	(+1.35)	(+5.83)

NB: + = significant increase in fatty acid

- = significant decrease in fatty acid

---- = Not available

% difference = Value in parenthesis is the difference between fatty acids Profile before and after transesterification

Others:

- i. Arachidic acid Methyl ester
- ii. Lauric acid Methyl ester
- iii. Capric acid Methyl ester
- iv. Elaidic acid methyl ester

Table 4.4. Fatty Acids Composition for *Jatropha curcas* Seed Oil Provenance from *Wasugu Maga Danko* (KBDW) Following Transesterification with Lipases (Industrial lipase, *Enterobacter aerogenes*, *Aspergillus niger* and *Aspergillus flavus*)

<b>Indices</b>	<b>Palmitoleic acid methyl ester</b>	<b>Palmitic acid methyl ester</b>	<b>Linoleic acid methyl ester</b>	<b>Oleic acid methyl ester</b>	<b>Stearic acid methyl ester</b>	<b>Others</b>
Fatty acids profile before transesterification (%)	1.39 ±0.01	17.37 ±0.01	35.43 ±0.01	34.49 ±0.01	9.90 ±0.00	1.31 ±0.01
Fatty acid profile after Lipase catalyzed Trans- esterification (%)						
- <b>Industrial lipase</b>	1.38 ±0.03	16.5 ±0.00	23.94 ±0.01	28.18 ±0.02	10.79 ±0.01	68.66 ±0.02
<i>Percentage (%) difference</i>	(-0.01)	(-00.82)	(-11.49)	(-6.31)	(+0.89)	(-67.35)
- <b>Enterobacter aerogenes</b>	1.77 ±0.01	19.50 ±0.01	27.87 ±0.00	33.65 ±0.02	11.5 ±0.00	5.66 ±0.01
<i>% difference</i>	(+0.38)	(+2.13)	(-7.56)	(-0.84)	(+7.57)	(-4.35)
- <b>Aspergillus niger</b>	1.44 ±0.00	18.54 ±0.01	26.98 ±0.01	32.82 ±0.00	9.51 ±0.01	10.71 ±0.03
<i>% difference</i>	(+0.05)	(+2.13)	(-8.45)	(-1.67)	(+0.39)	(+9.40)
- <b>Aspergillus flavus</b>	----	19.36 ±0.03	31.01 ±0.01	----	7.57 ±0.01	42.06 ±0.02
<i>% difference</i>	----	(+1.99)	(+4.42)	----	(-2.33)	(+40.75)

NB: + = significant increase in fatty acid  
 - = significant decrease in fatty acid  
 ---- = Not available

% difference = value in parenthesis is the difference between fatty acids profile before and after transesterification

Others:

- i. Arachidic acid Methyl ester
- ii. Lauric acid Methyl ester
- iii. Capric acid Methyl ester
- iv. Elaidic acid methyl ester

#### **4. 6 FATTY ACIDS PROFILE OF JATROPHA SEED OILS FOLLOWING TRANSESTERIFICATION WITH LIPASE OF *ASPERGILLUS NIGER*.**

The % fatty acids profiles for transesterified Jatropha seed-oil provenances are presented in Table 4.5. Linoleic, oleic, palmitic methyl ester, and stearic acids were detected as the dominant fatty acids in all the seed oil provenances. While, stearic and palmitic acids methyl esters were the major saturated fatty acids observed in the oils.

Table 4.5 Fatty Acids Profile for *Jatropha* Seed-oil Provenance Following Transesterification with Crude Lipase from *Aspergillus niger*.

Provenances	Palmitoleic acid methyl ester	Palmitic acid methyl ester	Linoleic acid methyl ester	Oleic acid methyl ester	Stearic acid methyl ester	Others
<b>Jigawa, Birnin Masaya (JGBM)</b>	0.91	17.39	30.34	42.86	8.12	0.39
-Before Transesterification (%)	±0.01	±0.02	±0.00	±0.03	±0.03	±0.01
-After Transesterification (%)	---	18.42 ±0.01	13.70 ±0.03	52.93 ±0.01	8.77 ±0.00	6.67 ±0.01
<b>-% difference</b>	---	<b>(+1.03)</b>	<b>(-16.64)</b>	<b>(+10.07)</b>	<b>(+0.65)</b>	<b>(+5.28)</b>
<b>Kebbi, J Birnin (KBJB)</b>	2.54	26.31	---	40.22	14.68	16.29
-Before Transesterification (%)	±0.02	±0.01	---	±0.03	±0.01	±0.01
-After Transesterification (%)	---	17.66 ±0.01	29.93 ±0.02	37.89 ±0.01	8.10 ±0.01	14.51 ±0.01
<b>-% difference</b>	---	<b>(-8.65)</b>	---	<b>(-2.33)</b>	<b>(-6.56)</b>	<b>(-1.78)</b>
<b>Kaduna, Gwari Jiba (KDGJ)</b>	0.97	15.80	9.96	34.33	8.37	10.57
-Before Transesterification (%)	±0.04	±0.03	±0.01	±0.01	±0.00	±0.02
-After Transesterification (%)	---	15.80 ±0.01	30.07 ±0.02	35.86 ±0.02	5.84 ±0.01	12.43 ±0.01
<b>-% difference</b>	---	<b>(0.00)</b>	<b>(-0.11)</b>	<b>(+1.53)</b>	<b>(-2.53)</b>	<b>(+1.86)</b>
<b>Kano, Welare Makoda (KNWM)</b>	1.21	14.58	34.06	25.35	10.49	15.43
-Before Transesterification (%)	±0.01	±0.01	±0.03	±0.00	±0.01	±0.01
-After Transesterification (%)	1.25 ±0.01	17.73 ±0.01	31.97 ±0.01	34.45 ±0.01	9.58 ±0.01	14.60 ±0.00
<b>-% difference</b>	<b>(+0.04)</b>	<b>(-3.15)</b>	<b>(-2.09)</b>	<b>(+9.10)</b>	<b>(-0.91)</b>	<b>(-0.83)</b>
<b>Katsina, Chanranchi Kuki (KTCK)</b>	2.02	23.44	---	41.10	13.89	19.54
-Before Transesterification (%)	±0.01	±0.01	---	±0.01	±0.00	±0.01
-After Transesterification (%)	---	15.97 ±0.01	28.43 ±0.01	37.86 ±0.00	7.82 ±0.01	23.33 ±0.01
<b>-% difference</b>	---	<b>(-7.47)</b>	---	<b>(-3.24)</b>	<b>(-6.07)</b>	<b>(+3.79)</b>
<b>Sokoto, Pompo Bodiga (SKPB)</b>	1.46	18.28	31.16	40.43	8.35	0.31
-Before Transesterification (%)	±0.03	±0.02	±0.03	±0.01	±0.03	±0.01
-After Transesterification (%)	---	16.87 ±0.02	28.97 ±0.01	42.22 ±0.01	7.15 ±0.01	4.80 ±0.01
<b>-% difference</b>	---	<b>(-1.41)</b>	<b>(-2.19)</b>	<b>(+11.79)</b>	<b>(-1.20)</b>	<b>(+4.49)</b>
<b>Zamfara, Dandotodaji (ZMDD)</b>	1.47	17.78	39.75	29.93	9.68	1.39
-Before Transesterification (%)	±0.02	±0.02	±0.00	±0.01	±0.01	±0.01
-After Transesterification (%)	---	15.55 ±0.01	26.48 ±0.01	30.12 ±0.00	9.45 ±0.01	18.39 ±0.01
<b>-% difference</b>	---	<b>(-2.23)</b>	<b>(-13.27)</b>	<b>(+0.19)</b>	<b>(-0.23)</b>	<b>(+17.0)</b>

NB:

<b>% difference = Value in parenthesis is the difference between fatty acid profile</b>
---

## CHAPTER 5

### 5.1 DISCUSSION

Previous studies reported that 99% of the oil in plants is embedded in the endocarps of the seed and this varies from seed to seed (Fairless, 2007). Our study observed some percentage oil output from the *Jatropha* seed provenances between 8.32-60.62%. Makkar *et al* (1997) reported that seed kernel of *Jatropha curcas* contain 40-60% (ww<sup>-1</sup>) oil. Also, Kumari *et al* (2009) found that the oil output of *Jatropha* seed ranges from 30-50% by weight whereas in kernel the oil output ranges from 45-60%. Our finding was consistent with these reports since most oil yield from the seed provenances were within this range. Variation in the percentage oil output observed in this study could be due to some climatic conditions or could be due to difference in soil composition.

It have been reported that growth of plants is dependent on soil fertility and rainfall especially the later and seed production respond to rainfall and nutrient. A poor level will lead to increased failure of seed development (Gubitz *et al.*, 1997). According to Tewari (1964) and Diwaker (1993) Reported that *Jatropha curcas* is a wild growing hardy plant, well adapted to harsh condition of soil and climate whereas the oil content varies with respect to soil site. Schgal *et al* (1989) report that seed oil content of plants such as *Pinus roxburghii* yielded higher percentage of oil at lower altitude than higher altitude.

Fatty acids composition was determined as an important characteristic for biodiesel. The properties of the triglyceride and the biodiesel are determined by the amount of each fatty acid that is present in the oil (Mittelbach and Remchimide, 2004). Plant oils are mostly composed of five common fatty acids, namely palmitate, stearate, oleate, linoleate and linolenate, although, depending on the particular species, longer or shorter fatty acids may also be major constituents (Timothy *et al.*, 2008). These fatty acids differ from each other in terms of acyl chain length and number of double bonds, leading to different physical

properties of the biodiesel. Timothy *et al* (2008) observed that fuel properties of biodiesel derived from a mixture of fatty acid depend on their composition.

Knothe (2005) and Robles *et al* (2009) reported that biodiesel containing high level of unsaturated fatty acid has characteristics that are most similar to conventional biodiesel. Literature also revealed that altering the fatty acid profile can therefore improve fuel properties of biodiesel such as cold temperature (cloud and pour points), oxidative stability and nitrous oxide emissions (Timothy *et al.*, 2008). Lipase-catalyzed transesterification of *Jatropha* seed oil was found to improve the oil quality via altering the saturated and unsaturated fatty acids of the seed oil. Alteration of fatty acids compositions following lipase-catalyzed transesterification of *Jatropha curcas* oil using crude lipase from *Aspergillus niger* agreed with the report that fungal lipases has better protein processing and wider range of activity (Gupta *et al.*, 2007).

In support of this, we found that lipase from *Aspergillus niger* and *Aspergillus flavus* were active in a wide range of pH (pH 6-8). This implies that fungal lipases (*Aspergillus niger* and *Aspergillus flavus*) can catalyse reaction in both acidic and alkaline media with optimal activity in the acidic medium. *Enterobacter areogenes* was only active in the alkaline medium. Most lipases of bacterial strain exhibited an optimum pH over the range of pH 7-9 (Senthilkumar and Selvakumar, 2008 and Ranjitha, *et al.*, 2009). In addition, lipases from *Aspergillus niger* and *Aspergillus flavus* has wider activity in different organic solvent. Lipases from *Aspergillus species* have been found appropriate as biocatalysts for the transesterification reaction step in biodiesel production (Shah *et al.*, 2003; Gupta *et al.*, 2004).

It was also reported that reducing the amounts of unsaturated fatty acid present in the plant oils improve their oxidative stability (Klopfentein, 1985; Stoumas *et al.*, 1995; Serderi *et al.*, 1999). Knothe and Dunn (2003) reported that oxidation of long chain unsaturated fatty

acids typically of those found in biodiesel result in the initial accumulation of hydroperoxides which eventually polymerize forming insoluble sediments that are capable of plugging filters, fouling injectors and interfering with engine performance. In this present study, transesterification of *Jatropha* seed oil with crude lipase of *Aspergillus niger* was observed to reduced the amount of unsaturated fatty acid esters which is an indication that the quality of the biodiesel has been improved via increasing its oxidative stability. Knothe and Dunn (2003) reported that increase oxidation stability of oil requires decreasing the amounts of unsaturated and polyunsaturated fatty acids. In addition, McCormick *et al* (2001) also reported that reducing unsaturated fatty acids content of plant oils result in lowering nitrous oxide emissions.

Altering the fatty acid composition of oil could also lead to the development of low viscosity plant oil. High viscosity of plant oils lead to poor fuel atomization, preventing their direct use as fuel in most modern diesel engines. Allen *et al* (1999) reported that viscosity increases with the number of acyl carbons and is decreased by the presence of double bonds. This implies that high content of low molecular weight triacylglycerides with length of 8-14 carbon atoms in oils could therefore be a source of such low viscosity (Graham, 1989). In our study, fatty acid esters with length of 8-14 carbon atoms were not found in high content in *Jatropha* seed oil before the transesterification. However transesterification of the oil with microbial lipase increase the fatty acids esters' content with length between 8-14 carbon atoms. This suggests that transesterification of *Jatropha* seed oil result to low viscosity oil which is an indication of high quality biodiesel. Even though, Timothy *et al* (2008) reported that there is no fatty acid profile that will provide a fuel for which all these parameters (cold temperature, oxidative stability, nitrous oxide emissions and viscosity) are optimal.

Since lipase-catalyze transesterification of vegetable oil occurred in the presence of alcohol and better yield of fatty acids composition in the transesterified oil confirm the catalytic ability of such enzyme. Vandana and Bera (2005) reported that microbial-lipase's activity decreased 30-65% in organic chemicals such as hexane, propanol and butanol. In this regard, inability of some of the microbial lipases used in the transesterification reaction of the *Jatropha* oil might be due to the inhibitory effect of the organic solvents on their activities. Khelmitzky *et al* (1988) have earlier reported that enzymes are generally denatured in the presence of organic solvents. The inhibitory effects of organic solvents on lipase enzyme vary with their sources. Fjerbaek *et al* (2009) found that lipase from *Pseudomonas* show more resistance to alcohol inhibition than lipases from *Thermomyces lanuginosis* and *Rhizomucor miehei*.

## CHAPTER 6

### SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

#### 6.1 SUMMARY

- i. The *Jatropha* seed provenances reveal some variation in their oil yield (8.32-60.62%).
- ii. Potentials of Biofuel has been recognized in *Jatropha curcas* seed oil. Identified microbial species possess good lipase activity. Optimization using the crude lipases was achieved via transesterification process
- iii. Changes in fatty acids compositions were observed following lipase-catalyzed transesterification of the *Jatropha* seed oil using crude microbial lipases, where a significant alteration of fatty acid profile was observed from *Aspergillus niger*.
- iv. Altering the fatty acid profile can therefore improve fuel properties of biodiesel such as cold temperature (cloud and pour points), oxidative stability and nitrous oxide emissions (Timothy *et al.*, 2008). Lipase-catalyzed transesterification of *Jatropha* seed oil was found to improve the oil quality via reducing the saturated and unsaturated fatty acids of the seed oil.
- v. Since enzymes are generally denatured by organic solvents, inactivity of some of the microbial lipases used in the transesterification reaction of the *Jatropha* seed oil could be as a result of inhibitory effect of the solvents on them.

## 6.2 CONCLUSIONS

- i. The oil yield of *Jatropha* provenances varies with climatic conditions such as soil profile, rain fall.
- ii. The study also reveals some fatty acid profile of the *Jatropha* seed oil which could be useful as biodiesel and for other industrial applications.
- iii. Transesterification of *Jatropha* seed oil by lipase from fungal origin should be utilized as better source for optimizing biodiesel production from seed oils.

### 6.3 RECOMMENDATIONS

- i. Study on microbial enzymes (lipase) of different sources for their ability to catalyze transesterification of *Jatropha* oil is recommended.
- ii. Optimizing biodiesel production using lipase-catalyzes transesterification of *Jatropha* seed oil in the presence of different organic solvents, various reaction times and /or in a solvent free system is also recommended.

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APPENDICES

Appendix 1.0 Titer values of 0.05M NaOH following the effect of temperature on microbial crude lipase activity on 5% (w/v) olive oil/gum Arabic substrate emulsion

	30 °C	40 °C	50 °C	60 °C	70 °C	80 °C
Blank titer value (no Lipase) (ml)	3.1	3	6	8	6.2	7.3
	3.05	2.99	6.1	7.95	6.15	7.28
Mean	<b>3.075</b>	<b>2.995</b>	<b>6.05</b>	<b>7.975</b>	<b>6.175</b>	<b>7.29</b>
SD	<b>0.035355</b>	<b>0.007071</b>	<b>0.070711</b>	<b>0.035355</b>	<b>0.035355</b>	<b>0.014142</b>
Lipase from <i>Enterobacter aerogenes</i> value (ml)	6	7	10.2	9.6	6.8	7.4
	5.9	7.1	10.1	9.7	6.85	7.3
Mean	<b>5.95</b>	<b>7.05</b>	<b>10.15</b>	<b>9.65</b>	<b>6.825</b>	<b>7.35</b>
SD	<b>0.070711</b>	<b>0.070711</b>	<b>0.070711</b>	<b>0.070711</b>	<b>0.035355</b>	<b>0.070711</b>
Lipase from <i>Aspergillus niger</i> value (ml)	8	10.8	12.8	11.4	9	8.6
	8.05	10.7	12.9	11.5	8.95	8.65
Mean	<b>8.025</b>	<b>10.75</b>	<b>12.85</b>	<b>11.45</b>	<b>8.975</b>	<b>8.625</b>
SD	<b>0.035355</b>	<b>0.070711</b>	<b>0.070711</b>	<b>0.070711</b>	<b>0.035355</b>	<b>0.035355</b>
Lipase from <i>Aspergillus flavus</i> value (ml)	7	11.2	13.5	12	10	10
	7.1	11.3	13.4	12.05	10.02	9.85
Mean	<b>7.05</b>	<b>11.25</b>	<b>13.45</b>	<b>12.025</b>	<b>10.01</b>	<b>9.925</b>
SD	<b>0.070711</b>	<b>0.070711</b>	<b>0.070711</b>	<b>0.035355</b>	<b>0.014142</b>	<b>0.106066</b>

Appendix 2.0 Titer values of 0.05M NaOH following thermostability effect on microbial crude lipase activity on 5% (w/v) olive oil/gum Arabic substrate emulsion

	30°C	40 °C	50 °C	60 °C	70 °C	80 °C
Blank titer value (no Lipase) (ml)	7.5	7	7.5	6.4	7.1	9.6
	7.55	7.1	7.45	6.5	7.15	9.7
<b>Mean</b>	<b>7.525</b>	<b>7.05</b>	<b>7.475</b>	<b>6.45</b>	<b>7.125</b>	<b>9.65</b>
<b>SD</b>	<b>0.035355</b>	<b>0.070711</b>	<b>0.035355</b>	<b>0.070711</b>	<b>0.035355</b>	<b>0.070711</b>
Lipase from <i>Enterobacter aerogenes</i> value (ml)	10	12.4	13.6	12	10.6	10.2
	10.06	12.3	13.5	12.05	10.7	10.15
<b>Mean</b>	<b>10.03</b>	<b>12.35</b>	<b>13.55</b>	<b>12.025</b>	<b>10.65</b>	<b>10.175</b>
<b>SD</b>	<b>0.042426</b>	<b>0.070711</b>	<b>0.070711</b>	<b>0.035355</b>	<b>0.070711</b>	<b>0.035355</b>
Lipase from <i>Aspergillus niger</i> value (ml)	13.4	13.2	12.6	9	8.4	10.5
	13.45	13.15	12.5	9.1	8.35	10.4
<b>Mean</b>	<b>13.425</b>	<b>13.175</b>	<b>12.55</b>	<b>9.05</b>	<b>8.375</b>	<b>10.45</b>
<b>SD</b>	<b>0.035355</b>	<b>0.035355</b>	<b>0.070711</b>	<b>0.070711</b>	<b>0.035355</b>	<b>0.070711</b>
Lipase from <i>Aspergillus flavus</i> value (ml)	11.1	12	10.6	7	6.6	7.2
	11.15	12.05	10.55	6.95	6.5	7.3
<b>Mean</b>	<b>11.125</b>	<b>12.025</b>	<b>10.575</b>	<b>6.975</b>	<b>6.55</b>	<b>7.25</b>
<b>SD</b>	<b>0.035355</b>	<b>0.035355</b>	<b>0.035355</b>	<b>0.035355</b>	<b>0.070711</b>	<b>0.070711</b>

Appendix 3.0 Titer values of 0.05M NaOH following the effect of pH on microbial crude lipase activity on 5% (w/v) olive oil/gum Arabic substrate emulsion

	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10
Blank titer value (no Lipase) (ml)	15.4	16.6	17	7.5	6.5	8.7	9.7
	15.3	16.5	17.05	7.45	6.6	8.75	9.8
Mean	<b>15.35</b>	<b>16.55</b>	<b>17.025</b>	<b>7.475</b>	<b>6.55</b>	<b>8.725</b>	<b>9.75</b>
SD	<b>0.070711</b>	<b>0.070711</b>	<b>0.035355</b>	<b>0.035355</b>	<b>0.070711</b>	<b>0.035355</b>	<b>0.070711</b>
Lipase from <i>Enterobacter aerogenes</i> value (ml)	15.7	17.6	18.6	10	9.3	10.15	10.6
	15.8	17.5	18.4	10.05	9.2	10.1	10.7
Mean	<b>15.75</b>	<b>17.55</b>	<b>18.5</b>	<b>10.025</b>	<b>9.25</b>	<b>10.125</b>	<b>10.65</b>
SD	<b>0.070711</b>	<b>0.070711</b>	<b>0.141421</b>	<b>0.035355</b>	<b>0.070711</b>	<b>0.035355</b>	<b>0.070711</b>
Lipase from <i>Aspergillus niger</i> value (ml)	16.5	18.1	20.3	10.7	8.3	9.4	11.8
	16.8	18.05	20.35	10.8	8.35	9.3	11.7
Mean	<b>16.65</b>	<b>18.075</b>	<b>20.325</b>	<b>10.75</b>	<b>8.325</b>	<b>9.35</b>	<b>11.75</b>
SD	<b>0.212132</b>	<b>0.035355</b>	<b>0.035355</b>	<b>0.070711</b>	<b>0.035355</b>	<b>0.070711</b>	<b>0.070711</b>
Lipase from <i>Aspergillus flavus</i> value (ml)	16.7	18	19.8	10	8.4	10	10.8
	16.6	17.9	19.75	10.04	8.45	10.02	10.9
Mean	<b>16.65</b>	<b>17.95</b>	<b>19.775</b>	<b>10.02</b>	<b>8.425</b>	<b>10.01</b>	<b>10.85</b>
SD	<b>0.070711</b>	<b>0.070711</b>	<b>0.035355</b>	<b>0.028284</b>	<b>0.035355</b>	<b>0.014142</b>	<b>0.070711</b>

Appendix 4.0 Titer values of 0.05M NaOH following pH stability effect of microbial crude lipase activity on 5% (w/v) olive oil/gum Arabic substrate emulsion

	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10
Blank titer value (no Lipase) (ml)	6.5	6.6	6.2	6.9	5.7	6	6.8
	6.5	6.5	6.25	7	5.8	5.95	6.8
Mean	<b>6.5</b>	<b>6.55</b>	<b>6.225</b>	<b>6.95</b>	<b>5.75</b>	<b>5.975</b>	<b>6.8</b>
SD	<b>0</b>	<b>0.070711</b>	<b>0.035355</b>	<b>0.070711</b>	<b>0.070711</b>	<b>0.035355</b>	<b>0</b>
Lipase from <i>Enterobacter aerogenes</i> value (ml)	7.7	7.8	8.6	8.6	8.3	7	7.6
	7.75	7.8	8.5	8.6	7.2	7.1	7.7
Mean	<b>7.725</b>	<b>7.8</b>	<b>8.55</b>	<b>8.6</b>	<b>8.25</b>	<b>7.05</b>	<b>7.65</b>
SD	<b>0.035355</b>	<b>0</b>	<b>0.070711</b>	<b>0</b>	<b>0.070711</b>	<b>0.070711</b>	<b>0.070711</b>
Lipase from <i>Aspergillus niger</i> value (ml)	10.4	11.4	11.6	12.5	8.6	8	7.4
	10.5	11.3	11.5	12.6	8.5	8.05	7.35
Mean	<b>10.45</b>	<b>11.35</b>	<b>11.55</b>	<b>12.55</b>	<b>8.55</b>	<b>8.025</b>	<b>7.375</b>
SD	<b>0.070711</b>	<b>0.070711</b>	<b>0.070711</b>	<b>0.070711</b>	<b>0.070711</b>	<b>0.035355</b>	<b>0.035355</b>
Lipase from <i>Aspergillus flavus</i> value (ml)	6.6	9.4	10.4	10	8.3	7.8	7.6
	6.7	9.5	10.3	9.95	8.2	7.7	7.6
Mean	<b>6.65</b>	<b>9.45</b>	<b>10.35</b>	<b>9.975</b>	<b>8.25</b>	<b>7.75</b>	<b>7.6</b>
SD	<b>0.070711</b>	<b>0.070711</b>	<b>0.070711</b>	<b>0.035355</b>	<b>0.070711</b>	<b>0.070711</b>	<b>0</b>

Appendix 5.0 Titer values of 0.05M NaOH following the effect of some organic solvents on microbial crude lipase activity on 5% (w/v) olive oil/gum Arabic substrate emulsion

	Aspergillus flavus titer value (ml)	Lipase activity (umole fatty acids/ml)	Relative activity (%)	Aspergillus niger titer value (ml)	Lipase activity (umole fatty acids/ml)	Relative activity (%)	Enterobacter aerogenes titer value (ml)	Lipase activity (umole fatty acids/ml)	Relative activity (%)
Blank	3			3			3		
	3.1			3.1			3.1		
Mean									
SD									
Positive control	10.2	0.036	100	10	0.035	100	11.2	0.041	100
	10.15	0.03525	100	10.05	0.03475	100	11.3	0.041	100
<b>Mean</b>			<b>100</b>			<b>100</b>			<b>100</b>
<b>SD</b>			<b>0</b>			<b>0</b>			<b>0</b>
Acetone	5.3	0.0115	31.94444	5.4	0.012	34.28571	5	0.01	24.39024
	5.2	0.011	31.20567	5.3	0.011	31.65468	5.05	0.00975	23.78049
<b>Mean</b>			<b>31.57506</b>			<b>32.9702</b>			<b>24.08537</b>
<b>SD</b>			<b>0.52239</b>			<b>1.860425</b>			<b>0.431163</b>
Methanol	7	0.02	55.55556	6.6	0.018	51.42857	5.7	0.0135	32.92683
	7.05	0.02025	56.25	6.5	0.017	48.92086	5.8	0.0135	32.92683
<b>Mean</b>			<b>55.90278</b>			<b>50.17472</b>			<b>32.92683</b>
<b>SD</b>			<b>0.491046</b>			<b>1.773217</b>			<b>1.59E-14</b>
Ethanol	6.2	0.016	44.44444	6.8	0.019	54.28571	6	0.015	36.58537
	6.3	0.0165	45.83333	6.7	0.018	51.79856	6.05	0.01475	35.97561
<b>Mean</b>			<b>45.13889</b>			<b>53.04214</b>			<b>36.28049</b>
<b>SD</b>			<b>0.982093</b>			<b>1.758683</b>			<b>0.431163</b>
Butanol	5.29	0.01145	31.80556	5.2	0.011	31.42857	4.8	0.009	21.95122
	5.3	0.0115	31.94444	5.3	0.0115	33.09353	4.85	0.00875	21.34146
<b>Mean</b>			<b>31.875</b>			<b>32.26105</b>			<b>21.64634</b>
<b>SD</b>			<b>0.098209</b>			<b>1.1773</b>			<b>0.431163</b>
Hexane	5.9	0.0145	40.27778	6	0.015	42.85714	5.6	0.013	31.70732
	6	0.015	41.66667	6.05	0.01475	42.44604	5.5	0.012	29.26829
<b>Mean</b>			<b>40.97222</b>			<b>42.65159</b>			<b>30.4878</b>
<b>SD</b>			<b>0.982093</b>			<b>0.290691</b>			<b>1.724651</b>

Appendix 6.0 Values for fatty acids profile of Jatropha seed oil provenances from JGBK and KMDW before and after transesterification with lipases (Industrial lipase, Aspergillus niger, Aspergillus flavus and enterobacter aerogenes)

Fatty Acids Profile of JGBK Seed oil	Palmitoleic acid methyl ester	Palmitic acid methyl ester	Linoleic acid methyl ester	Oleic acid methyl ester	Stearic acid methyl ester	Others
Before Transesterification (%)						
	1.08	16.42	30.09	36.1	8.55	6.9
	1.06	16.45	30.11	36.1	8.58	6.92
Mean	<b>1.07</b>	<b>16.435</b>	<b>30.1</b>	<b>36.1</b>	<b>8.565</b>	<b>6.91</b>
SD	<b>0.014142</b>	<b>0.021213</b>	<b>0.014142</b>	<b>0</b>	<b>0.021213</b>	<b>0.014142</b>
After Transesterification (%)						
- <b>Industrial lipase</b>	0	6.44	6.94	9.88	2.49	74.27
	0	6.4	6.97	9.84	2.5	74.26
Mean	<b>0</b>	<b>6.42</b>	<b>6.955</b>	<b>9.86</b>	<b>2.495</b>	<b>74.265</b>
SD	<b>0</b>	<b>0.028284</b>	<b>0.021213</b>	<b>0.028284</b>	<b>0.007071</b>	<b>0.007071</b>
- <i>Enterobacter aerogenes</i>	0	23.86	8.55	33.15	0	34.46
	0	23.8	8.51	33.15	0	34.49
Mean	<b>0</b>	<b>23.83</b>	<b>8.53</b>	<b>33.15</b>	<b>0</b>	<b>34.48</b>
SD	<b>0</b>	<b>0.042426</b>	<b>0.028284</b>	<b>0.000</b>	<b>0</b>	<b>0.021</b>
- <i>Aspergillus niger</i>	0	4.52	6.59	0	2.33	86.5
	0	4.48	6.57	0	2.35	86.52
Mean	<b>0</b>	<b>4.5</b>	<b>6.58</b>	<b>0</b>	<b>2.34</b>	<b>86.51</b>
SD	<b>0</b>	<b>0.028284</b>	<b>0.014142</b>	<b>0</b>	<b>0.014142</b>	<b>0.014142</b>
- <i>Aspergillus flavus</i>	0	18	25.54	34.77	9.9	11.72
	0	17.96	25.5	34.77	9.92	11.76
Mean	<b>0</b>	<b>17.98</b>	<b>25.52</b>	<b>34.77</b>	<b>9.91</b>	<b>11.74</b>
SD	<b>0</b>	<b>0.028284</b>	<b>0.028284</b>	<b>0.000</b>	<b>0.014142</b>	<b>0.028284</b>

<b>Fatty Acids Profile of KMDW Jatropha seed oil</b>	<b>Palmitoleic acid methyl ester</b>	<b>Palmitic acid methyl ester</b>	<b>Linoleic acid methyl ester</b>	<b>Oleic acid methyl ester</b>	<b>Stearic acid methyl ester</b>	<b>Others</b>
Before transesterification (%)						
	1.4	17.36	35.44	34.48	9.8	1.3
	1.38	17.38	35.42	34.49	10	1.32
Mean	<b>1.39</b>	<b>17.37</b>	<b>35.43</b>	<b>34.485</b>	<b>9.9</b>	<b>1.31</b>
Sd	<b>0.014142</b>	<b>0.014142</b>	<b>0.014142</b>	<b>0.007071</b>	<b>0.141421</b>	<b>0.014142</b>
After Transesterification (%)						
- <b>Industrial lipase</b>	1.36	16.5	23.93	28.16	10.79	68.68
	1.4	16.5	23.95	28.2	10.78	68.64
Mean	<b>1.38</b>	<b>16.5</b>	<b>23.94</b>	<b>28.18</b>	<b>10.785</b>	<b>68.66</b>
SD	<b>0.028284</b>	<b>0.000</b>	<b>0.014142</b>	<b>0.028284</b>	<b>0.007071</b>	<b>0.028284</b>
- <i>Enterobacter aerogenes</i>	1.77	19.56	27.87	33.67	11.53	5.65
	1.76	19.54	27.87	33.64	11.53	5.67
Mean	<b>1.765</b>	<b>19.55</b>	<b>27.87</b>	<b>33.655</b>	<b>11.53</b>	<b>5.66</b>
SD	<b>0.0070</b>	<b>0.014142</b>	<b>0.000</b>	<b>0.021</b>	<b>0.000</b>	<b>0.014142</b>
- <i>Aspergillus niger</i>	1.44	18.53	26.99	32.82	9.51	10.72
	1.44	18.55	26.97	32.82	9.53	10.68
Mean	<b>1.44</b>	<b>18.54</b>	<b>26.98</b>	<b>32.82</b>	<b>9.52</b>	<b>10.7</b>
SD	<b>0.000</b>	<b>0.014142</b>	<b>0.014142</b>	<b>0</b>	<b>0.014142</b>	<b>0.028284</b>
- <i>Aspergillus flavus</i>	0	19.34	31	0	7.58	42.07
	0	19.38	31.02	0	7.56	42.04
Mean	<b>0</b>	<b>19.36</b>	<b>31.01</b>	<b>0</b>	<b>7.57</b>	<b>42.055</b>
SD	<b>0</b>	<b>0.028</b>	<b>0.014142</b>	<b>0</b>	<b>0.014142</b>	<b>0.0212</b>

Appendix 7.0 Values for Fatty acids composition of Jatropha curcas seed oil provenance

	<b>Palmitoleic acid methyl ester</b>	<b>Palmitic acid methyl ester</b>	<b>Linoleic acid methyl ester</b>	<b>Oleic acid methyl ester</b>	<b>Stearic acid methyl ester</b>	<b>Others</b>
Jigawa, <i>Birnin Masaya</i> (JGBM)	0.9	17.40	30.34	42.82	8.14	0.38
	0.92	17.37	30.34	42.86	8.1	0.4
<b>Mean</b>	<b>0.91</b>	<b>17.385</b>	<b>30.34</b>	<b>42.84</b>	<b>8.12</b>	<b>0.39</b>
<b>SD</b>	<b>0.014142</b>	<b>0.021213</b>	<b>0</b>	<b>0.028284</b>	<b>0.028284</b>	<b>0.014142</b>
Kebbi, <i>J Birnin</i> (KBJB)	2.52	26.32	0	40.2	14.68	16.28
	2.55	26.3	0	40.24	14.7	16.3
<b>Mean</b>	<b>2.535</b>	<b>26.31</b>	<b>0</b>	<b>40.22</b>	<b>14.69</b>	<b>16.29</b>
<b>SD</b>	<b>0.021213</b>	<b>0.014142</b>	<b>0</b>	<b>0.028284</b>	<b>0.014142</b>	<b>0.014142</b>
Kaduna, <i>Gwari Jiba</i> (KDGJ)	0.94	15.6	9.96	34.34	8.37	10.55
	1	15.76	9.97	34.32	8.37	10.58
<b>Mean</b>	<b>0.97</b>	<b>15.8</b>	<b>9.965</b>	<b>34.33</b>	<b>8.37</b>	<b>10.565</b>
<b>SD</b>	<b>0.042426</b>	<b>0.028284</b>	<b>0.0071</b>	<b>0.014142</b>	<b>0.000</b>	<b>0.021213</b>
Kano, <i>Welare Makoda</i> (KNWM)	1.22	14.56	34.04	25.35	10.5	15.42
	1.2	14.6	34.08	25.35	10.48	15.44
<b>Mean</b>	<b>1.21</b>	<b>14.58</b>	<b>34.06</b>	<b>25.35</b>	<b>10.49</b>	<b>15.43</b>
<b>SD</b>	<b>0.014142</b>	<b>0.028284</b>	<b>0.028284</b>	<b>0.000</b>	<b>0.014142</b>	<b>0.014142</b>
Katsina, <i>Chanranchi Kuki</i> (KTCK)	2.01	23.43	0	41.09	13.89	19.53
	2.03	23.45		41.1	13.89	19.55
<b>Mean</b>	<b>2.02</b>	<b>23.44</b>	<b>0</b>	<b>41.095</b>	<b>13.89</b>	<b>19.54</b>
<b>SD</b>	<b>0.014142</b>	<b>0.014142</b>		<b>0.0070</b>	<b>0.000</b>	<b>0.014142</b>
Sokoto, <i>Pompo Bodiga</i> (SKPB)	1.48	18.3	31.14	40.42	8.37	0.3
	1.44	18.27	31.18	40.44	8.32	0.32
<b>Mean</b>	<b>1.46</b>	<b>18.285</b>	<b>31.16</b>	<b>40.43</b>	<b>8.345</b>	<b>0.31</b>
<b>SD</b>	<b>0.028284</b>	<b>0.021</b>	<b>0.028284</b>	<b>0.014142</b>	<b>0.035</b>	<b>0.014142</b>
Zamafara, <i>Dandotodaji</i> (ZMDD)	1.45	17.79	39.75	29.92	9.67	1.38
	1.48	17.76	39.75	29.94	9.69	1.4
<b>Mean</b>	<b>1.465</b>	<b>17.775</b>	<b>39.75</b>	<b>29.93</b>	<b>9.68</b>	<b>1.39</b>
<b>SD</b>	<b>0.021</b>	<b>0.021213</b>	<b>0.000</b>	<b>0.014142</b>	<b>0.014142</b>	<b>0.014142</b>

Appendix .8.0 Values for fatty acids composition of *Jatropha curcas* seed oil provenance following transesterification with lipases (Industrial lipase, *Enterobacter aerogenes*, *Aspergillus niger* and *Aspergillus flavus*)

	<b>Palmitoleic acid methyl ester</b>	<b>Palmitic acid methyl ester</b>	<b>Linoleic acid methyl ester</b>	<b>Oleic acid methyl ester</b>	<b>Stearic acid methyl ester</b>	<b>Others</b>
Jigawa, <i>Birnin Masaya</i> (JGBM)	0	18.43	13.68	52.92	8.77	6.66
	0	18.41	13.72	52.94	8.77	6.68
<b>Mean</b>	<b>0</b>	<b>18.42</b>	<b>13.7</b>	<b>52.93</b>	<b>8.77</b>	<b>6.67</b>
<b>SD</b>	<b>0</b>	<b>0.014142</b>	<b>0.028284</b>	<b>0.014142</b>	<b>0</b>	<b>0.014142</b>
Kebbi, <i>J Birnin</i> (KBJB)	0	17.67	29.92	37.88	8.11	14.52
	0	17.65	29.95	37.9	8.09	14.5
<b>Mean</b>	<b>0</b>	<b>17.66</b>	<b>29.935</b>	<b>37.89</b>	<b>8.1</b>	<b>14.51</b>
<b>SD</b>	<b>0</b>	<b>0.014142</b>	<b>0.021213</b>	<b>0.014142</b>	<b>0.014142</b>	<b>0.014142</b>
Kaduna, <i>Gwari Jiba</i> (KDGJ)	0	15.79	30.08	35.85	5.83	12.44
	0	15.81	30.05	35.88	5.85	12.43
<b>Mean</b>	<b>0</b>	<b>15.8</b>	<b>30.065</b>	<b>35.865</b>	<b>5.84</b>	<b>12.435</b>
<b>SD</b>	<b>0</b>	<b>0.014142</b>	<b>0.021213</b>	<b>0.021213</b>	<b>0.014142</b>	<b>0.007071</b>
Kano, <i>Welare Makoda</i> (KNWM)	1.26	17.74	31.98	34.46	9.59	14.6
	1.24	17.72	31.96	34.44	9.57	14.6
<b>Mean</b>	<b>1.25</b>	<b>17.73</b>	<b>31.97</b>	<b>34.45</b>	<b>9.58</b>	<b>14.6</b>
<b>SD</b>	<b>0.014142</b>	<b>0.014142</b>	<b>0.014142</b>	<b>0.014142</b>	<b>0.014142</b>	<b>0</b>
Katsina, <i>Chanranchi Kuki</i> (KTCK)	0	15.98	28.44	37.86	7.83	23.34
	0	15.96	28.42	37.86	7.81	23.32
<b>Mean</b>	<b>0</b>	<b>15.97</b>	<b>28.43</b>	<b>37.86</b>	<b>7.82</b>	<b>23.33</b>
<b>SD</b>	<b>0</b>	<b>0.014142</b>	<b>0.014142</b>	<b>0</b>	<b>0.014142</b>	<b>0.014142</b>
Sokoto, <i>Pompo Bodiga</i> (SKPB)	0	16.86	28.97	42.21	7.16	4.79
	0	16.89	28.96	42.23	7.14	4.81
<b>Mean</b>	<b>0</b>	<b>16.875</b>	<b>28.965</b>	<b>42.22</b>	<b>7.15</b>	<b>4.8</b>
<b>SD</b>	<b>0</b>	<b>0.021213</b>	<b>0.007071</b>	<b>0.014142</b>	<b>0.014142</b>	<b>0.014142</b>
Zamafara, <i>Dandotodaji</i> (ZMDD)	0	15.56	26.47	30.11	9.46	18.38
	0	15.54	26.49	30.12	9.44	18.4
<b>Mean</b>	<b>0</b>	<b>15.55</b>	<b>26.48</b>	<b>30.12</b>	<b>9.45</b>	<b>18.39</b>
<b>SD</b>	<b>0</b>	<b>0.014142</b>	<b>0.014142</b>	<b>0.000</b>	<b>0.014142</b>	<b>0.014142</b>

Table 2.1. Fatty acid profile of oils and fats used for biodiesel production (Akoh *et al.*, 2007; Marchetti *et al.*, 2007)

<b>Oil/fats</b>	<b>Arachidic (20:0)</b>	<b>Linoleic (18:2)</b>	<b>Linolenic (18:3)</b>	<b>Oleic (18:1)</b>	<b>Palmitic (16:0)</b>	<b>Palmitoleic (16:1)</b>	<b>Stearic (18:0)</b>	<b>Other</b>
Canola		22.3	8.2	64.4	3.5		0.9	0.7
Coconut				6.0	5.0		3.0	86.0
Cotton seed		57.5		13.3	28.3		0.9	
Groundnut		26.0		51.6	8.5		6.0	7.9
Jatropha	0.2	36.2		37.0	16.4	1.0	6.2	3.0
Karanj	1.6	17.7	3.6	51.8	10.2		7.0	8.1
Microalgae		2.2	0.9	1.3	15.5	17.3	0.3	62.5
Olive	0.4	8.5	0.7	74.2	11.8	1.5	2.6	0.3
Palm Oil		10.1	0.2	40.5	42.6	0.3	4.4	1.9
Peanut	1.3	32.0	0.9	48.3	11.4		2.4	3.7
Rapeseed		22.3	8.2	64.4	3.5		0.9	0.7
Safflower seed		77.0		13.5	7.3	0.1	1.9	0.2
Soybean	0.3	53.8	9.3	20.8	11.4		4.4	
Sunflower	0.3	62.4		25.5	7.1		4.7	
Tallow				44.5	29.0		24.5	2.0