UNIVERSITY OF NAIROBI

TWO-STAGE CHEMICAL AND ENZYMATIC STRATEGIES FOR THE PREPARATION OF BIODIESEL FROM *CROTON MEGALOCARPUS* OIL AND EVALUATION OF ITS ENGINE PERFORMANCE AND OXIDATION STABILITY

BY

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DECLARATION

I declare that this thesis is my original work and has not been submitted elsewhere for examination, award of a degree or publication.

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ABSTRACT

Croton oil was extracted from dry *Croton megalocarpus* seeds by a mechanical pressing machine then filtered. The optimum conditions for preparation of biodiesel from the oil through a two-stage chemical process in reactor were determined by varying parameters such as temperature, oil: alcohol mole ratio and amount of catalysts. The optimum conditions established were temperatures of 50 and 60 ^{o}C for esterification and transesterification, respectively, methanol: oil mole ratio of 3:1 and 6:1 for esterification and transesterification, correspondingly and a base catalyst mass of 1% (w/w) of the Croton oil for transesterification. The optimum reaction times established were 1 and 2 hours for transesterification and esterification correspondingly. A maximum yield of 88% biodiesel which had an acid value of 0.336 mg KOH/g, a density of 0.8858 g/cm^3 and viscosity of 4.51 cs at 40 ^{o}C was obtained. The flash point of the biodiesel was greater than 200 ^{o}C, which made it safer to store and transport as compared to diesel which had a flash point of 65 ^{o}C. Both the cloud and pour points of the biodiesel were lower than that of petrodiesel, thus making its blends more suitable for lower temperature operations.

An enzymatic procedure for transesterification of Croton oil was also investigated. Croton biodiesel was prepared using commercial lipase from *Thermomyces lanuginosus.* The enzymatic transesterification of Croton oil produced a high yield of 93% biodiesel at optimum conditions of 35 ^{o}C, oil: alcohol molar ratio of 1:6, catalyst volume of 8% of croton oil and reaction time of 1 hour. All the physico-chemical properties of Croton biodiesel prepared using the two-stage chemical and commercial enzyme were found to be similar. Thin layer chromatography (TLC) analysis showed distinct differences between chromatograms of Croton oil and that of biodiesel while Gas chromatograph – Mass spectrometer (GC – MS) analysis showed that the Croton

biodiesel was composed of both saturated and unsaturated methyl esters with carbon atom chain lengths ranging from C-9 to C-19.

Extracellular lipase producing microorganisms were enriched by separately incubating 100 ml portions of sterile salt media (pH 8) containing 4% Croton oil inoculated with selected water and soil samples in a reciprocating shaker at 37 ^{o}C and 100 rpm for 10 days. The lipase producing microorganisms were then grown on agar plates containing minimum salt media and 4% Croton oil in an incubator. The lipase activities were screened by monitoring orange halos formed around the colonies on Rhodamine B/agar plates under ultraviolet (UV) light at 350 nm after 12 hours in an incubator. Pure colonies of lipase producing microorganisms were produced through submerged fermentation (SmF) in LB media for 48 hours in a reciprocating shaker at 37^{o}C and 100 rpm. Aqueous solutions of lipase enzymes were obtained from fermentation mixture by collecting the supernatant liquid after centrifugation of the LB media solutions. TLC and GC-MS analysis showed that the extracted lipase solutions poorly catalysed transesterification of the Croton oil using methanol under the optimum conditions established for *T. lanuginosus*.

Emission, performance and combustion characteristics of Croton biodiesel blends were tested in a computerized direct injection single cylinder four stroke diesel engine test rig. The use of biodiesel blends led to a reduction in exhaust smoke emissions ranging from 10 to 41% at maximum engine load of 10 Kg as compared to petrodiesel, while a slight increase in NO_x emissions was observed with increase in concentration of biodiesel in the blends. Similar general increase in brake thermal efficiency (BTE), temperature of exhaust gas emissions and fuel flow rate for both petrodiesel and biodiesel blends were observed with increasing engine load. The difference between BTE for petrodiesel and biodiesel blends ranged from 2 to 5%. The brake specific energy consumption (BSEC) decreased with increasing engine load for both petrodiesel

and biodiesel blends. Both engine pressure and heat released increased with increase in concentration of biodiesel in the blends. The difference in maximum engine pressure ranged from 1.05 bars at 0 Kg to 3.77 bars at 10 Kg load. The greatest difference in maximum engine pressure was recorded between petrodiesel and B50 blend.

The effects of antioxidants and storage on oxidation stability of Croton biodiesel and its blends were determined using PetroOxy equipment. The biodiesel and blends with and without antioxidants were stored in a metallic locker at room temperature for 8 weeks. The oxidation stability indices of the biodiesel and blends were monitored after every 2 weeks. The oxidation stability index for neat biodiesel depreciated at a very fast rate of 45% while that for biodiesel with 1000 ppm antioxidants depreciated by 16, 12 and 21% for pyrogallol (PYG), propyl gallate (PRG) and butylhydroxyanisole (BHA) correspondingly during the 8 weeks storage period. A more rapid decline in oxidation stability was therefore noted in biodiesel and blends without antioxidants than those with antioxidants and the oxidation stability rose with increase in concentration of the antioxidants. The use of appropriate concentrations of suitable antioxidants can therefore improve oxidation stability of biodiesel by preventing extensive and deleterious oxidative deterioration on storage.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF REACTION EQUATIONS

LIST OF ABBREVIATIONS, SYMBOLS AND ACRONYMS

ASAL	Arid and semi-arid lands
ASTM	American Society for Testing and Materials
ATP	Adenosine triphosphate
B5	5% biodiesel, 95% diesel blend
B10	10% biodiesel, 90% diesel blend
B15	15% biodiesel, 85% diesel blend
B20	20% biodiesel, 80% diesel blend
B50	50% biodiesel, 50% diesel blend
B100	100% biodiesel
BGM	Bacteria growth media
BHA	Butylhydroxyanisole
BHT	Butylated hydroxytoluene
CFPP	Cold filter plug point
CI	Compression ignition
CO	Carbon monoxide (Carbon (II) Oxide)
CP	Cloud Point
DG	Diglycerides
DHA	Docosahexaenoic acid
DI	Direct injection
EGR	Exhaust gas recirculation
EN	European standard
EPA	Eicosapentaenoic acid
EU	European Union

FAME	Fatty acid methyl ester
FAAE	Fatty acid alkyl ester
FFA	Free fatty acid
FID	Flame ionization detector
GC	Gas chromatography
HC	Hydrocarbons
H_2SO_4	Sulphuric acid
IDI	Indirect injection
IF	Inorganic fraction
IUPAC	International Union of Pure and Applied Chemistry
KOH	Potassium hydroxide
LA	Linolenic acid
LB	Lysogeny broth
MAG	Monoacylglyceride
MG	Monoglycerides
MS	Mass spectrometer
MSS	Minimum salt solution
NACOSTI	National Council of Science, Technology and Innovation
NaOH	Sodium hydroxide
NO_x	Nitrogen oxides
OSI	Oxidation stability index
PAH	Polycyclic/Polynuclear aromatic hydrocarbons
PM	Particulate matter
PP	Pour Point

ppm	Parts per million
PRG	Propyl gallate
PUFA	Polyunsaturated fatty acids
PYG	Pyrogallol
rpm	revolutions per minute
RSC	Royal Society of Chemistry
SI	Spark ignition
SmF	Submerged Fermentation
SOF	Soluble organic fraction
SSF	Solid state Fermentation
SVO	Straight vegetable oil
TBARS	Thiobarbiturate acid reagents
TBHQ	*tert*-butylhydroquinone
TAG	Triacylglyceride
TDG	Transportation of dangerous goods
TG	Triglyceride
TLC	Thin layer chromatography
UoN	University of Nairobi
UPES	University of Petroleum and Energy Studies
UV	Ultraviolet

LIST OF APPENDICES

CHAPTER ONE

INTRODUCTION

1.1 Background information

Diesel engines are extensively used because of their efficiency and cost effectiveness. Their efficacy is due to high compression within the engine which generates heat that causes spontaneous ignition of the fuel. Diesel-powered engines are commonly used in cars, trucks, buses, locomotives, ships, agricultural machinery and electrical power generators [Robert, 2001].

The ever-growing fuel demand and pollution resulting from automobile and industrial emissions have made biodiesel to be viewed as an important substitute for petrodiesel. Since the global fossil fuel sources are finite, the quest for alternative fuel is presently going on, all over the world. The erratic and mostly ever rising cost of petroleum fuels, eminent depletion of reserves and strict world protocols on emissions from internal combustion engines have also generated the necessity for replacement of petroleum fuels with alternative fuels that are less polluting, readily available and non-edible renewable sources of fuels for automobile and industrial engines (Sahoo and Das, 2009).

Strict world treaties on controlling levels of exhaust emissions such as the Copenhagen Summit of December, 2009 agreed to keep the global rise in temperature at a maximum of 2 ^{o}C this 21st century. This has made many countries to come up with specific measures, aimed at reducing the levels of certain contaminants from internal combustion engines. The European Union (EU) has given each member state a target of at least 10% of transport fuel to be obtained from renewable sources by 2020 [Smith, 2015].

The incessant recognition of shrinking petroleum fuel reserves coupled with the possibility of using readily available low cost non-edible and waste oils as sources of environment-friendly renewable substitutes have created great interest in biodiesel research [Agarwal and Das, 2001]. Biodiesel, which is composed of alkyl esters of fatty acids, are usually prepared using animal fats and plant or restaurant waste oils [Canakci and Van Gerpen, 2001]. Base-catalysed transesterification reactions employing potassium hydroxide (KOH) or sodium hydroxide (NaOH) are extensively employed for commercial preparation of biodiesel because of their fast rates. These reactions are however, adversely affected by free fatty acids (FFAs) and presence of water [Van Gerpen *et al*., 2004]. The fatty acid methyl esters (FAME), which are the widely utilized biodiesel, are normally produced by transesterification of oils or fats using methanol and alkaline catalyst [Upadhyay and Sharma, 2013].

The widespread use of fuels derived from crude oil has caused a drop in world's petroleum deposits. Since global fuel requirement is constantly escalating, the growing demand can be appropriately met by finding alternative sustainable and environment-friendly sources of fuels [Taher *et al.*, 2011]. The insufficiency of traditional fossil fuel reserves, growing concern of pollutants emitted from combustion reactions and the rising costs is making alternative green energy sources very attractive. The limited fossil fuel reserves which are located in a few regions of the world, most of which are unstable politically, are also on the brink of attaining their highest production [Dermibas, 2008]. Kenya is heavily dependent on fossil fuels for its transport and industrial sectors. According to Kenya's Economic Survey of 2011, the use of fuels derived from crude oil in the Country increased from 2.9 million Tons of Oil Equivalent (TOE) in 2004 to 3.6 million TOE in 2009 [Ministry of Energy, 2012].

Over a century ago, Rudolf Diesel demonstrated the appropriateness of edible vegetable oil as a fuel in diesel engine. However, since the edible vegetable oils were relatively more expensive as compared to petroleum fuels, the concept of using them as alternative fuels were shelved. With increased concerns about future supply of petroleum fuel and continued rising cost, the use of biodiesel has attracted much attention [Taher *et al.*, 2011]. Azam *et al.*, [2005] explored the prospects of using FAME of 26 non-conventional plant oils as potential biodiesel and found that they met all the main specifications of biodiesel to be used in diesel engine. Subramanian *et al.*, [2005] reported the availability of more than 300 dissimilar types of plants that yield oil-bearing seeds. Thus, there is considerable amount of feedstock that can be used for preparation of biodiesel. Some of the plants that yields non-edible oil bearing seeds such as *Jatropha carcus* grow readily in marginal (Arid and Semi-Arid Lands, ASAL) and wastelands where there is no likelihood of land use contending with food production. Pant *et al.*, [2006] reported the variation of oil content from Jatropha trees based on the species, climatic conditions and altitude where they are grown.

Despite ready availability of large quantities of non-edible plant and waste oils such as Croton, kitchen waste and Nile perch viscera oil that can be used as sources of biodiesel feedstocks in many developing countries such as Kenya, no intensive effort has been made to prepare and use biodiesel on large scale. However, some of oil feedstocks possess high FFA content which makes the widely employed alkali-catalyzed transesterification reactions inappropriate. Optimum conditions for conversion of each specific non-edible oil feedstock with high FFA should therefore be established using the two-stage chemical and enzymatic processes that may be employed in saleable production of biodiesel. In this study, optimum conditions for preparing biodiesel from Croton oil using two-stage chemical and enzymatic processes were investigated.

The physicochemical properties and performance characteristics of Croton biodiesel were also tested and compared with recommended ASTM values and those of petrodiesel.

1.2 Biodiesel

Biodiesel is an unconventional fuel that can be prepared from plant or animal feedstocks. Biodiesel is attracting much consideration globally either for blending purposes or direct substitute for conventional diesel in compression ignition (CI) engines [Dermibas, 2009]. The American standard specification (ASTM 67-02) describes biodiesel as a fuel composed of methyl or ethyl esters of fatty acids derived from renewable plant oil or animal fat feedstocks. The European Union Specification (EN14214) also accepts this definition [Minodora *et. al.*, 2010]. Biodiesel may be prepared from edible, non-edible and waste plant or animal oils or fats. Owing to diminishing world's crude oil deposits, and food security issues, non-edible plant and waste oils have attracted increased interest as possible sources of raw materials for production of alternative fuel. Some of the processes that have been developed for biodiesel production involve reaction of the oils with alcohol under supercritical temperatures or in the presence of chemical or enzyme catalysts [Kumari *et al.*, 2009].

Biodiesel is mainly produced on a large scale by reacting animal fats or vegetable oils with alcohols, mainly methanol, to provide fatty acid methyl esters (FAME). A catalyst like potassium or sodium hydroxide is usually used and glycerol is produced as a by-product [Van Gerpen *et al.*, 2004]. Biodiesel can work in any internal CI engine either in its pure form or blended in various proportions with petrodiesel since they have similar characteristics [Dermibas, 2009]. Biodiesel has become quite important owing to great demand on the current fossil fuel reserves and its lower emission of hazardous gases as compared to petrodiesel fuelled engines [Park *et al*., 2008]. Biodiesel made from non-edible oils, restaurant waste and animal fats can therefore be used as an

alternative low-cost fuel which is globally acceptable and economically competitive [Modi *et al*., 2007].

Biodiesel is at the moment regarded as an important substitute fuel and is being seen as a suitable alternative to petrodiesel. Some of the advantages of biodiesel in comparison to petrodiesel includes non-toxicity, biodegradability, renewable, non-contribution to global warming, has negligible content of aromatic compounds and sulfur, higher cetane number and flash point [Taher *et al.,* 2011]. Additional profits of biodiesel include improved lubricating abilities and reduced hazardous exhaust emissions in comparison to petrodiesel [Dermibas, 2008].

The use of agricultural crops such as sunflower oil, safflower oil, corn oil, coconut oil palm oil, and soyabean oil as sources of biodiesel feedstocks, however, contends with their use as food and can therefore not effectively fulfil the world's requirement for biodiesel. The direct use of vegetable or plant oils also has some negative effects, such as increased viscosity and low volatility, which may result in partial ignition in CI engines, and hence deposition of carbon. Extensive work has been done to come up with derivatives of biodiesel that have properties resembling those of petrodiesel [Taher *et al.,* 2011]. This project targeted the use of non-edible plant seed oil, *Croton megalocarpu*s for biodiesel production using enzyme and two-stage process.

Schumacher *et al.*, [2001] stated that the use of biodiesel in automobile engines leads to 4, 47 and 66% decline in total hydrocarbon (HC), CO and smoke or particulate matter (PM) emissions respectively, while Dermibas, [2008] reported a reduction of 42 and 55% in CO and PM emissions correspondingly, in comparison to petrodiesel. The observations were explained by the presence of oxygen in biodiesel molecules and their higher cetane number. Although biodiesel

environmental considerations with regards to emissions are very positive, its use normally leads to higher emission of nitrogen oxides. Reports have, however, shown that the levels of NO_x emissions for biodiesel fuelled engines can be reduced by modifying injection timing and combustion temperatures [Taher *et al.*, 2011].

1.3 Glycerol

Glycerol is an odourless, colourless, viscous and simple polyol liquid compound that is extensively employed in pharmaceutical formulations. Glycerol contains three -OH groups that are accountable for its ability in to dissolve in water and absorb moisture. The glycerol backbone is found in all lipids called triglycerides [University of Minnesota, 2011]. Crude glycerol is the main primary by-product during transesterification process in a biodiesel plant, accounting for about 10% (w/w) of the biodiesel product. The rapid growth of large-scale biodiesel production around the world is expected to create a huge surplus of glycerol in the market, significantly reduce its price and lead to closure of many traditional glycerol production plants [Nanda *et al.*, 2014].

Glycerol can be removed from the mixture of transesterification products by using a special enzyme that breaks down phytol and starch. The effective separation and purification of the co-product glycerine can greatly improve the purity of biodiesel and lower the production cost [University of Minnesota, 2011]. Many techniques for purifying glycerol such as ion exchange resin, nano-cavitation technology, membrane separation technology (MST), simple distillation under reduced pressure, acidification, followed by neutralization and solvent extraction have been developed [Nanda *et al*., 2014]. The use of traditional techniques of distillation and ion exchange to purify glycerol are limited due the cost involved and lower yields which make glycerol relatively expensive to generate. Recent combination treatment techniques which produce large

quantities of glycerol-rich layers that require simple processes to give high yields of glycerol at low operational costs have been developed [Saifuddin *et al.*, 2014].

High purity glycerol is an important raw material for various applications in food, cosmetic and pharmaceutical industries. Glycerol may be ingested to aid in weight loss, improve exercise capability, assist the body replenish water lost as a result of diarrhoea and vomiting as well as reduce pressure inside the eye for people suffering from glaucoma. Similarly, glycerol can be used by athletes to minimize dehydration. Health workers occasionally administer glycerol intravenously to lower pressure inside the brain in illnesses such as Reye's syndrome, Menengitis, stroke, central nervous system trauma, cerebri, pseudotumor, tumor and encephalitis. Glycerol is also administered to reduce brain volume during neurosurgical processes and treatment of loss of consciousness on prolonged standing as a result of reduced blood flow to the brain [University of Minnesota, 2011].

1.4 Fatty acids

Fatty acids or fats are carboxylic esters derived from the alcohol, glycerol and are commonly referred to as triglycerides (TG). Since each fat is composed of glycerides from different carboxylic acids with proportions varying from one fat to the other, fats from different sources, have their characteristic composition. Except for a few cases, all fats are straight chain molecules ranging from three to eighteen carbons. Apart from the fats with three and five carbon atoms, most naturally occurring fats have an even number of carbon chains. The even numbers are a result of the usual biosynthesis of fats from acetate units, in stages that nearly resemble malonic ester synthesis. Both unsaturated and saturated fatty acids, having one or more double bonds per molecule occur naturally [Morrison and Boyd, 1987].

The major component of oils and fats are triglycerides which consist of a glycerol backbone in which the hydroxyl groups are substituted by fatty acid radicals. The different fatty acid units linked to the glycerol backbone regulates the properties of the specific glyceride [Canakci and Van Gerpen, 2001]. Fatty acids are carboxylic acids with long aliphatic tails which are either saturated or unsaturated [IUPAC, 2007]. Fatty acids are either derived from triglycerides or phospholipids. Fatty acids that are not directly attached to other molecules are known as free fatty acids (FFA). The fatty acids are very significant sources of energy in living organisms since they produce large amounts of adenosine triphosphate (ATP) on metabolism. Majority of cells in living organisms use either glucose or fatty acids for this purpose. Heart and skeletal muscle particularly prefer fatty acids [Campbell and Farell, 2006].

1.5 Statement of the problem

The widespread use of fossil fuels, especially in highly industrialized cities has lead to a build up of hazardous fumes such as oxides of nitrogen and sulphur. The threat of global warming and climate change caused by internal combustion engine emissions highlight the energy crisis and challenges facing the world today. The build-up of pollutant gases can lead to low air quality which can either result in human health problems or cause adverse effects on lives of microorganisms and animals. The emergence of newly industrialized countries, limited discovery of new crude oil reserves and inadequate supply of fossil fuels has also caused increased global demand for fossil fuels and unpredictable inflation. Widespread use of biodiesel which is biodegradable can both reduce the demand of fossil fuels and mitigate pollution problems caused by spillage of crude oil and refined fuels on land and water bodies.

The issues cited above provide adequate reasons and incentives to find alternative sources of renewable energy from non-edible and waste plant and animal oils. Biodiesel can play a vital role

as a substitute fuel that can enormously reduce the pollution effects, lower the demand and stabilize price of fuels. This study focussed on enzymatic and two-stage chemical preparation of biodiesel from *Croton megalocarpu*s oil.

1.6 Objectives of the study

1.6.1 General Objective

The general objective of this study was to establish optimum conditions for production of biodiesel from *Croton megalocarpu*s oil using two-stage chemical process and enzyme-based technologies and to determine characteristics of the biodiesel.

1.6.2 Specific Objectives

The specific objectives of this study were:

i) To determine optimum conditions for two-stage transesterification of *Croton megalocarpus* seed oil.

ii) To develop an enzymatic procedure for transesterification of *Croton megalocarpus* oil and characterize the biodiesel produced.

iii) To determine properties of Croton biodiesel and blends and compare engine performance, emissions and combustion characteristics of biodiesel blends with petrodiesel.

iv) To determine the effects of antioxidants and storage on oxidation stability of Croton biodiesel.

1.7 Justification and significance of the study

1.7.1 Justification of study

The increased industrialization and world population has resulted in extra energy demands. However, due to the limited discovery of new fossil fuel reserves, the increased demands has led to an unpredictable increase in oil price as well as inflation in the price of basic commodities. The rate at which fossil fuels are currently being used is more rapid than they can be renewed and this has led to fears of their depletion and unsustainability. The emergence of newly industrialized nations and increased world population has also led to excessive emission of pollutants into the environment that cannot be readily assimilated. Therefore, it is important to come up with alternative renewable environment-friendly sources that can be sustainable on a long term basis. The increased hazardous emissions from internal combustion engines and corresponding environmental problems can be mitigated by substituting fossil fuels with green renewable energy sources. Production of biodiesel from non-edible sources such as Croton does not pose food security issues as compared to food sources such as soy bean and vegetable oils.

1.7.2 Significance of study

Petroleum-derived diesel is the most commonly used source of energy in the transport sector as well as domestic and commercial engine driven appliances such as generators. The attainment of Kenya's vision 2030 of becoming a newly industrialized, middle-income nation will result in increased demand and consumption of fuel. The establishment of large-scale production and use of biodiesel from locally available non-edible feedstocks can lead to huge savings in foreign exchange, reduce emissions of certain harmful gases, improve rural income through diversification of rural economies, contribute to conservation of ecosystem, improve biodiversity conservation efforts and ensure environmental sustainability. Consequently, this will also result

in increased fuel supply, provide energy security and avert fuel crises that might disrupt economic growth targets and increase inflation.

Since enzymes are very precise in the reactions they catalyse, the development of low-cost enzyme-based technologies for commercial preparation of biodiesel can greatly improve the yield. The employment of immobilized commercial lipases or those isolated from readily available microorganisms for commercial preparation of biodiesel from non-edible and waste oils and fats may help in lowering the overall price of biodiesel. Additionally, isolated lipases can also be used in the treatment of waste oil effluents from oil processing industries, automobile garages and hotel kitchen wastes. In this study, the two-stage preparation of biodiesel and ability of commercial lipase from *T. lanuginosus* to catalyse methanolysis of Croton oil was investigated. This project also evaluated the performance, emission and physicochemical characteristics as well as the impacts of antioxidants on storage and oxidation stability of *Croton megalocarpu*s biodiesel.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Croton megalocarpus*

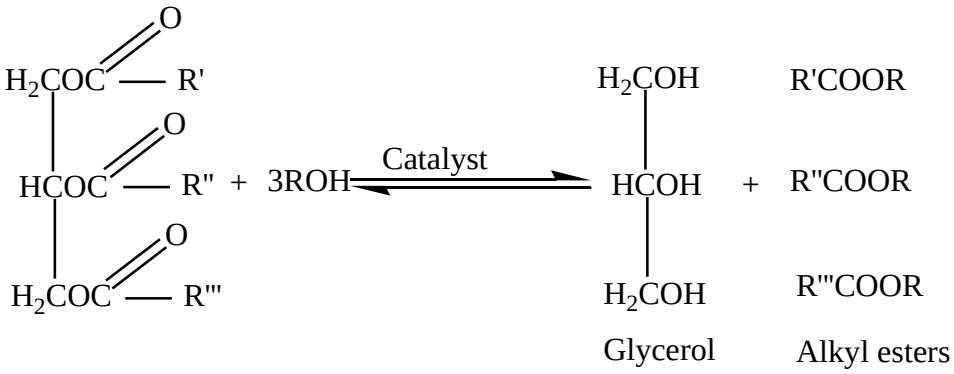
Croton megalocarpus Hutch. belongs to the family *Euphorbiaceae*. Since the plant grows readily in many parts of East Africa at altitude ranges of 4,000 to 6,700 feet, it is commonly used as a shade tree in coffee plantations and homes. It may reach a height of 120 feet, with a clear cylindrical pole measuring between 40 to 60 feet in length and trunk diameters of 2 to 4 feet. The plant may start bearing fruits at 3 years and matures at about 11 years (Bolza and Keating, 1972). Croton oil can be easily extracted from its dry nuts using a mechanical pressing machine to obtain straight vegetable oil (SVO) which can be used for biodiesel preparation while the press cake may be used in making chicken feeds or for biogas production. In Kenya, the plant is widely distributed in many forests, farms and homes.

2.2 Transesterification

Transesterification is the conversion of large triglycerides (TGs) into short chain alkyl ester molecules whose size and physical properties are similar to those of petrodiesel (Sinha *et al.*, 2008). Although every molecule of a triglyceride stoichiometrically needs three molecules of alcohol for complete reaction, surplus alcohol is normally added to alter the equilibrium of the reversible reaction and improve the yield of biodiesel. The mole ratio employed depends on the oil feedstock, catalyst used, reaction time and temperature. Some of the most widely used alcohols for transesterification include methanol, ethanol and propanol. The yield of biodiesel does not depend on the nature of alcohol used and the choice is usually based on the cost [Sharma *et al.*, 2008].

Excessive amount of alcohol causes increased emulsification and makes separation of glycerol quite challenging. The most suitable oil: alcohol ratio must therefore, be determined experimentally for each oil feedstock [Christie, 1989]. During transesterification, the ester bonds are first broken then followed by cleavage of hydroxyl bond. However, in esterification process, hydroxyl bonds are broken before the ester bonds resulting in the formation of glycerol as a co-product in transesterification and water in esterification [Al-Zuhair *et al.*, 2007].

Transesterification can be carried out using different catalysts such as alkali, acid or enzymes (lipases) which promote hydrolysis of triglycerides to fatty acid alkyl esters (FAAE) and glycerol. The biodiesel is then purified from glycerol through a separation step that involves washing with warm water a number of times (Taher *et al.*, 2011). The general chemical equation for transesterification can be represented as shown in Reaction equation 2.1:



Reaction equation 2.1 Balanced General equation for transesterification

2.2.1 Kinetics and mechanism of transesterification

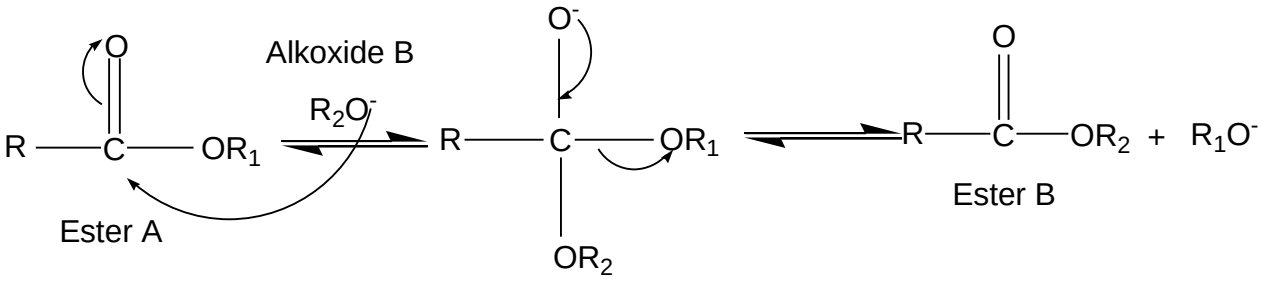
Transesterification of triglycerides involves two-step mechanism that proceeds through hydrolysis of the ester units to liberate glycerol and fatty acids, followed by esterification process using an alcohol such as methanol. The mechanism for transesterification of triglycerides that is

widely accepted is the ping-pong bi-bi, where each product is formed upon the addition of reagents, although a simplified Michaelis-Menten kinetics is applied when showing the formation of the products [Minodora *et al*., 2010]. However, none of the models used explicitly explains the formation of the mono- and diglycerides, the effects of temperature on deactivation of enzymes or equilibrium limitation for the conversion [Fjerbaek *et al*., 2009].

2.2.2 Alkali-catalysed transesterification

In alkali catalysed transesterification of vegetable oils, the alkali catalyst is dissolved in alcohol and the solution mixed with oil feedstock in a biodiesel reactor with vigorous stirring for 1 to 2 hours at about 340K [Dermibas, 2009]. A positive transesterification reaction yields two separate layers, ester and crude glycerol [Dermibas, 2008]. Alkali-catalysed transesterifications proceed much quicker as compared to acid-catalyzed reactions. The first stage in base catalysed reaction involves formation of an alkoxide and a protonated catalyst as a result of the reaction between the base and alcohol. This is followed by the nucleophilic attack of the alkoxide at the carbonyl group of the triglyceride to produce an intermediate. Subsequently, the alkyl ester and the respective anion of the diglyceride are formed from the intermediate. The diglyceride and monoglyceride intermediates are eventually transformed through a similar process into a combination of FAAE's and glycerol [Schuchardt *et al.*, 1998].

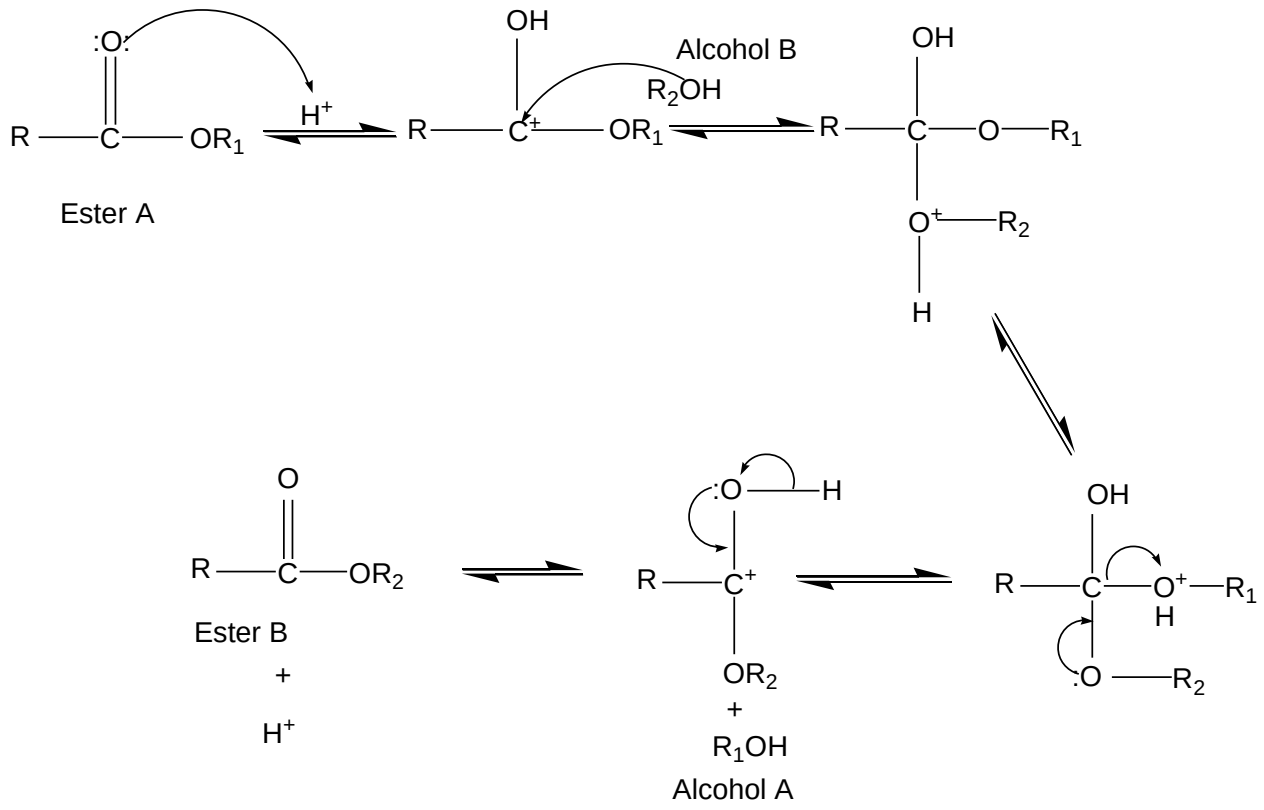
Alkaline metal alkoxides like $CH_3O^-Na^+$ are quite effective in improving the rates of transesterification reactions and normally give excellent yields (>98%) within a short duration of less than 1 hour even at marginal quantities (0.5 mol%). However, they require complete dearth of water which renders them unattractive for usual commercial procedures where complete elimination of water translates to higher cost [Schuchardt *et al.*, 1998]. Reaction equation 2.2 shows the mechanism for alkali catalysed transesterification.



Reaction equation 2.2: Mechanism of alkali-catalysed transesterification of vegetable oils.

2.2.3 Acid-catalysed transesterification

The most commonly used acid catalysts include sulphuric, hydrochloric and sulfonic acids. The catalyst is dissolved into methanol under vigorous stirring then mixed with oil in a biodiesel reactor [Dermibas, 2008]. Although acid catalysts produce very high yields of FAAEs, the reactions rates are relatively slow. One of the major issues that affect the rate of transesterification is the alcohol: vegetable oil mole ratio. The ideal alcohol: oil ratio however, must be determined experimentally, based on every reaction [Christie, 1989]. Reaction equation 2.3 shows the mechanism of acid-catalysed transesterification of vegetable oils.



Reaction equation 2.3 Mechanism of acid-catalysed transesterification of vegetable oils.

2.2.4 Lipase-catalysed transesterification

Lipases can be used to catalyse transesterification of vegetable oils to FAAEs, which are valuable intermediates in oleochemical studies and very good alternatives to diesel fuel [Gupta *et al*., 2011]. Studies on transesterification of *Jatropha carcus* oil in solvent free systems using lipases from different sources such as porcine pancreas, *Candida rugosa* and C*hromobacterium viscosum*, have shown that only lipase from *C. viscosum* gives appreciable yield of biodiesel [Shah *et al.*, 2014]. Immobilization of lipase from *C. viscosum* on celite-545 increased the biodiesel yield to 71% from the 62% achieved when free tuned enzyme preparation was used at a temperature of 113K and 8 hours reaction time [Dermibas, 2008]. Enzyme (lipase) catalysed

transesterification of oils is a better substitute to chemically catalysed reactions due to its lower temperature requirements environmental friendly and precise nature [Kumari *et al.*, 2009].

Although lipase-catalysed transesterification processes have not been commercially developed, recent articles and patents have shown that the process is feasible. The effective use of enzymes in transesterification requires appropriate optimization of reaction parameters such as solvent used, pH, temperature and nature of microorganism which produces the lipase in order to identify the right conditions for a manufacturing procedure. Currently, yields obtained from enzyme catalysed reactions are still quite low compared to base-catalysed systems [Mudge and Pereira, 1999]. Since the hydrolytic enzymes such as lipases are readily availability and can be easily handled, they are widely used organic synthesis [Dermibas, 2008].

Some of the advantages of enzymatic transesterification includes formation of products with high purity and low reaction temperatures of between 35 to 45 ^{o}C [De Paola *et al,* 2009]. In contrast to chemical catalysis, the use of enzymes does not result in the formation of soaps and they normally catalyse esterification of FFA and TG in one step. The major disadvantages of enzymatic transesterification are high cost of commercial enzymes, slower rate of reaction and possibility of enzyme deactivation by methanol [Robles-Medina *et al.*, 2009]. Transesterification of oils using methanol gives poor yields due to its severe harmful impact on activity of enzymes. A mole ratio of methanol to oil above 1:1 normally results in austere deactivation of the activity of most enzymes. This problem can however, be overcome by substituting methanol with methyl acetate as the acyl acceptor. This normally results to higher yields of up to 92% methyl ester at a mole ratio of methyl acetate: oil of 12:1. Methyl acetate does not display any negative effect on enzymatic activity. Moreover, the co-product obtained, triacetylglycerol is an essential reagent with a greater worth than glycerol [Du *et al*., 2004].

2.2.5 Kinetics of lipase-catalysed transesterification

Kinetic investigations on lipase-catalysed transesterification using short chain alcohol has shown that only alcohol inhibition affects the rate of the reaction which can be described by a Ping-Pong kinetic model with competitive inhibition by the alcohol as shown in equation 2.1.

$$\upsilon = \frac{\mathrm{Vmax}}{1+\frac{\mathrm{KA}}{[\mathrm{A}\}}\left(1+\frac{[\mathrm{B}]}{\mathrm{KiB}}\right)+\frac{\mathrm{KB}}{[\mathrm{B}]}} \qquad \text{(Equation 2.1)}$$

where υ is the initial speed of reaction, V_{max} the maximum speed of reaction, K_A and K_B are the binding constants for the fatty acid, A and the alcohol, B, and K_{iB} is the inhibition constant for the alcohol. Although the obstruction effect of alcohol is generally accepted, the impact of concentration of fatty acid and amount of water in mixture are still under dispute [Al-Zuhair *et al*., 2007]. Krishna and Karanth, [2001] suggested that in micro-aqueous media, both fatty acids and alcohol produce an inhibition effect, particularly in reactions where short fatty acid chains are involved. If fatty acid inhibition is taken into consideration, equation 2.1 is transformed into equation 2.2.

$$\upsilon = \frac{\mathrm{Vmax}}{1+\frac{\mathrm{KA}}{[\mathrm{A}]}\left(1+\frac{[\mathrm{B}]}{\mathrm{KiB}}\right)+\frac{\mathrm{KB}}{[\mathrm{B}\}}\left(1+\frac{[\mathrm{A}]}{\mathrm{KiA}}\right)} \qquad \text{(Equation 2.2)}$$

where, K_{iA} is the inhibition constant for the acid while other representations remain identical to those in equation 2.1. In case there is no fatty acid inhibition, the term $[A]/K_{iA}$ becomes negligible and equation 2.2 transforms back to equation 2.1, with only alcohol inhibition. This assumption was confirmed by Krishna and Karanth, [2001] from experimental results of esterification of butyric acid with methanol using immobilized lipase from *Rhizomucor miehei* in *n*-hexane micro-aqueous media. The findings showed that preliminary speed of reaction reduced as the

concentration of butyric acid was increased from 0.5 to 4M at various methanol concentrations. A comparable observation was stated by Yadav and Lathi, [2004] on the esterification of lauric acid with cintronellol in the presence of Novozyme SP 435 in *n*-hexane micro-aqueous media.

2.2.6 Oxidation of biodiesel

Although many reports have shown that engines fuelled with biodiesel are causes slighter pollution than petrodiesel, biodiesel readily undergoes oxidation. The oxidation leads to increased acidity and formation of insoluble gums and sediments that can block fuel filters, corrode or damage engines [Monyem & Van Gerpen, 2001, Osawa *et al.*, 2015]. Biodiesels from many feedstocks and their corresponding blends are more vulnerable to oxidation than petrodiesel unless treated with antioxidants. Hence, biodiesel dealers are anxious that it can develop dregs during storage while machine operatives fear that dregs and gum may develop in the course of their use and destroy engines [McCormick *et al*., 2007].

The vulnerability of biodiesel to oxidation during storage is as a result of different levels of unsaturation [Karavalakis *et al*., 2010]. The rate at which biodiesel undergoes oxidation is dependent on both the number of double bonds and their locations within the molecule [Abderrahim *et al*., 2007]. Oxygen readily attaches itself to the bis-allylic site and starts autoxidation chain reaction sequence. Thus, the oxidative stability of lipids such as biodiesel is largely influenced by the number of bis-allylic sites in the unsaturated compound [Shah *et al*., 2009b]. The overall oxidation stability of biodiesel is also influenced by conformational cis-trans isomerization. Despite the higher stability of trans-unsaturation as compared to the cis-unsaturation, conjugated trans-unsaturations are more delicate to oxidation as compared to cis-unsaturations [Fang and McCormick, 2006, Osawa *et al.*, 2015].

Biodiesel oxidation is instigated by production of radicals at bis-allylic sites which readily isomerize to form stable intermediate compounds that reacts with oxygen to form peroxides [Devinder *et al*., 2009, Osawa *et al*., 2015]. The fatty acid alkyl esters (biodiesel) are then destroyed and the secondary oxidation products formed include carbonyl compunds, acids with low molecular mass and volatile organic compounds. The oxidation products finally polymerize to form a sludge which makes the fuel inappropriate for use and can damage engines [Sendzikiene *et al*., 2005]. Oxidation of lipids such as biodiesel, therefore, decreases their quality by forming hazardous secondary products during storage [Frankel, 1980]. Some of the factors that determines the rates of oxidation of biodiesel include, temperature, light, radiation intensity and availability of natural antioxidants. Antioxidants, however, bind free radicals hence stabilizing fatty peroxyradicals and stopping oxidation chain reactions [Shah *et al*., 2009b]. The duration over which lipid-containing compounds such as biodiesel and polyunsaturated compounds can be safely stored before undergoing oxidative deterioration is dependent on their chemical stability. However, lipids can also be defended against oxidation by addition of appropriate quantities of suitable synthetic or natural antioxidants straightaway after preparation [Mbatia *et al*., 2010, Osawa *et al*., 2015].

Terry *et al.*, [2006] stated that advanced extents of oxidation may make biodiesel blends to isolate into two layers which may create complications in the injector and fuel pump. Sarin *et al.*, [2010] observed that synthetic antioxidants were more potent in boosting the oxidation stability of *Jatropha* methyl ester than natural antioxidants. Hess *et al.*, [2005] reported that apart from boosting the oxidation stability of biodiesel, introduction of certain commercial antioxidants led to reduction in NO_x engine emissions. Karavalakis *et al.*, [2010] reported a sharp decrease in fuel stability of a commercial biodiesel over a ten week storage period. They observed that addition of

antioxidants greatly boosted the stability of biodiesel. Kivevele *et al.*, [2011a] reported that, although addition of antioxidants greatly improved the oxidative stability of Croton biodiesel, it had little effects on engine exhaust emissions.

The most commonly used synthetic antioxidants in cooking oils to prevent oxidation are essentially phenols such as Butylated hydroxyanisole, *tert*-Butyl hydroquinone, Butylated hydroxytoluene, Propyl gallate and pyrogallol [Marie-Elizabeth *et al.*, 1992]. The synthetic phenols disrupt the free-radical oxidative chain reactions by donating hydrogen from the phenolic hydroxyl groups thereby producing stable free radicals that impedes oxidation of lipids. It is therefore important to add the phenolic antioxidants as early as possible in the finished product or during the manufacturing process since they neither have the ability to converse the oxidation of the oils nor suppress hydrolytic oxidation which involves enzymatically catalyzed hydrolysis of fats [Anderson *et al*., 2007].

2.2.7 Antioxidants

An antioxidant is a substance that is added to oxidation prone compounds at low concentrations in order to delay or prevent oxidation of the compounds. Oxidative deterioration is a problem of great economic importance in the production and storage of lipid-containing compounds. Apart from producing offensive odours and flavours, oxidation of lipids also decreases its quality and safety owing to the formation of secondary reaction products during use or storage [Frankel, 1980]. Lipid-containing compounds such as biodiesel can be readily protected from oxidation by adding appropriate concentrations of natural or synthetic antioxidants. [Mbatia *et al.,* 2010].

Since recent reports have shown that commonly employed artificial antioxidants like butylated hydroxytoluene (BHT) are regarded as potential promoters of carcinogenesis, the employment of

natural phenolics as antioxidants in boosting the shelf life of lipid-containing commodities has attracted much interest [Stamatis *et al.,* 1999]. Most of the naturally occurring molecules with antioxidative impacts are acid-phenols or flavonoids and their corresponding esters [Cuvelier *et al.,* 1992]. The antioxidant capability of phenolic compounds depends on their capacity to rummage free radicals. Apart from their antioxidant effects, natural phenolics such as rutin are also linked with numerous health advantages like antimicrobial, anti-inflammatory, anti-tumour and anti-angiogenic activities [Foteini *et al.,* 2006].

The application of natural phenolics in lipid-based systems is, however, currently restricted because of their low solubility, which may reduce their capacity to inhibit oxidation of unsaturated fatty acids at oil-water or oil-air interfaces [Eric *et al.,* 2005]. Acylation of natural phenols with lipophilic moieties with a target of increasing hydrophobicity of phenolics has been employed as an approach of eliminating the drawback. Lyophilisation of natural phenols such as rutin and Vanillyl alcohol with fatty acids of different chain lengths has been stated [Viskupicova *et al.,* 2010, Kunduru *et al.,* 2011]. Linolenic acid (C18:3) esterified to rutin has been reported to protect fish oil and emulsion systems from oxidation [Viskupicova *et al.,* 2010].

2.3 Lipases

Lipases, which is a member of the group of hydrolytic enzymes called hydrolases, are enzymes that break down fats so that it can be digested and absorbed by organisms. Lipases, which can be readily isolated from microorganisms like bacteria, yeast and fungi can catalyse transesterification reactions of TGs and FFAs to esters. Lipase enzymes extracted from *Mucor miehei, Rhizopus oryzae, Mucor miehei* and *Pseudomonas cepacia* have been widely used as enzymes in various processes [Fjerbaek *et al,* 2009]. In biological organisms, lipases hydrolyse

triglycerides to fatty acids and glycerol. Lipases operate in moderate situations and can catalyse transesterification of TGs from different sources [Marchetti *et al.*, 2007].

Lipases have vital biological functions and a vast industrial potential. Physiologically, lipases hydrolyse TGs into DGs, MGs, fatty acids and glycerol. Apart from their usual role of hydrolysing carboxylic ester bonds, lipases also catalyse the reverse esterification, amidation or transesterification reactions in aqueous and anhydrous organic solvents [Qian, 2007]. Lipases can catalyse diverse types of reactions owing to their broad substrate specificity, stability in organic solvents and usually high regio- and/or stereo-selectivity. These characteristics have made lipases to be some of the most preferred catalysts for many uses in industries and organic synthesis [Krishna and Karanth, 2002]. Some of the various uses of lipases comprise syntheses of organic compounds, hydrolysis of fats and oils, alteration of fats, improvement of food flavour, resolution of racemic mixtures, and chemical analyses [Sharma *et al*., 2001].

The interest in the use of lipases has developed recently owing to their outstanding catalytic abilities and various industrial uses such as supplements in detergents and dietetic foods. Additionally, lipases are widely employed in the preparation of bioactive molecules in medicine industry, chemical synthesis of pure optical compounds and alteration of fats and lipids by hydrolysis and esterification reactions [Falony *et al*., 2006]. Most lipases have different specificity for the triglycerides that they break down. The precision with which lipases catalyse substrates depends either on the position of the unsaturated group or fatty acid chain length [Bendikiene *et al.,* 2005]. The use of lipases can bring about new and exciting applications that can provide benefits in various industrial processes such as reduced environmental pollution, greater yield, enhancement of product's properties, lower production costs and energy consumption [Novozymes, 2008].

Both extracellular and intracellular lipases are widely used as biocatalysts [Ranganathan *et al.*, 2008]. Extracellular lipases are those that are readily extracted from the microorganism by centrifugation of the aqueous growth media, while intracellular lipases are the enzymes that remain inside the producing cell walls of microorganisms [Robles-Medina *et al.*, 2009]. Suitable lipases for transesterification should be non-specific so that it can transform different glycerides (tri-, di- and mono-) and free fatty acids of varying lengths found in natural lipids to alkyl esters. The yield of biodiesel in lipase catalysed reactions depends on factors such as the origin of lipase, its formulation, alcohol used, alcohol: oil ratio, reaction temperature, optimal water activity, reaction time and enzyme lifetime [Minodora *et al*., 2010].

2.4 Sources and production of lipases

Although many microbes and higher eukaryotes produce lipases, the most commonly used and economically viable are those extracted from microbial sources, especially bacterial and fungal. The microbes that generate lipases can be found in various surroundings like industrial wastes, vegetable oil and dairy refining plants, soil polluted with oil, oilseeds, decaying fatty foods, manure stacks and hot springs. Lipases are commonly extracted from microbes such as bacteria, fungi, yeasts and actinomyces. The microbial lipases can be generated by either solid-state or submerged culture fermentation methods [Sharma *et al*., 2001].

In solid-state fermentation (SSF), the microorganisms are grown on moist insoluble porous substance that serves both as a backing as well as source of food in the absence of free flowing water. The water and nutrients present on the solid substrate support the growth of microorganisms which secrete useful enzymes [Kumar and Kanwar, 2012]. Submerged fermentation (SmF) on the other hand involves growing the microorganisms in aqueous media. Some of the advantages of SSF over SmF include the efficient utilization of room needed for

fermentation, simplification of fermentation media, higher yields and non-prerequisite of intricate equipment. Despite these advantages, SSF has some drawbacks such as limited types of microorganisms that can grow under the controlled situations and difficulty in controlling and monitoring growth variables like humidity, pH, air flow rate and temperature [Falony *et al*., 2006].

2.4.1 *Thermomyces lanuginosus*

Thermomyces lanuginosus, formally referred to as *Humicola lanuginosa* is a fungus that can be readily isolated from decaying plant matter. Various species of the fungus have been shown to give high levels of L-xylanase both in shake-flask and bioreactor preparations. The gene encoding *T. lanuginosus* xylanase has been cloned and sequenced and has been found to be a member of family 11 glycosyl hydrolases. The ability of *T. lanuginosus* to liberate high yields of cellulase-free stable xylanase has made it a very appealing source of thermostable xylanase which has a great prospect as a blanch-improving agent in pulp and paper industry and as a supplement for the baking industry [Singh *et al*., 2003].

The lipase from *T. laguginosus* is a basophilic and relatively thermostable enzyme which is available in the market in both solution and immobilized versions. Although this enzyme was originally commonly used in the food industry, it is currently widely used in various applications such as biodiesel production, alteration of fats and oils, resolution of racemic mixtures, enantioselective hydrolysis of pro-chiral esters and regio-selective procedures involving sugar preparations [Fernandez-Lafuente, 2010].

2.5 Immobilization of lipase

Enzymes naturally perform their biological functions in aqueous solutions. There is however, great applicability for non-aqueous enzymology in synthesis as shown by recently reported research findings. Immobilization of lipases has therefore become an invaluable technique for improving the activity of enzymes in organic solvents [Kumar and Kanwar, 2011]. Immobilization of enzymes can be performed in various ways, which can be categorized into physical and chemical methods. Various immobilization techniques are normally used in biodiesel enzymatic production [Taher *et al.*, 2011].

Du *et al.*, [2004] employed adsorption on macro porous resin, Noureddini *et al.*, [2005] operated with hydrophobic sol-gel backing by entrapment while Orcaire *et al.*, [2006] worked on silica aerogel by encapsulation. Other common techniques used in immobilization of enzymes include the use of a silica matrix activated with ethanolamine then cross-linked with 1% glutaraldehyde as well as the use of solvents which can reduce the adverse effects caused by the alcohol reagents and glycerol produced during the reaction [Kumari *et al.*, 2009].

Immobilized Biocatalysts promote enzymatic conversions in organic solvents and has more advantages as compared to reactions done in aqueous systems. Organic based reaction systems possess a number of benefits such as increased solubility of organic compounds in non-aqueous media, possibility of performing novel reactions that cannot be performed in water-based systems due to kinetic or thermodynamic constraints, higher stability of enzymes and comparative simplicity of separating product from organic solvents than water [Hirakawa *et al.*, 2005].

Immobilized lipases have been reported to provide effectual ways of achieving hydrolysis/esterification, easy separation of end product(s) and potent separation of biocatalyst

for diverse enzymatic reactions. Since a large number of proteins show poor solubility in organic solvents, it is therefore crucial to immobilize the enzyme onto an appropriate absorbent material that affords improved surface area, easy removal of catalyst and its recyle. From commercial considerations, immobilized biocatalysts can provide cost-effective inducements such as enhanced simplicity of handling, easy separation and recyle relative to those that are not immobilized [Kanwar *et al.*, 2004].

2.6 Chemical analysis

In this study, the final products were identified using a gas chromatograph coupled with a mass spectrometer (GC-MS). Thin layer chromatography (TLC) plates were used to determine the extent of transesterification of the plant oil. The working principles of GC and MS are briefly described in the preceding sub-sections.

2.6.1 Gas chromatography

In gas chromatography, the analyte is vaporized then injected onto the head of a chromatographic column. The analyte vapour is then eluted by an inert gaseous mobile phase such as argon, helium or nitrogen. Contrary to many other kinds of chromatography, no interaction occurs between the mobile phase and analyte particles. The only role of the mobile phase is to transfer the analyte along the column. The vaporized sample is transported by the carrier gas whose flow rate is measured by a rotometer [Vining, 2004]. The mobile phase is usually an inert gas while the stationary phase may be either a column packed with a solid of large surface area or a thin layer of liquid adhering to a suitable backing material through which a mobile phase is able to flow. As the moving stream of vaporized gaseous mixture of substances together with the carrier gas moves along the column, various sample components of the analyte are drawn at differently to the stationary phase and are therefore isolated. Gas chromatography is a small-scale, rapid

process of isolating and identifying components in a sample. It may also be used to analyse mixtures since the time taken by particular substances at a specific temperature to move through the column to the detector (retention time) is constant and can, therefore, be used in their identification [Ebbing and Gammon, 2009].

Gas-liquid chromatography is widely used for the separation of components that are adequately volatile. It is one of the most potent techniques for fast and expedient examination of components of mixtures of organic compounds. It is based on partitioning of the constituents between a mobile gas phase and the stationary liquid layer adsorbed on a suitable solid backing medium. For routine analytical work, the supporting medium impregnated with stationary phase is packed into a metal or glass column. The column is usually approximately 2 to 3 m long, 2 to 4 mm internal diameter. The column is usually in the form of a circular spiral of three or four turns and is located in a temperature controlled oven [Vogel, 1989].

The mobile phase enters the column at one end which also incorporates an injection port for the introduction of the sample. The components of the sample are carried through the column and emerge in turn into the detector which is attached at the end of the column. The separation of the components of the sample depends upon their partition co-efficient between the stationary and mobile phase. The signal from the detector is suitably amplified and fed to a pen recorder. The quantitative and qualitative composition of the mixture can be assessed from an examination of the graphical traces produced [Vogel, 1989]. The direct coupling of GC to MS provides a very powerful analytical tool to the organic chemist. In GC – MS systems, the components of the mixture emerging from the column pass straight into the MS for analysis [Arene & Kitwood, 1979].

2.6.2 Mass spectrometer

In a mass spectrometer (MS), organic molecules are first converted into singly charged positive gaseous ions. The beam of positively charged gaseous ions is accelerated by positively charged electrode then passed through a variable magnetic or electric fields in a high vacuum to deflect them according to their masses and cause their separation [Kemp, 1989]. Non-volatile samples are vaporized by injecting them into a heated chamber then ionized. The most commonly used methods of ionisation include electron ionisation, chemical ionisation, field desorption, plasma desorption, fast atom bombardment, electrospray ionisation and matrix-assisted laser desorption/ionisation [Przybylski *et al.*, 2003]. A suitable detector measures the relative abundance of each isotope present since the charge on the ions produces an electric current at the detector. The presence of more ions of the same mass in a sample results in a higher current and the corresponding peak observed. A mass spectrum shows a plot of relative abundance of the ions against the mass/charge ratio (m/z) [Kemp, 1989].

Recent developments in analytical techniques and progress in equipment improvement have made mass spectrometry play a central role in contemporary biopolymer examination. The developments have made it feasible to ascertain the relative formula masses of biomacromolecules at very high levels of accuracy [Przybylski *et al.*, 2003]. Complex mixtures of substances are normally analysed by coupling mass spectrometer to chromatographic instruments such as gas chromatograph (GC-MS) or a liquid chromatograph (LC-MS). The mass spectrometer is widely used in the analysis of both known and unknown compounds. In cases of known compounds, a computer search is usually conducted by comparing the spectrum of the compound being analysed with a library of mass spectra. In case of an unidentified substance, the

molecular ions, fragmentation trends and corroboration from other types of spectrometry such as IR and NMR can be incorporated to identify the new compound [Silverstein *et al.*, 2005].

2.7 Characteristics of diesel fuels

Diesel fuels produce power when atomized, mixed with air in the cylinder and burned at a high pressure in a compression ignition (CI) engine. For the fuel to burn effectively, it should undergo ignition at an appropriate time, release adequate energy on combustion and provide a large amount of energy output per unit volume. The diesel fuel should flow freely and burn readily even at low temperatures, be non-corrosive to the engine and contain no sediments that can plug filters or block orifices. The fuel should also not cause excessive pollution on burning, be relatively safe to handle in terms of flammability and toxicity and its viscosity should be specified within a fairly narrow range [Van Gerpen *et al.*,2004]. Some of the essential characteristics of diesel fuels are briefly described in the preceding sections

2.7.1 Saponification and acid values

The saponification value or number of a fatty acid or ester is the amount of KOH needed to neutralise one gram of the oil or fat. The saponification value is thus inversely proportionate to the relative formula masses of the fatty acids obtained from the esters. The knowledge of saponification value can be employed to determine the amount of alcohol required to react with the TG during transesterification reactions [Vogel, 1989]. The acid value is the amount of KOH needed to react completely with free fatty acids available in a plant or animal oil/fat. The acid value is normally expressed as mg KOH/g of the oil/fat or % FFA present in the TG. The acidity of biodiesel can cause poor fuel stability, corrosion of fuel tanks, fuel transmission pipes and engine cylinders. The acidity can also result in production of sediments in some types of fuel injection systems [http://www.ufa.com/petroleum/resources/fueel/diesel_fuel_resources.html].

2.7.2 Flash point

The flash point is the temperature at which the fuel forms an inflammable vapour combination with air at normal pressure. Since the flash point of diesel fuel is at least 55 ^{o}C, it is much safer to transport and store than petrol or kerosene whose flash points are -40 and 30 ^{o}C respectively [Stone, 1999]. Flash point can be established by heating up the fuel in equipment with a sealed chamber such as a Pensky cup apparatus until it produces a vapour that readily catches fire when a flame is placed over the liquid fuel. The flash point of a fuel does not have any effect on its operation in an engine or auto combustion abilities. However, it can provide a valuable indicator on the presence of pollutants, since the presence of small quantities of contaminants greatly affects its value. [http://www.ufa.com/petroleum/resources/fueel/diesel_fuel_resources.html].

Flash point is a very important measure in determining the safety requirements for transportation and storage of fuels. The value of flash point is used in the classification of fuels and other liquids during transport such as the Transportation of Dangerous Goods (TDG)) regulations. It is also used in setting safety measures needed in fuel handling and storage and requirement for insurance and fire regulations [http://www.ufa.com/petroleum/resources/fueel/diesel_fuel_resources.html].

2.7.3 Density

Density of a fuel is the ratio of its mass to volume at a particular temperature. Densities of fuels are usually determined at a standard temperature of 15 °C using a density meter. Knowledge of density can be used for determination of quantity calculations and evaluating ignition quality. The density of plant oils is contingent on their source and constitution. Generally, the density of plant oils is normally greater than that of their corresponding biodiesel and petrodiesel fuel [Lujaji *et al.*, 2010]. Density is a very important property of fuel, since the quantity of fuel that should be delivered into engine cylinders by injection systems such as pumps and injectors for

proper combustion in the engines is determined by their densities. Density values of biodiesel are also very essential in designing biodiesel production systems such as reactors, distillation units, separation process, storage tanks and process piping [Pratas *et al.*, 2011].

Suitable fuels should have adequate amounts of less dense and volatile components for easy starting of vehicles under cold environments and fast warm-up. The fuels should also possess denser constituents that provide power and fuel economy once the engine attains maximum operating temperature. The fuel density requirement depends on the size of engine, design, nature of speed, load variations, starting and atmospheric situations. Engines used in fast changing load and speed operations like buses and trucks may require less dense and more volatile fuels for improved operation, especially in relation to smoke emission. However, the use of denser fuels such as diesel usually results in better fuel economy since they have higher energy content than the less dense fuels [http://www.ufa.com/petroleum/resources/fueel/diesel_fuel_resources.html].

2.7.4 Kinematic viscosity

Viscosity is a measure of internal fuel friction which opposes dynamic changes in motion of fluid at a specific temperature. Although straight vegetable oils generally display high viscosity values, their corresponding biodiesel and mixtures with diesel, have similar viscosity values to diesel [Lujaji *et al.*, 2010]. Generally, different fluids have their specific temperature-viscosity relationship and since viscosity depends on temperature, all values of viscosity should be stated with corresponding values of temperature. Viscosity is measured using a viscometer, and the most commonly used unit is Stokes or Centistokes. Kinematic viscosity is a vital characteristic of fuels because it determines the extent of prior heating needed for handling, storage and sufficient atomization. Fuels that are highly viscous can be hard to pump, undergo poor atomization and

take more time to burn. Pre-heating is normally done to improve atomization, since insufficient atomization can lead to deposition of carbon on burner tips or cylinder walls [Shaha, 1974].

High viscosity fuel can damage engine or fuel pump system by increasing gear train and causing corrosion on the system due to greater injection force required. Fuels with high viscosity also atomize less efficiently and can, therefore, cause difficulty in starting the engine. Viscosity affects both atomization and rate of fuel delivery to the combustion chamber [https://www.ufa.com/petroleum/resources/fueel/diesel_fuel_resources.html]. The suitable ranges of kinematic viscosity are listed in different standards such as ASTM. Kinematic viscosity of diesel is commonly measured at 40 °C, as recommended in ASTM D445. The kinematic viscosity usually rises as the chain length of the organic liquids increases. The rise in kinematic viscosity with increase in carbon chain length is less significant in straight chain hydrocarbons than in compounds derived from fatty acids [Knothe and Steidley, 2005].

Although the kinematic viscosity of unsaturated fatty compounds is dependent on the nature and number of double bonds, the position of the double bond has little effect on viscosity. Terminal double bonds present in aliphatic hydrocarbons usually have relatively little viscosity-lowering impact. Studies done on the effect of branching in the alcohol structure has shown that it does not considerably influence viscosity as compared to the straight-chain analogues [Knothe and Steidley, 2005]. Fuels with viscosities greater than 5.5 centistokes at 40 °C are restricted to use in slow moving engines and can sometimes call for prior heating before injection. The minimum viscosity is normally specified for some engines to prevent power loss owing to injection pump and injector leakage. The highest viscosity of fuel is however determined by factors such as fuel temperature, features of injection structure, design and size of engine [https://www.ufa.com/petroleum/resources/fueel/diesel_fuel_resources.html].

2.7.5 Cetane number

Cetane (ignition) number indicates the ignition delay time of fuel in internal combustion engines and can therefore be used to evaluate the characteristics of fuel. The cetane rating of a fuel can be used to explain its ignition properties [Lujaji *et al*., 2010]. Fuels which have too low cetane numbers can lead to diesel knock due to very rapid combustion caused by long ignition delay periods. This is because ignition only takes place after particular limits of temperature and pressure have been attained for sufficient time while fuels with high cetane numbers readily undergo self-ignition. In the Cetane scale of 0 - 100, the value of 0 is assigned to methylnaphthalene ($C_{10}H_7CH_3$), with poor self-ignition quality while the value 100 is assigned to n-Cetane ($C_{16}H_{34}$) [Stone, 1999].

Fuels with high cetane numbers generally take short time intervals between their injection and combustion in the engine cylinders. Cetane number therefore, measures the ease with which a fuel ignites and is very important during start of vehicles at low temperature conditions, engine warm up, idling and smooth, uniform ignition. The cetane number of a fuel to be used in an engine is governed by its design, size, speed, load variations, starting and environment conditions. Cetane number can be determined in a single cylinder test engine with adjustable compression ratio. Cetane mixed with alphamethyl naphthalene in various proportions is used as standard fuels. Whereas cetane has a very short delay in ignition, alphamethyl naphthalene has a long ignition delay. The percentage of cetane in the reference fuel is defined as the cetane number of the test fuel [http://www.ufa.com/petroleum/resources/fueel/diesel_fuel_resources.html].

Fuels with low cetane number cause delay of combustion in the engine which leads to problems in starting engines and may result in engine knock. Delays in ignition also cause high consumption of fuel, power loss and eventually engine damage. Fuels with low cetane number

can result in production of white smoke from the exhaust as well as fuel odour during start-up in cold weather. The white smoke produced from the exhaust is composed of fuel vapours and carbonyl compounds formed due to incomplete ignition in the engine. The ignition delay is caused by insufficient heat in the combustion chamber for complete combustion. Cetane number can be improved by using supplements like hexyl nitrate or amyl nitrate. [http://www.ufa.com/petroleum/resources/fueel/diesel_fuel_resources.html].

2.7.6 Calorific value

The ability of fuel to undergo complete combustion is one of the main parameters used to determine its efficiency. The combustion process of the most commonly used organic fuels normally produce water vapour, whose energy content may be recovered by condensing it. Moisture, however, has a negative effect on the combustion efficiency and hence its level in the fuel should be as low as possible [Platace *et al.*, 2013]. Thermal studies are usually used to obtain details on combustion properties of fuels, such as calorific value. Calorific value can be defined as the amount energy liberated when fuel is burnt in an engine and is commonly determined using the bomb calorimeter. Biodiesel generally has lesser total calorific value than diesel and the blends [Lujaji *et al*., 2010].

2.7.7 Cloud and pour point

The cloud point (CP) is the temperature at which visible wax in diesel or biowax in biodiesel begins to form, giving the fuel a hazy look. Large crystals combine form clusters that finally become widespread enough to inhibit free flow of the fluid at lower temperatures than CP. If a fuel is used at temperatures below its CP, the crystals formed may plug filters or form sediments at the bottom of a storage tank. Cloud point thus provides an indicator of the tendency of the fuel

to plug filters or block miniature openings at low temperature working conditions [https://en.wikipedia.org/wiki/Cloud_point].

The pour point (PP) is the temperature at which the diesel fuel freezes to become a semi-solid and ceases to flow freely. The PP thus provides a measure of the least temperature at which the fuel may still be readily transferred in liquid state, before it turns into a gel. Fuels with high pour points are more difficult to use in areas or conditions where lower temperatures are involved because it must be kept warm by some methods such as electric heaters with insulated tanks [http://en.wikipedia.org/wiki/ Pour_point].

CP and PP are important properties of fuel since the presence of solid waxes can clog filters and adversely affect engine performance. A traditional manual laboratory method for measurement of cloud and pour points involves cooling the fuel and recording the temperature at suitable intervals of time at which visible particles begin to form, and the free flow of fuel ceases respectively. In regions with extreme cold weather conditions diesel fuels are usually blended with suitable additives to deliver vital energy and performance and eliminate challenges associated with low temperature [https://www.ufa.com/petroleum/resources/fueel/diesel_fuel_resources.html].

2.7.8 Cold filter plug point

Cold Filter Plug Point (CFPP) of a fuel refers to the temperature at which it cannot easily pass through a fuel strainer. CFPP lies nearly midway between the CP and PP. The effects of cold weather conditions on diesel operations depends mainly on the architecture of systems, especially in relation to the fuel line bore, lack of bends, size and position of filters and extent of warm fuel recirculation as well as the quantity and kind of wax crystals. Additives called wax crystal

modifiers can be added to diesel to provide satisfactory fuel flow at temperatures between 9 to -27 °C [https://www.ufa.com/petroleum/resources/fueel/diesel_fuel_resources.html].

2.7.9 Fuel lubricity

Fuels used in engines should have satisfactory lubricity to lubricate their injection systems and provide ample defence from extreme fuel injection system wear. Some of the processes employed for removing sulphur from diesel (desulphurization) can reduce its natural lubricating qualities. Certain commercially available additives that are employed to increase the lubricity of diesel fuels possess certain negative impacts when used at appropriate quantities or blended with other additives. The high lubricity standard outlined by the High-Frequency Reciprocating Test Rig (ASTM D6079) ensures that engines' injection systems have sufficient lubrication under most operating conditions [http://www.ufa.com/petroleum/resources/fueel/diesel_fuel_resources.html].

2.8 Biodiesel specifications

Biodiesel fuels are commonly characterized by properties such as density, viscosity, distillation range, cetane number, flash points, carbon residue, acid value, copper corrosion, higher heating value, cloud and pour points. These variables are listed in the ASTM D6751 biodiesel standards which indicates the acceptable limits for the variables that neat biodiesel (B100) should satisfy for it to be employed either as a direct substitute or blended with petrodiesel. Table 2.1 shows the EN specifications for biodiesel, B100 while Table 2.2 shows the most commonly used ASTM D6751 - 02 requirements. These physicochemical properties of biodiesel are comparable to those of petro-diesel [Dermibas, 2008].

Table 2.1 Biodiesel B100, specifications (EN14214) for vehicle use

Property	**Method**	**Limits**	**Units**
Density (15^{o}C)	EN ISO 3675	860 - 900	Kg/m^{3}
Flash point	EN ISO 3679	120 min	^{o}C
Water content	EN ISO 12937	500 max	mg/Kg
Kinematic viscosity at 40^{o} C	EN ISO 3104	3.5 - 5.0	mm^{2}/s
Sulfated ash	EN ISO 5165	0.02 max	%(mol/mol)
Sulfur content	EN ISO 20846	10.0 max	mg/Kg
Copper strip corrosion	EN ISO 2160	1	degree of corrossion
Cetane number	EN ISO 5165	51 min	
Cold filter plugging point	EN 116	-	^{o}C
Carbon residue	EN ISO 10370	0.30 max	%(mol/mol)
Acid value	EN 14104	0.50 max	mg KOH/g
Free glycerine	EN 14105	0.02 max	%(mol/mol)
Total glycerine	EN 14105	0.025 max	%(mol/mol)
Phosphorus content	EN 14107	10 max	mg/Kg
Methanol content	EN 14110	0.20 max	%(mol/mol)
Oxidative stability (110^{o}C)	EN14112	6.0 min	hours
Iodine value	EN 14111	120 max	g I_2/100g
Total contamination	EN 12662	24 max	mg/Kg
MAG content	EN 14105	0.80 max	%(mol/mol)
DAG content	EN14105	0.20 max	%(mol/mol)
TAG content	EN14105	0.20 max	%(mol/mol)
Group I metals (K+Na)	EN14108	5.0 max	mg/Kg
Group II metals (Ca + Mg)	prEN14538	5.0 max	mg/Kg
Linolenic acid content	EN 14103	12.0 max	%(mol/mol)
Ester content	EN14103	96.5 max	%(mol/mol)
Content of FAME with ≥4 double bonds		1 max	%(mol/mol)

Source: Knothe, 2006.

Table 2.2 Biodiesel B100, specifications (ASTM D6751 - 07 requirements)

Property	Method	Limits	Units
Flash point	D 93	130 min	^{o}C
Water and sediment	D 2709	0.050 max	% volume
Kinematic viscosity at 40^{o} C	D 445	1.9 - 6.0	mm^2/s
Sulfated ash	D 874	0.020	% mass
Total sulfur, (S500 grade)	D 5453	0.05 max	% mass
Total Sulfur (S15 grade)	D 5453	0.0015	% mass
Copper strip corrosion	D 130	No. 3 max	
Cetane number	D 613	47 min	
Cloud point	D 2500	Report	^{o}C
Carbon residue	D 4530	0.050 max	% mass
Acid number	D 664	0.80 max	mg KOH/g
Free glycerine	D 6584	0.020	% mass
Total glycerine	D 6584	0.240	% mass
Phosphorus	D 4951	10 max	ppm
Vacuum distillation end point	D 1160	360 ^{o}C max at 90% distilled	^{o}C

Source: American Society for Testing and Materials (ASTM), 2007.

2.9 Internal combustion engines

An internal combustion engine transforms the chemical energy contained in fuels to mechanical energy through ignition of fuel inside the engine cylinders. The transfer of energy that provides mechanical power output occurs directly between the air-fuel mixture before ignition, the burned products after combustion and the mechanical components of the engine. There are two main types of internal combustion engines. These are spark ignition (SI) and compression ignition (CI). The SI engines, also known as Otto or petrol or gasoline engines have spark plugs located at the top of the cylinders to produce sparks which ignites the fuel [Heywood, 1988]. The CI engine, also known as diesel engine and is an example of an auto-ignition engine in which high

temperatures generated by compression of the air-fuel mixture inside the engine causes spontaneous ignition in the combustion chamber. Thus, CI engine uses heat generated during compression to convert chemical energy in diesel fuel to mechanical energy of motion [Bosch, 2005].

The initiation of combustion by injection of liquid fuel into air heated by only compression tremendously improves efficiency in comparison to the internal combustion engines in which the air-fuel mixture is burned by use of spark since much greater expansion ratios is achieved without detonation or knock [Heywood, 1988]. There are two main classes of CI engines. The first type is those with direct injection (DI) into the main chamber. The second type is those with indirect injection (IDI) into a divided chamber. The less air motion in DI engines as compared to IDI is normally compensated for by employing high injection pressures of up to 500 bar with numerous-hole nozzles. Injection needs for IDI engines are however less challenging, employing single-hole injectors with pressures of about 300 bar [Stone, 1999].

CI engines possess a number of advantages over SI engines. The CI engines require less fuel than SI engines in executing equal amount of work, owing to greater ignition temperature and improved compression and expansion ratio achieved in the engine. The non-use of spark plugs eliminates the requirement for high-tension electrical ignition system and this result in increased dependability and ready adjustment to moist situations. The absence of coils and spark plug wires also eradicates the production of radio frequencies that may affect navigation and communication equipment, mainly in marine and aircraft applications. CI engines can produce more power on a uninterrupted basis than petrol engines and they can last nearly twice as long as petrol engines due to improved strength of parts used. The lubrication ability of diesel is also better than that of petrol and is less dangerous to store and transport than petrol due to its higher flash point. The

lower vapour pressure of diesel makes it quite suitable for use in marine environments, where accumulation of explosive fuel-air mixtures can readily lead to fire hazards [Singer and Rapper, 1978].

2.9.1 Two-stroke and four-stroke engines

During each crankshaft revolution there are two strokes of the piston, and both CI and SI engines can be designed to operate either in a four-stroke or two-stroke of the piston. The four-stroke operating cycle consist of separate induction, compression, power (expansion) and exhaust strokes of the piston and hence produces one power stroke for every two revolutions of the piston. The two-stroke cycle eradicates the need for a separate induction and exhausts strokes and consists of compression and power strokes. During the compression stroke, the underside of the piston simultaneously draws in fuel/air mixture through a spring-loaded non-return inlet valve. As the piston approaches the end of the power stroke, the exhaust port opens and a blow down occurs. When the piston reaches the bottom dead centre, the transfer port is also open, and the compacted fuel/air mixture in the crankcase expands into the cylinder thereby displacing any residual exhaust gases in the cylinder [Stone, 1999].

Since the two-stroke engines have twice as many power strokes per unit time than the four stroke engines, they are more powerful than four-stroke engines for a particular engine capacity working at a specific speed. Four stroke engines are however, more effective in fuel utilization than the two stroke engines. The inefficiency of the two stroke engines is caused by the charge dilution of the exhaust gas residuals during the simultaneous induction and exhaust processes. The inefficient fuel consumption of the two-stroke DI engines can be reduced by supercharging which ensures that the air pressure at the inlet to the crankcase is greater than the exhaust back pressure [Stone, 1999].

2.10 Engine emission and combustion

Internal combustion engine emissions produced through the exhaust is one of the major courses of urban air pollution. The engine exhaust emissions are mainly composed of oxides of nitrogen (NO_x), carbon monoxide (CO), unburned or partly burned hydrocarbons (HC) and particulate matter (smoke) [Heywood, 1988]. Although recent research and developments have significantly reduced engine pollutant emissions, the growing population, increased number of automobiles and industrialization has however led to increased overall engine pollutant emissions [Pulkrabek, 2004].

Engine discharges of CO, NO_x and HC depend on parameters like design, speed, load, temperature, ignition timing and fuel: air ratio. The emission of pollutants from the exhaust is more pronounced in SI than CI engines. The worries about engine emissions was developed in the 1960's when atmospheric conditions resulted in the formation of photochemical smog. Various concerns have been recently raised about the possible existence of carcinogens in engine exhaust emissions, though it is still unclear whether they originate from diesel fuels or combustion process [Stone, 1999].

The most commonly used parameters to determine combustion characteristics of engines include exhaust gas temperature, fuel flow rate, brake thermal efficiency, brake specific energy consumption, engine pressure and heat released by the engine. Emission characteristics are normally analyzed by measuring the levels and properties of engine exhaust emissions.

2.10.1 NO_x emission

The high temperatures generated in the diesel engine cylinders during the power stroke causes oxygen and nitrogen in air to react and form nitrogen oxides [Resitoglu *et al.*, 2014]. Nitrogen

oxides (NO_x) is composed of a combination of nitric oxide (NO) and nitrogen dioxide (NO_2). NO is the most abundant nitrogen oxide produced in the course of fuel ignition in the engine. Successive oxidation results in the formation of NO_2 in the environment which eventually reacts with non-methane hydrocarbons in the presence of UV light to produce photochemical smog [Stone, 1999]. The formation of NO_x is relatively intricate because it is dependent on a sequence of reactions like the Zeldovich mechanism shown in reaction equation 2.4.

$$O_2 \rightleftharpoons 2O$$
$$O + N_2 \rightleftharpoons NO + N$$
$$N + O_2 \rightleftharpoons NO + O$$

Reaction equation 2.4: Zeldovich mechanism

It has been shown through chemical kinetics that the production of NO and other oxides of nitrogen increases with rising flame temperature. Thus, the highest concentration of NO_x engine emissions occurs at the highest flame temperatures with lean fuel mixtures. Since the formation of NO_x also depends on flame speed, lower flame speeds with lean fuel mixtures which offer a longer time for NO_x formation implies that its emission increases at lower engine speed [Stone, 1999].

Higher levels of NO_x discharges from engines fuelled with biodiesel blends can be mitigated by reducing the combustion temperature or duration of combustion. Alternatively, the peak pressure and temperature may be reduced by retarding ignition although this has a negative impact on power output and fuel economy. Nevertheless, NO_x emissions may be reduced by increasing the concentration of residual gases in the cylinder by exhaust gas recirculation (EGR). EGR effectively reduces flame temperature and speed but provides substantial drop in NO_x. However,

EGR can reduce the efficiency at full load and reduce lean ignition limit. Catalysts may also be employed to lower the NO_x emission [Stone, 1999].

2.10.2 Smoke/Particulate matter emission

Particulate matter productions fron engine exhaust consists of a conglomeration of minute particles of partially burned fuel and lube oil, ash content of fuel oil, cylinder lube oil or sulfates and water [Maricq, 2007]. The main components of PM emission from diesel engines include soot, soluble organic fraction (SOF) and inorganic fraction (IF). Soot comprises over 50 % of the total PM discharges that appears as black smoke and mainly composed of heavy hydrocarbons adsorbed or condensed on the soot. The SOF are usually quite high at low engine loads when exhaust temperatures are low (Sarvi *et al.,* 2011).

Smoke, which is emitted as typical grey or black soot, is one of the most serious emissions from CI engines. It emanates from carbon particles produced by breaking of large hydrocarbon molecules in the fuel-rich side of the reaction zone during combustion. The carbon particles agglomerate until they reach the fuel-lean zone where they can be oxidised. The ultimate speed of soot release is dependent on the difference between the speed of production and oxidation. Smoke emission can be minimized by improving the injection timing or intrducing a finer spray of fuel through the use of higher injection pressures or finer nozzles [Stone, 1999].

Studies on the influence of PM discharges on the sorrounding and human health have shown that their inhalation can lead to adverse health complications like asthma, lung cancer, and other cardiovascular problems which can lead to premature death. These emissions also gives rise to air, water and soil pollution, soiling of buildings, global climate change, reduction in visibility and agricultural productivity (Englert, 2004).

2.10.3 CO emission

Carbon monoxide emission occurs due to incomplete combustion of carbon in the diesel fuels and its concentration in the exhaust gas mainly depends on air/fuel mixture [Resitoglu *et al.*, 2014]. Rich fuel mixtures often lead to the formation of CO due to air deficiency which results in incomplete combustion. Although lean fuel mixtures also result in the formation of CO, the concentration reduces with decreasing combustion temperatures [Stone, 1999]. The producttion of CO in diesel engines with lean fuel mixtures mainly results from too large droplets of fuel spray or inadequate turbulence or swirl in the ignition chamber [Resitoglu *et al.*, 2014].

2.10.4 Hydrocarbon (HC) emission

Unburnt hydrocarbons in a well-regulated CI engines may originate from the perimeter of the reaction zones where the mixture is too lean to burn [Stone, 1999]. HC may also originate from the fuel retained in cold boundaries such as piston grooves, the nozzle sac and spray holes, where the temperature of the air–fuel blend is much less as compared to the middle of the cylinder [Correa and Arbilla, 2008]. The fuel retained in the piston grooves, nozzle sac and spray holes usually undergo incomplete combustion since it enters the chamber towards the end of the combustion cycle hence resulting in HC emission. HC emission is more pronounced in DI than IDI engines especially at light loads when there is an appreciable delay in ignition in DI engines [Stone, 1999].

Hydrocarbon emission can be lowered by advancing injection timing, which however increases NO_x emission and engine noise. HC emission increases at partial load, because the delay in ignition duration raises and the amount of lean fuel mixture at the perimeter of the reaction zone increases. Over-fuelled diesel engines also result in appreciable HC emissions. Concern has been raised about the existence of carcinogenic, polycyclic/polynuclear aromatic hydrocarbons (PAH)

in HC emission which may result in increased incidences of Cancer [Stone, 1999]. Trier *et al.*, [1990] proposed that the PAH are most likely formed during combustion.

Higher combustion temperature and pressure leads to shorter ignition time and decline in combustion noise and HC emission. Higher ignition temperatures, however, result in an increase in NO_x emission and decrease in CO emission due to increased oxidation. However, a turbo-charged engine fitted with an inter-cooler reduces temperature and NO_x emission. Both CO and HC exhaust emissions could be minimized by using lean fuel mixtures, though this has the shortcoming of lowering power output of the engine. It is also challenging to warrant even supply of fuel/air mixture to every cylinder in a multi-cylinder engine. Additionally, exhaust gas catalytic reactors could be employed to complete the oxidation of fuels and if required, extra air may be admitted into the cylinders [Stone, 1999].

2.11 Previous related studies

Wanjala, [2011] showed that FAME obtained from *C. megalocarpus* seeds as well as its blends with petrodiesel has similar physical properties with diesel. A conversion of 85% plant oil to FAME in transesterification reactions using an alkali catalyst was reported. In this work, however, Croton FAME was prepared using lipase and a two stage process and the operation of the biodiesel in a single cylinder, four stroke DI engine investigated. Sahoo and Das, [2009] reported that feedstocks with high FFA content such as Polanga oil produced lower yields of fuel grade biodiesel when direct alkali-catalysed transesterification was used due to soap formation. They also reported that the yield of fuel grade biodiesel could be greatly improved by using two-step chemical process for *Jatropha* and *Karanja*; and triple-step chemical process for Polanga oils. Canakci and Van Gerpen, [2001] reported that the acid values of plant and waste oils containing high free fatty acids could be lowered to less than 2 mg KOH/g through acid-catalysed

pre-treatment reactions. They also noted that increasing the amount of acid catalyst was very effective in lowering the acid values of the oil feedstocks.

Lyberg and Adlercreutz, [2007] performed selective transesterification of saturated squid oil using immobilized lipase from *T. lanuginosus*. Ethyl esters were isolated from the unsaturated squid oil by short path distillation then converted to ω-3 essential oils such as (Eicosapentaenoic acid) EPA and Docosahexaenoic acid) DHA. Canakci and Van Gerpen, [2001] developed a two-stage chemical procedure for preparing biodiesel from oil feedstocks with high content of FFAs which involve reduction of FFAs through acid-catalysed esterification followed by base catalysed transesterification. Royon *et al*., [2006] produced biodiesel by reacting methanol with cottonseed oil using immobilized *Candida antarctica* lipase as catalyst in *t*-butanol solvent. They observed that the inhibition of enzyme by methanol was eradicated by addition of t-butanol to the mixture hence resulting to a high increase in reaction rate and yield of the methyl ester. They also determined the effects of temperature as well as concentrations of t-butanol and methanol on the rate of the reaction. They obtained a methanolysis yield of 97% after 24 hours at 50^{o}C and optimum concentrations of the reagents and catalyst.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection and processing of Croton seeds

*Croton megalocarpu*s nuts were collected from University of Nairobi, Chiromo Campus and around Thika town in Kenya. The nuts were dried for one week after which the seeds were removed manually from the shells. The seeds were dried further for two days to remove any surface adsorbed moisture. Croton oil was extracted from the dry seeds using a mechanical pressing machine at Jomo Kenyatta University of Agriculture and Technology. The oil was filtered using gravity filter bags to remove solid impurities, kept in clean, dry air tight containers and stored. Figures 3.1, 3.2a and b show a *Croton megalocarpus* tree at Chiromo Campus (University of Nairobi), fruits and seeds, respectively while Figure 3.3 shows the mechanical oil pressing machine that was used to extract oil from the Croton oil seeds.

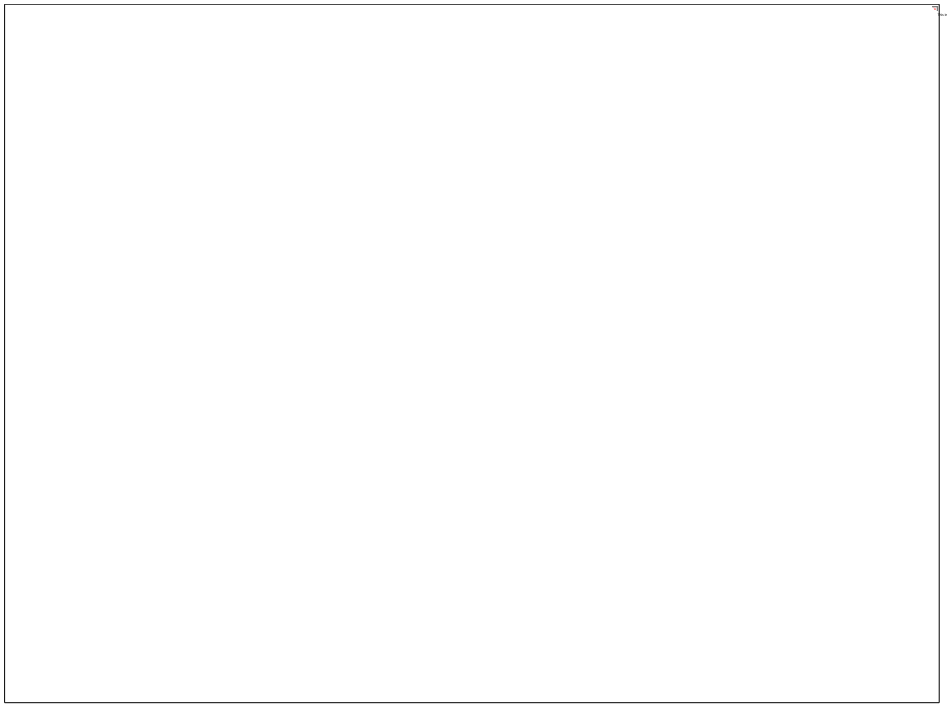
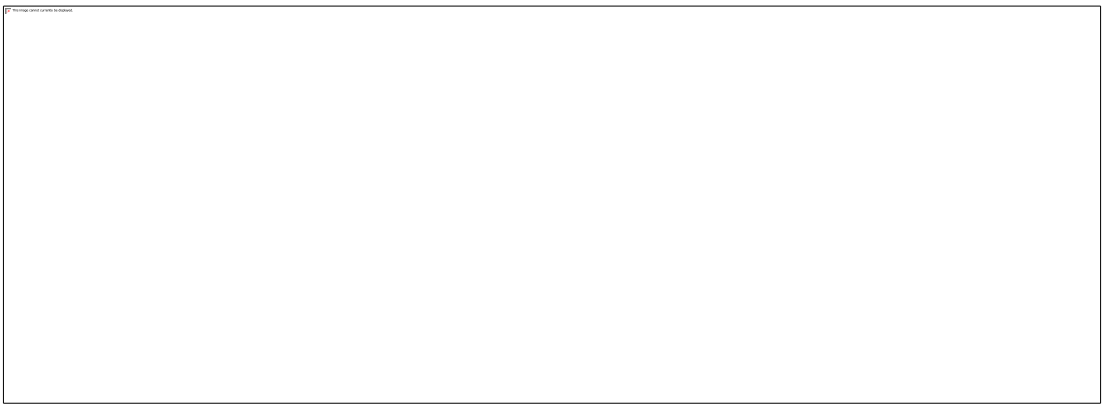


Figure 3.1 Croton megalocarpus tree at Chiromo Campus, University of Nairobi, Kenya.



3.2(a) **3.2(b)**

Figure 3.2(a) *Croton megalocarpus* seeds and **3.2(b)** *Croton megalocarpus* fruits

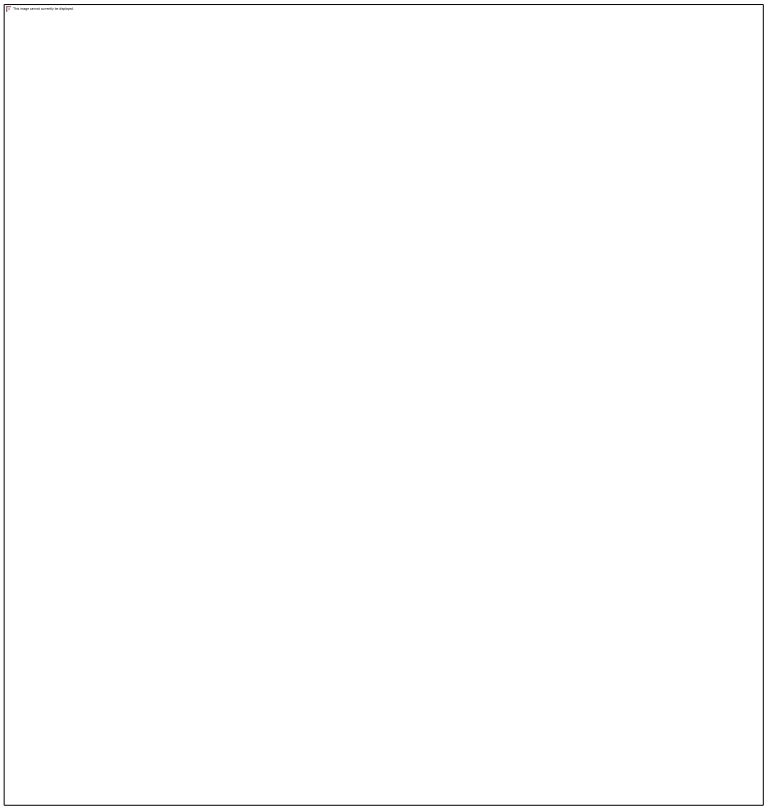


Figure 3.3 Mechanical oil pressing machine

3.2 Equipment and reagents

3.2.1 Equipment

The following equipment used in this work included Mechanical oil pressing machine, Grindomix - GM 200 grinder, GC-MS, PetroOxy equipment, Pensky Martins Flash Point Cup Apparatus (DM 93), Density meter (DM 300), Biodiesel reactor, Pour and Cloud point apparatus, Fungilab viscometer (V300003) connected to thermostatic water bath, Gallenkamp orbital incubator shaker, Computerized single cylinder four stroke direct injection engine and Bomb calorimeter, Exhaust gas and smoke analyzer.

3.2.2 Reagents

The reagents used in this project included *Croton megalocarpus* seeds, diesel, thermostatic water bath, methanol, ethanol, diethyl ether, n-hexane, cyclohexane, acetone, acetonitrile, acetic acid, chloroform, sodium hydroxide, hydrochloric acid, iodine tank, sulphuric acid, potassium hydroxide, P-nitrophenylbutyrate, p-nitrophenol, Pyrogallol, Propyl gallate, butylated hydroxytoluene (BHT), heptane, ethyl acetate, butyl hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ), lipase from *T. lanuginosus* (≥100 000 U/g), silica gel drying agent, silica gel 60 TLC plates from Merck (Darmstadt, Germany) and FAME standard (C_4 to C_{24}). All reagents were of analytical grade procured from Sigma Aldrich, Merck or Rankem Chemicals.

3.3 Preparation of biodiesel using two stage process

3.3.1 Determination of saponification and acid value of Croton oil and biodiesel

The saponification value of Croton oil was determined using the method described by Vogel, [1989]. Approximately 0.5M solution of KOH was prepared by dissolving 14 g of KOH in 10 ml of distilled water and the solution made to the mark with 95% ethanol in a 500 ml volumetric flask. The solution was left to decant for 24 hours after which it was filtered. 25 ml portions of

the solution were titrated with 0.5M HCl using phenolphthalein indicator, and a mean titre value of 20.20 ml was obtained.

2 g of croton oil was accurately weighed into a 500 ml flask with a ground glass joint and 25 ml of the KOH solution added. The reflux condenser was attached and the flask heated with its contents on a steam bath for 1 hour with intermittent shaking. Phenolphthalein indicator was added while the solution was still hot and the excess KOH titrated with 0.5M HCl. The titration was carried out in triplicate in both experiments and the mean titre used to determine the saponification and acid values respectively.

The acid value of both the croton oil and biodiesel were determined using a method published by the American Oil Chemists Society (AOCS), [1980]. Briefly, a 2.5 g sample was dissolved in 50 ml of 95% ethanol and four drops of phenolphthalein indicator added. The mixture was warmed until a homogenous solution was obtained. The solution was titrated with 0.5N KOH until the first pink colour which persisted for 30 sec was obtained.

3.3.2 Optimization of reaction parameters for biodiesel preparation

Since the FFA content of Croton oil was greater than 2%, the biodiesel was made through a two-stage chemical procedure, which involved acid catalysed esterification of FFAs followed by base catalysed transesterification of triglycerides. Acid treatment not only prevents soap and emulsion formation which causes difficulties in biodiesel/glycerol separation, but also improves biodiesel yield by transforming the FFAs to esters [Canakci and Van Gerpen, 2001]. Both esterification and transesterification reactions were performed in a thermostatic biodiesel reactor furnished with a mechanical stirrer. The reactor temperature was kept constant using a heated oil bath. The oil was first heated at 60 ^{o}C for 1 hour to eliminate traces of moisture. The optimal reaction

conditions were examined by changing variables like temperature, time, mass of potassium hydroxide catalyst and mole ratio of reagents. The parameters were changed one at a time while maintaining the others constant and their effects determined. Each reaction was done in duplicate and the mean values recorded.

3.3.4 Acid catalysed esterification

The effect of concentrated sulphuric acid catalyst on esterification of free fatty acid (FFA) was determined by adding different volumes of acid to a mixture of Croton oil and methanol in a biodiesel reactor. The volume of methanol required to react completely with FFA in Croton oil was calculated from the acid value. Since the esterification is a reversible reaction, a FFA:methanol ratio of 1:3 was employed to favour the forward reaction and improve the yield of ester. 2 ml of 99% pure methanol was added to different portions of 100 g of Croton oil in a reactor and mixed for 5 min by stirring at 350 rpm. A graduated pipette was used to add different volumes of 98% concentrated sulfuric acid to each mixture. The mixture was continuously stirred while maintaining the temperature constant. The acid value of the organic layer was tested after regular intervals of time in duplicates and the mean values recorded. Similarly, the influence of temperature on rate of esterification was determined using an acid volume of 0.4 ml at 30, 40, 50 and 60 ^{o}C.

3.3.5 Base catalysed transesterification

The effect of base catalyst, Potassium hydroxide (KOH) and amount of methanol on the rate of transesterification of *Croton* oil was investigated by separately varying the mass of base or volume of methanol added to different portions of the reaction mixture containing 100 g *Croton* oil and determining their effects on the percentage yield of biodiesel relative to the mass of Croton oil. The KOH was mixed with methanol then poured into the reaction mixture in a

biodiesel reactor at a constant temperature and stirred continuously at 350 rpm. The influence of temperature and volume of methanol on the speed of transesterification were also investigated by separately varying the temperature and volume of methanol and determining their effects on the percentage yield of biodiesel. Figure 3.4 shows the biodiesel reactor that was used for the two-stage process.

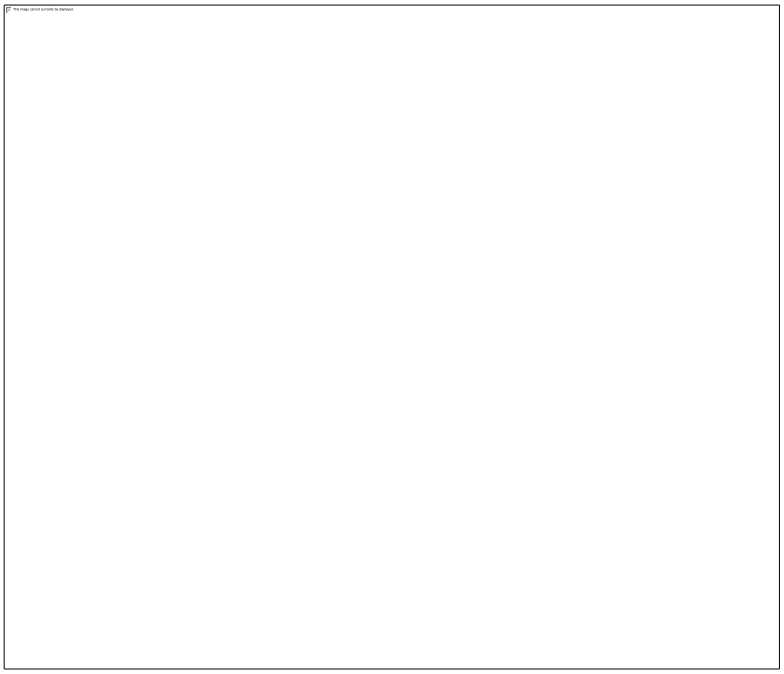


Figure 3.4 Biodiesel reactor

3.3.6 Lipase enzyme catalysed transesterification of plant oils

Transesterification of the Croton oil was carried out using a commercial lipase from *Thermomyces lanuginosus* (1000 U/g) in a sealed jacketed reactor. The optimum reaction conditions were investigated by varying factors such as oil:alcohol ratio, temperature and volume of enzyme each at a time while maintaining other variables constant. The oil: alcohol ratio was varied from 1:3 to 1:8, the temperature was varied from 30 to 50 ^{o}C in steps of 5 ^{o}C while the volume of lipase catalyst was varied from 2 to 15% of the Croton oil. The effects of the reaction

parameters were determined by calculating the percentage yield (v/v) of the biodiesel with respect to the Croton oil.

3.3.7 Biodiesel washing and drying

The biodiesel was repeatedly washed in a separating funnel with approximately 100 ml portions of warm water (45 ^{o}C) to remove traces of alkali, soap and glycerine until clear water separated out. The aqueous and biodiesel layers were allowed to settle after every washing for between 30 min to 1 hour after which the aqueous layer was drained out. The volume of biodiesel produced was accurately measured and the mean percentage yield recorded. The biodiesel was transferred into a clean bottle where it was dried with fused silica gel for 24 hours. The dry biodiesel was finally filtered and kept in dry plastic containers with air-tight lids.

3.3.8 Properties of Croton oil and biodiesel

The properties of Croton oil and biodiesel were analyzed using standard procedures and apparatus. The values obtained were compared with both the recommended American Society for Testing and Materials (ASTM) values and those of petrodiesel. The equipment used for determination of the pysico-chemical properties included Pensky-Martins Flash Point Cup Apparatus (DM 93), density meter (DM 300), Pour and Cloud point apparatus, Fungilab Viscometer (V300003) connected to a thermostatic water bath and Bomb calorimeter. Figures 3.5 to 3.10 show some of the equipment that were used in determination of physico-chemical properties of Croton biodiesel.

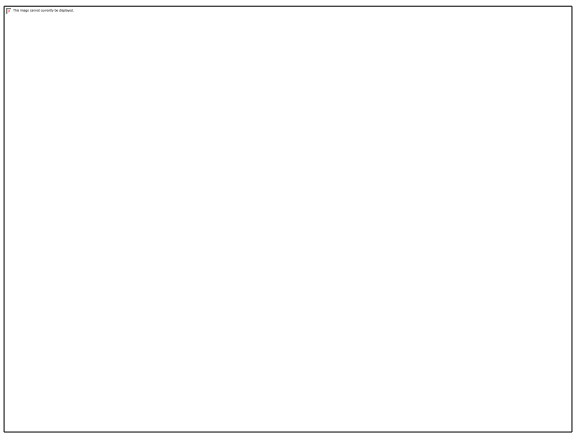


Figure 3.5 Bomb Calorimeter

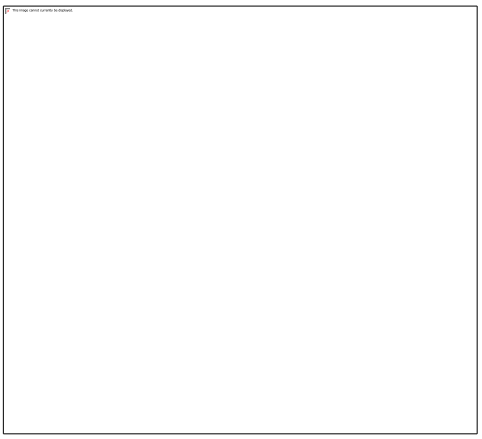


Figure 3.6 Pensky cup apparatus

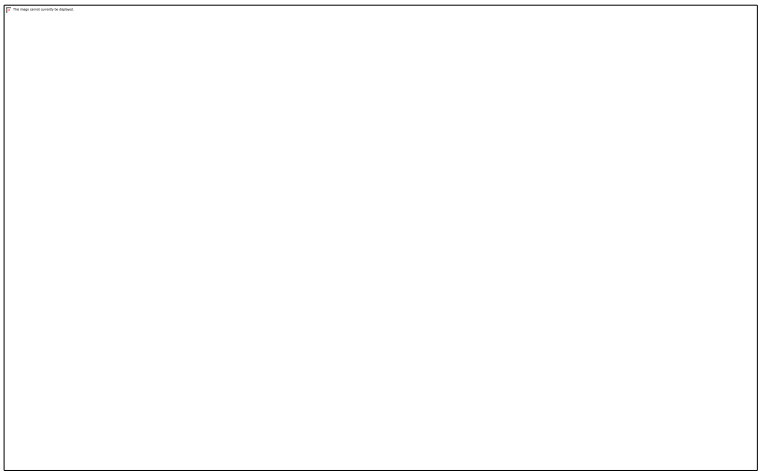


Figure 3.7 Viscosity meter

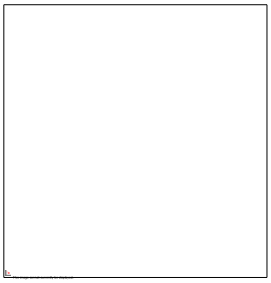


Figure 3.8 Portable density meter

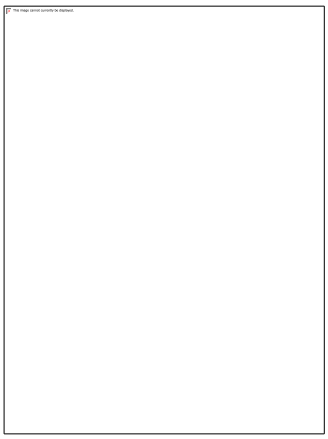


Figure 3.9 Density meter

Figure 3.10 CP and PP meter

3.4 Isolation, identification and use of lipase in preparation of biodiesel

Water and soil samples that were screened as sources of extracellular lipase-producing microorganisms were collected from Lake Sonachi, Kitengela, oil contaminated soil from Thika solid waste disposal site and soil contaminated with fish oil from fish frying sites. Both the soil and water samples were first mixed with sterile minimum salt medium then left to settle for 12 hours.

3.4.1 Enrichment of microorganisms and preliminary test for lipase production

Lipase-producing microorganisms were enriched by inoculating the water and clear supernatant liquid from the soil samples in 50 mL of sterile aqueous media composed of 4% Croton oil and minimum salt solution containing 0.001% $FeSO_4$, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.08 % KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.02% $CaCl_2 \cdot 2H_2O$, 0.25% NaCl, 0.05% yeast and the pH adjusted to 8. The mixture was incubated in a reciprocal shaker for ten days at 37 ^oC and 100 rpm. The growth of the microorganisms was monitored by observing the turbidity of the solution.

3.4.2 Plate assay and screening of lipase producing microorganisms

The enriched microorganisms were cultivated in an incubator for 24 hours at 37 ^{o}C on a plate of autoclaved bacteria growth media (BGM) composed of minimum salt solution (MSS), 3% Croton oil, 2.5% agar and 0.2% yeast at a pH of 8. The pure colony of lipase producing microorganism was harvested using a sterile wire loop and re-plated on a plate composed of the same media and cultured in an incubator at 37 ^{o}C for 48 hours. The plates containing the lipase producing microorganisms were kept in a cold room at 4 ^{o}C for further investigation. Additionally, the pure colonies of lipase producing microorganisms were spotted on 0.02% Rhodamine B agar plates containing BGM and 1% sucrose and incubated at 37 ^{o}C for 24 hours and lipase production monitored under UV light at 350 nm. The colony of microorganisms that displayed the greatest growth intensity was chosen for further investigation.

3.4.3 Characterization and identification of lipase-producing microorganisms

The lipase-producing microorganism was characterized using Gram's stain reaction procedure. The fixed bacterial smear was viewed under a microscope to determine whether it was a gram positive or gram negative bacteria. Lipase-producing microorganism was freshly cultured in nutrient broth in a reciprocal shaker at 37 ^{o}C for 16 hours. The DNA was extracted as described by Wilson, [1987]. The extracted DNA was subjected to gel electrophoresis, viewed on UV at 350 nm, analyzed on polymerase chain reaction (PCR), amplified and identified.

3.4.4 PCR conditions and sequencing

PCR was performed using AccuPower® PCR PreMix, comprising 1U Taq DNA polymerase, 250 µM of each dNTP, 10 mM Tris-HCl, 40 mM KCl, 1.5 mM $MgCl_2$ and tracking dye for electrophoresis. PCR amplification was done on a GeneAmp PCR system 9700 thermocycler (Applied Biosystems) using the following program: 94 ^{o}C for 5 min, tailed by 40 cycles of 94 ^{o}C for 20 sec, 58 ^{o}C for 20 sec, and 72 ^{o}C for 1 min, followed by 72 ^{o}C for 10 min. The amplified PCR products were run on a 2% pre-stained ethidium bromide agarose gel to confirm amplification.

Sequencing reactions were performed using the BIGDYE® Terminator Cycle Sequencing (Applied Biosystems). The reactions were performed in a 10 µl volume comprising of 4 µl of water; 2 µl of diluted PCR products (2.5 ng/µl); 2µl of reverse or forward primer (5 pmoles/µl); 1.0 µl of BIGDYE and 1.0 µl of 5X sequencing buffer. The sequencing reaction was set for 45 cycles of 96 ^{o}C for 10 sec; 58 ^{o}C for 5sec; and 60 ^{o}C for 4 min, followed by a final hold at 4 ^{o}C. PCR products were purified by ethanol precipitation according to instructions from the manufacturer (Applied Biosystems). The samples were sequenced on an ABI 3730 Genetic Analyzer (Applied Biosystems). The sequencing trace files were aligned into contigs with slight modifications of the default conditions using Sequencer® software version 4.6 (Gene Codes Corporation, USA). All chromatograms and sequences were visually inspected and sequences edited, and only high-quality sequences were further used.

3.4.5 Extraction of extracellular lipases and determination of its catalytic activity

Lipase-producing microorganisms harvested from the plates were cultivated in a reciprocal shaker at 37 ^{o}C and 100 rpm for 48 hours in 50 ml conical flasks containing autoclaved medium of LB broth at pH 8. The lipase solution was obtained by centrifuging the culture media for 10

min to remove the bacterial cells. The supernatant lipase solution was carefully transferred into a clean falcon tube and kept in a fridge at 4 ^{o}C. The activity of the lipase solution was determined by a titrimetric method as described by [Rajan and Nair, 2011] with slight alteration. A reaction mixture containing 2 ml croton oil, 5 ml of 0.1 M Tris-HCl buffer at pH 8 and 1 ml of the culture supernatant was incubated at 37 °C for 20 min with agitation at 100 rpm. The reaction was stopped by adding 10 mL of acetone. The free fatty acid produced was titrated with 0.1 M KOH in the presence of phenolphthalein indicator. Blank tests were done by titrating the incubated mixture of croton oil and Tris-HCl buffer. All titrations were done in triplicate. The ability of the extracted lipase to catalyse transesterification of Croton oil was then determined.

3.5 Analysis of biodiesel

3.5.1 Thin layer chromatography (TLC)

The Croton oil and biodiesels prepared using both extracted and commercial enzymes were spotted on TLC silica gel 60 plate (Merck, Darmstadt, Germany) and separated with a mobile phase comprising cyclohexane/diethyl ether/acetic acid in the ratio 50:50:1. The chromatogram was visualized by using either UV lamp or placing the plate in an iodine tank [Lyberg and Adlercreutz, 2007, Mbatia *et al.,* 2010].

3.5.2 GC-MS Analysis

The Croton oil and prepared biodiesel (methyl esters) were analysed using a GC-MS method as described by Lyberg and Adlercreutz, [2007]. A 1-µL sample was injected into a Varian gas chromatograph 3400 (Varian Instrument Group, Walnut Creek, CA, USA), furnished with a flame-ionisation detector (FID). The equipment was equipped with a septum-programmable injector. The column used was an SP-2380 fused silica capillary column (30 m x 0.32 mm, 0.20 µm film thickness; Supelco, Bellefonte, PA, USA).

The temperature of the detector was maintained at 250 ^{o}C, while the injector and column were predetermined with respect to temperature. The injector temperature was first maintained at 70 ^{o}C for 6 seconds, then raised at a speed of 60 ^{o}C/min to 250 ^{o}C, where it was kept for 2 min. The column temperature was first kept at 70 ^{o}C for 2 min, and then raised at a speed of 20 ^{o}C/min to 160 ^{o}C. The temperature was maintained for 10 min then finally raised to 220 ^{o}C at a rate of 4 ^{o}C/min. The final temperature of 220 ^{o}C was maintained for 3 min.

The instrument was calibrated using a standard mixture of fatty acid methyl esters of known concentrations. The response factors of the different fatty acid methyl esters were obtained from examination of the standard mixture as described by Braithwaite and Smith, [1996]. The identities of the isolated compounds were determined by comparing the spectrum of the compounds being analysed with those in the library of mass spectra in the system. The GC-MS data was based on duplicate measurements.

3.6 Assessment of engine performance emission and combustion characteristics

The emission, combustion and performance qualities of a test engine fuelled with petrodiesel and different blends of freshly prepared biodiesel and petrodiesel were separately investigated in a computerized single cylinder, four stroke direct injection diesel engine test rig. The schematic experimental set up for engine testing is shown in Figures 3.11 and 3.12 while Figure 3.13 shows the diesel engines and the computer control room in the engine testing laboratory. The manufacturer's specifications for the test engine used for investigation are summarized in Table 3.

Figure 3.11 Schematic diagram of test engine arrangement

Figure 3.12 Display of engine layout and test parameters

Figure 3.13 Test engine laboratory and control room

Table 3 Manufacturer's specifications for test engine from manual

Particulars	Specifications
Engine Make	AV1 Kirloskar
Engine type	Direct injection single cylinder four stroke diesel engine
Engine weight without flywheel (Kg)	114
Weight of flywheel (Kg)	33
Bore diameter (metres)	0.08
Stroke length (metres)	0.11
Connecting rod length (metres)	0.235
Compression ratio	16.7:1
Engine cooling water flow (mL/sec)	60
Engine speed (rpm)	1500

The test engine, mounted on a stand was subjected to a maximum load of 10 Kg (100%) at intervals of 2 Kg (20%) through dynamometer. The engine was operated at an optimum speed of 1500 rpm. The combustion and performance variables such as fuel flow rate, airflow rate, exhaust gas temperature, brake power, brake thermal efficiency, heat release, pressure and crank angle at each load were determined electronically and the data stored in specific files in the computer. Each parameter was determined in triplicates on loading and unloading of the engine, and the mean values recorded.

The nature and levels of exhaust gas emissions were determined using exhaust gas analyser (AVL DIGAS 444) while the particulate matter (PM) or smoke in the exhaust emissions was determined by a smoke meter (AVL 437). The exhaust gases analysed included Nitrogen oxides (NO_x) Carbon II Oxide and hydrocarbons (HC). Both smoke and exhaust gas emissions were recorded in triplicates on loading and unloading of the engine and the mean values determined. Figures 3.14 and 3.15 show the smoke and exhaust gas analysers that were used for investigations in this study.

Figure 3.14 Smoke analyser

Figure 3.15 Exhaust gas analyser

3.7 Oxidation and storage stability of biodiesel

Samples of biodiesel blends were made by combining petrodiesel with newly prepared dry Croton biodiesel in various proportions. The samples were separately combined with accurately weighed antioxidants to form 100 and 1000 ppm antioxidant solutions. Reference samples without antioxidants were also prepared. The impact of antioxidants on oxidation stability of Croton biodiesel was initially tested using 1000 ppm solutions of five commercial antioxidants in different portions of newly prepared biodiesel samples. The samples with antioxidants added as well as a reference sample were separately subjected to oxidative stability test in a PetroOxy equipment. 5 ml of each sample was separately injected into the sealed test chamber where it was mixed with oxygen at a high pressure of 700 kPa and temperature of 140 °C in a PetroOxy equipment. These conditions initiated a fast synthetic aging procedure, which was determined by a pressure drop in the test compartment. The oxidative stability was determined by measuring the time between the start of the test and the time at which a pressure drop of 10% below the

maximum pressure was detected (induction period, IP). The molecular structures of the five synthetic analytical grade commercial phenolic antioxidants, Pyrogallol (PYG), Propyl gallate (PRG), Butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT) and *tert*-Butylhydroquinone (TBHQ) that were employed in this study are shown in Figure 3.16. Subsequently, the three antioxidants that showed the best results were selected for further studies on storage stability of the biodiesel and blends [Osawa *et al.*, 2015].

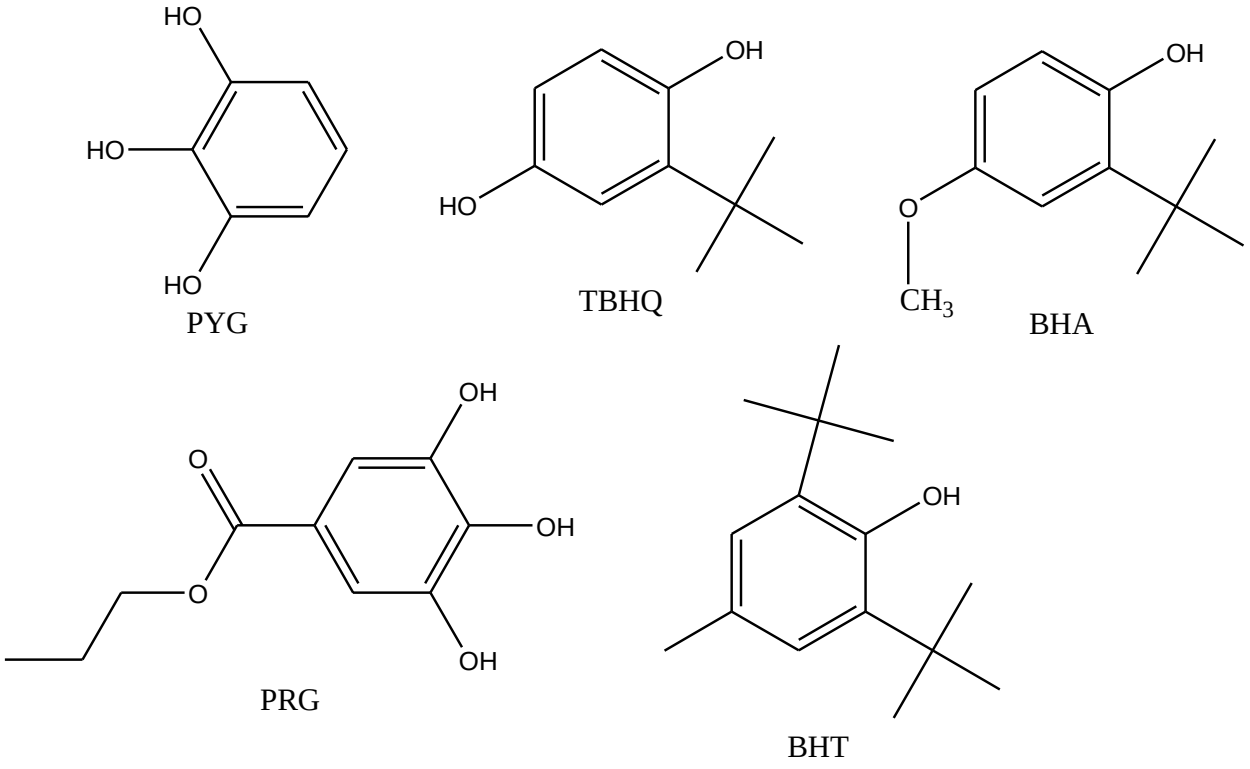


Figure 3.16 Structures of some common synthetic antioxidants

The biodiesel and blends containing varying concentrations of the selected three antioxidants that gave the best preliminary antioxidant activities were kept in a metallic locker at room temperature for 8 weeks. The storage condition displayed normal environment in which biodiesel is usually kept, either in metallic or plastic tanks before use. The impacts of storage and addition of varying concentrations of antioxidants on the oxidation stability of the samples were

determined for 8 weeks at intervals of 2 weeks [Osawa *et al.*, 2015]. Figure 3.17 shows the PetroOxy equipment that was used for the determination of oxidation stability.

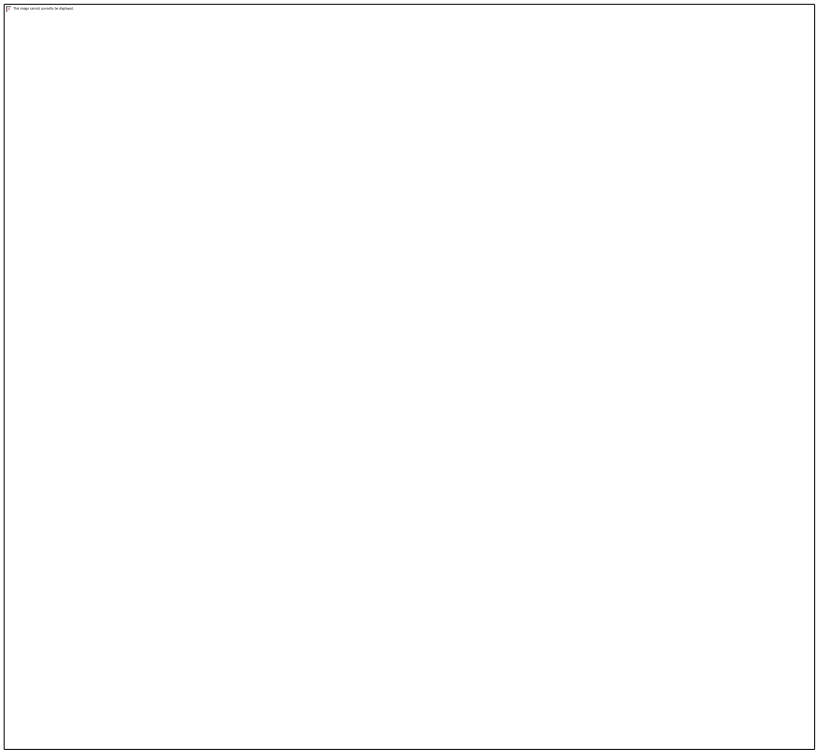


Figure 3.17 PetroOxy Equipment

CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.1 Oil extraction and properties of Croton oil and biodiesel

The mechanical oil extraction produced 17 litres of Croton oil from 48 kg of dry seeds which represented a yield of 35.42% (v/w). The density, kinematic viscosity and acid value for Croton oil of 0.9262 g/cm^3, 30.54 cs and 7.518 mg KOH/g respectively obtained during this study were all higher than the recommended standards for biodiesel shown in tables 2.1 and 2.2. This therefore implied that the straight vegetable oil (SVO) was unsuitable for direct use as biodiesel. However, both enzymatic and the two-stage chemical process of esterification followed by transesterification led to enormous transformations in the corresponding values for the biodiesel which were within the range of recommended ASTM and EN values for biodiesel standards and in some cases comparable to those of petrodiesel. Both the biodiesel prepared using two-stage and lipase catalyst processes displayed similar physicochemical properties. Table 4.1 shows the selected characteristics of Croton biodiesel and blends that were investigated in this study.

Table 4.1 Properties of Croton methyl ester and blends

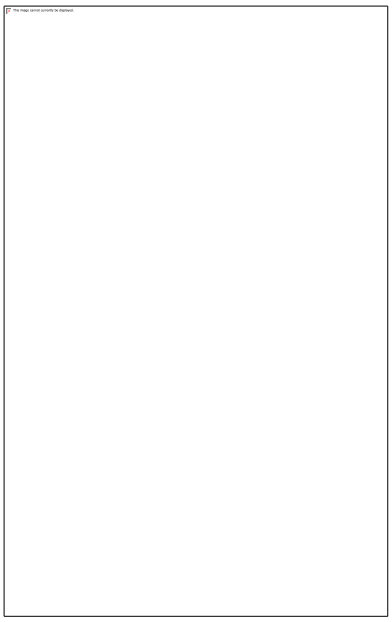
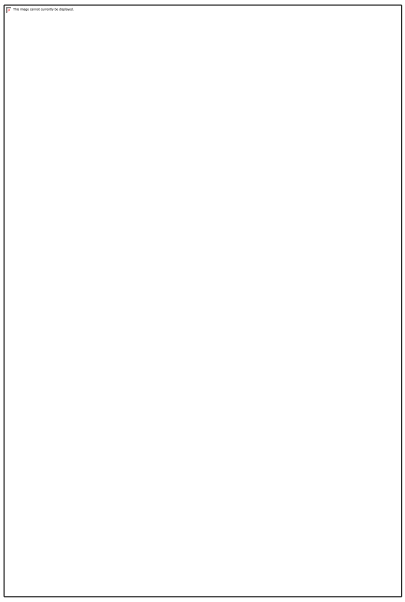
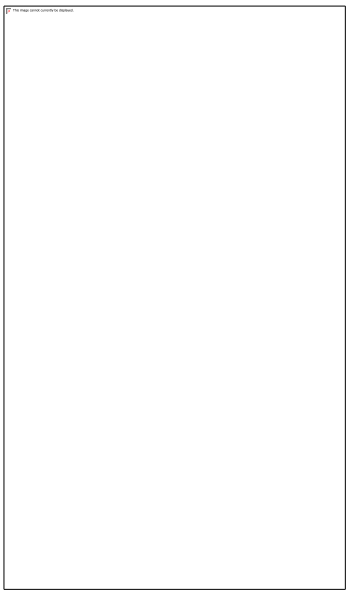
Property	**Method**	**Diesel**	**B100**	**B50**	**B20**	**B15**	**B10**	**B5**
Density (g/cm^3)	ASTM D15	0.8231	0.8858	0.8545	0.8347	0.8318	0.8293	0.8262
Kinematic Viscosity (40^oC, cs)	ASTM D445	2.87	4.51	3.63	3.14	3.08	3.03	2.97
Calorific Value (j/g)	ASTM D2015-85	44648	39179	40506	41445	42320	42957	43596
Cloud point (^oC)	ASTM D2500-91	4.0	-1.5	1.5	2.0	2.0	2.4	3.0
Pour point (^oC)	ASTM D97-94	-2.0	-6.5	-4.3	-3.0	-2.5	-2.3	-2.0
Flash point (^oC)	ASTM D93	64	>170	88	74.5	72	70	68
Acid number (mg KOH/g)	ASTM D664	ND	0.336	0.170	0.065	0.050	0.032	0.002

All the values of selected physicochemical properties of Croton biodiesel analysed were within recommended American (ASTM D6751) and European (EN 14214) standard values for biodiesel. Both the cloud and pour point of Croton biodiesel were lower than that of petrodiesel. These properties showed that the biodiesel and its blends were more suitable for low-temperature operations than petrodiesel. The biodiesel blends also had higher flash points than petrodiesel, hence making them safer to transport and store.

The large differences between properties of Croton oil and biodiesel can be attributed to conversion of large and branched triglyceride molecules into lighter straight chain methyl ester

(biodiesel) molecules. The results from physicochemical properties showed that the use of both commercial lipase and two-stage process greatly enhanced the fuel properties of Croton oil in relation to acid value, density and viscosity. Although the biodiesel had inferior total calorific value as compared to petrodiesel and blends, both the viscosity and density values of methyl esters and blends were comparable to those of diesel. Similar observations were made by Lujaji *et al.*, [2010]. They also observed that SVO had lower calorific value, higher viscosity and density than biodiesel and vegetable oil/butanol-diesel blends.

The glycerol produced during enzymatic transesterification of Croton oil was relatively clear hence could be easily recovered. However, the glycerol obtained through the two-stage process using chemical catalyst was dark brown which made its recovery more difficult. The dark brown colour was probably due to formation of a potassium salt. Figures 4.1a and b shows the biodiesel before and after washing respectively for base catalysed reaction while Figure 4.1c shows the glycerol layer and biodiesel layer for lipase catalysed reaction.

4.1a **4.1b** **4.1c**

Figure 4.1(a) Crude glycerol (lower layer) and biodiesel (upper layer) in base catalysed transesterification before washing, (**b)** Water (lower layer) and biodiesel (upper layer) after washing, (**c)** Glycerol (lower layer) and biodiesel (upper layer) in enzyme catalysed transesterification.

4.2 Optimization of reaction parameters for two stage process

4.2.1 Effect of temperature and acid catalyst volume on esterification

The acid value of the organic layer decreased with increase in reaction time. The lowest acid value of 0.3 mg KOH/g was attained after 2 hours at both 50 and 60 ^{o}C reaction temperatures and acid volumes of 0.4, 0.5 and 0.6 ml. A more drastic initial decrease in acid value of the organic layer was observed with increase in both reaction temperature and volume of acid catalyst added to the reaction mixture. These observations could be attributed to improved attainment of equilibrium at higher temperatures and shifting of the equilibrium of the reaction equation 4.1, to the right with increased acid catalyst concentration.

$$RCOOH + R_1OH \underset{}{\overset{H^+}{\rightleftharpoons}} RCOOR_1 + H_2O$$

Reaction equation 4.1 General esterification equation

Figure 4.2 shows the variation of acid value of the organic layer with temperature while Figure 4.3 shows the effects of volume of concentrated sulphuric acid catalyst used on acid value of organic layer at regular intervals of time at 50 ^oC.

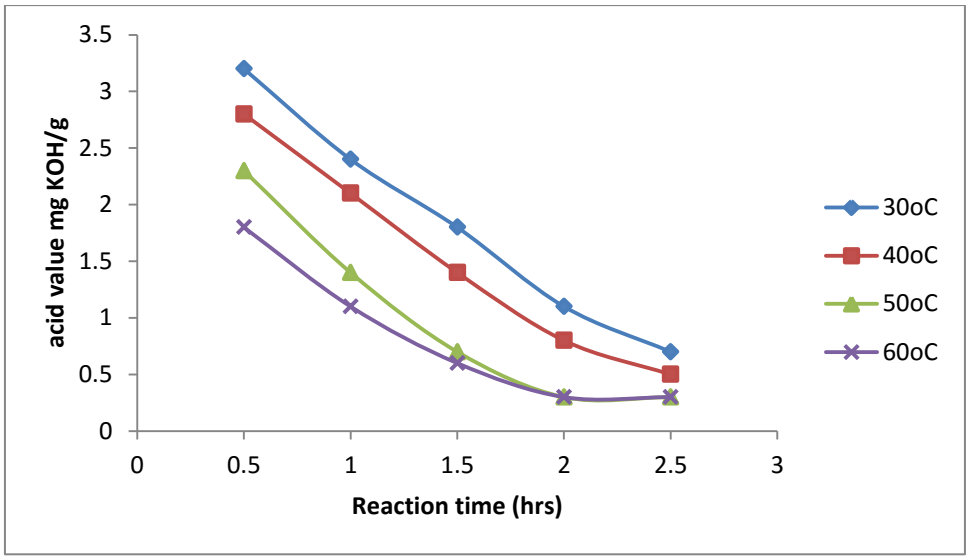


Figure 4.2 Effect of temperature on esterification of free fatty acids.

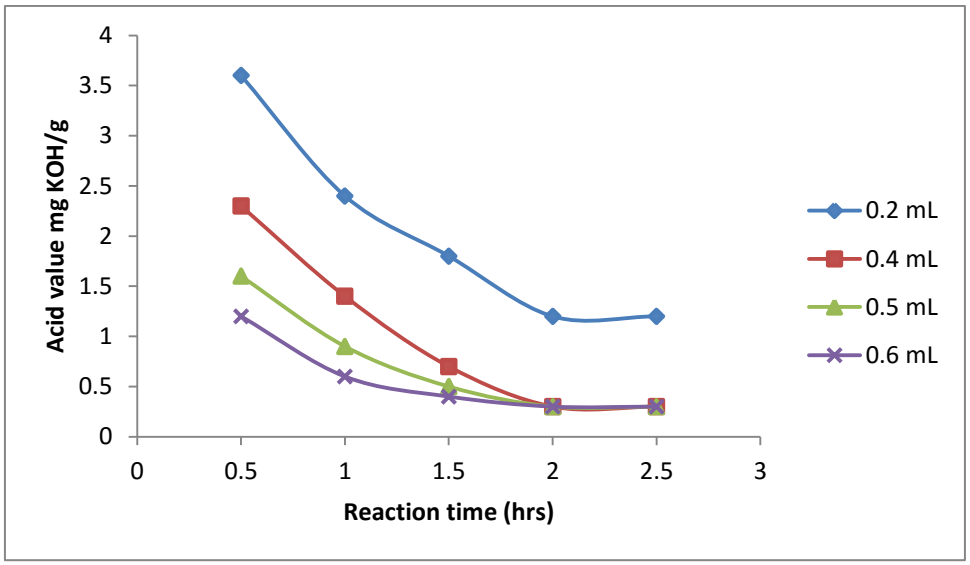


Figure 4.3 Effect of volume of H_2SO_4 acid catalyst on acid value of Croton oil.

4.2.2 Effect of mass of base catalyst on transesterification

The percentage yield of biodiesel initially increased with increase in mass of base catalyst until a maximum yield was obtained at a base concentration of 1 g KOH/100g *Croton* oil (1% w/w). Further increase in mass of base catalyst added to the reaction mixture caused a decline in percentage yield of biodiesel. A rise in formation of emulsion was observed at lower catalyst concentration which necessitated the need for extra washing. At higher concentrations (>1.0%), both soap and emulsion formation occurred and a lower production of biodiesel was obtained. Figure 4.4 shows the impact of mass of KOH added on the percentage yield of biodiesel.

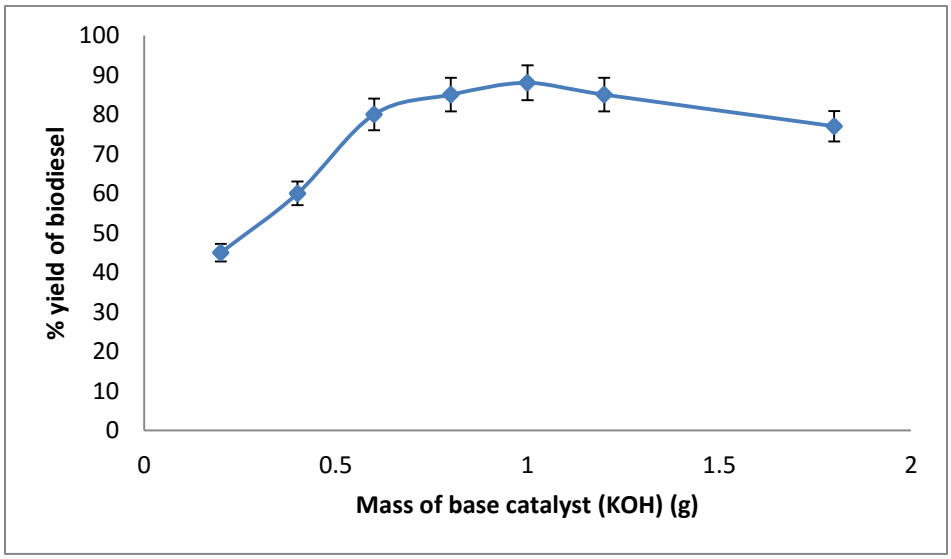


Figure 4.4 Effect of mass of KOH catalyst on yield of biodiesel from 100 g *Croton* oil.

4.2.3 Effect of methanol volume on biodiesel yield

The percentage yield of biodiesel improved steadily with increase in volume of methanol. The maximum yield was obtained at methanol volume of 22 ml, which corresponded to oil: methanol molar ratio of approximately 1:6. Further increase in volume of methanol added to the reaction mixture led to decrease in percentage yield of biodiesel. At lower methanol volume, increased emulsion formation was observed while higher methanol volume resulted in increased soap formation. Figure 4.5 shows the variation of mean percentage yield of Croton biodiesel with volume of methanol used for transesterification.

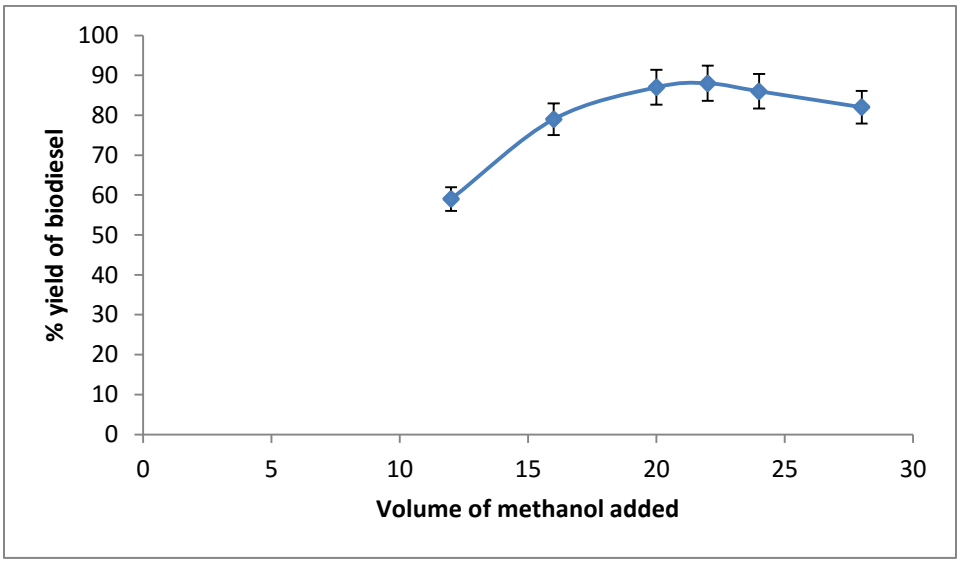


Figure 4.5 Effect of volume of methanol on yield of *Croton* biodiesel.

4.2.4 Effect of temperature and time on esterification

The percentage yield of biodiesel improved with increase in temperature of reaction. The maximum production of biodiesel was achieved after 1 hour at 60 ^{o}C. Although the maximum yield of biodiesel improved with increase in reaction time at 30, 40 and 50 ^{o}C, no significant increase in yield of biodiesel was observed at 60 ^{o}C after 1 hour. At the optimal conditions, the two stage chemical process resulted in a high biodiesel yield of 88% (v/v) with an acid value of 0.336 mg KOH/g. Figure 4.6 shows the variation of yield of Croton biodiesel as a function of time at different reaction temperatures.

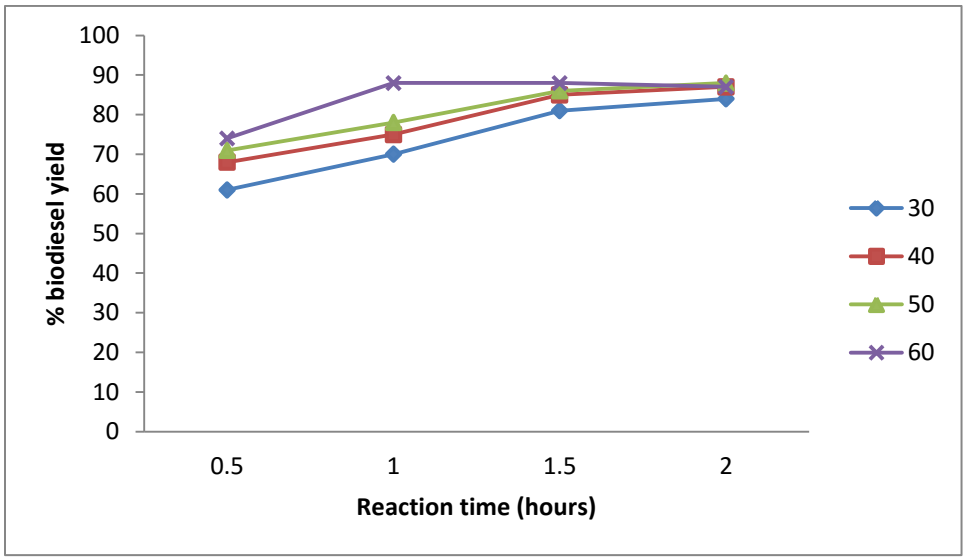


Figure 4.6 Effect of temperature and reaction time on yield of Croton biodiesel

4.3 Optimization of transesterification parameters for lipase catalysed reaction

4.3.1 Effect of volume of lipase catalyst on transesterification

A general increase in yield of biodiesel was observed with increase in volume of lipase catalyst added to the reaction mixture until a maximum yield was achieved at a catalyst concentration of 7.5% (v/v) of Croton oil. Further increase in lipase concentration did not have any effect on the yield of biodiesel. Since lipases normally display their catalytic actions in living organisms, the physiological temperature of 37 ^{o}C, oil:methanol volume of 1:3 was arbitrarily chosen and the reactions allowed to proceed for 2 hours in an orbital shaker. Figure 4.7 shows the influence of volume of lipase catalyst on percentage yield of biodiesel.

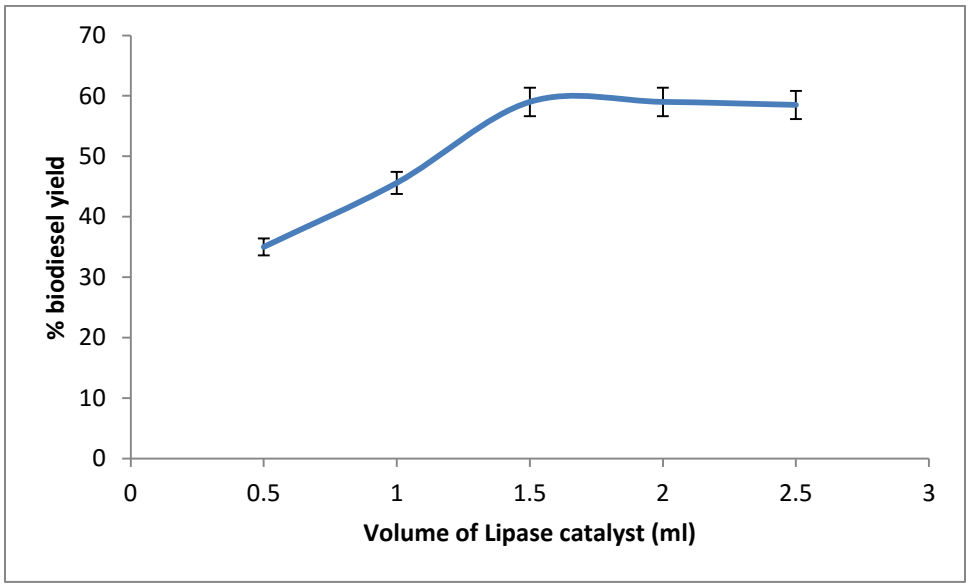


Figure 4.7 Effect of lipase volume on yield of Croton biodiesel

4.3.2 Effect of amount of methanol used on yield of biodiesel in lipase catalysed transesterification

The yield of biodiesel improved with increase in amount of methanol used for tranesterification. The highest production of biodiesel was attained at an oil: methanol mole ratio of approximately 1:6, with further addition of methanol having no appreciable effect on the yield of biodiesel. The physiological temperature of 37 ^{o}C was again employed in these reactions. Although the triglycerides in Croton oil stoichiometrically react with methanol in the ratio of 1:3, the higher ratio can be elucidated by the fact that the reaction is reversible hence according to Le-Chatelier's principle, higher concentration of alcohol is one of the factors that favour the forward reaction. Figure 4.8 shows the variation of percentage yield of Croton biodiesel with volume of methanol used for transesterification.

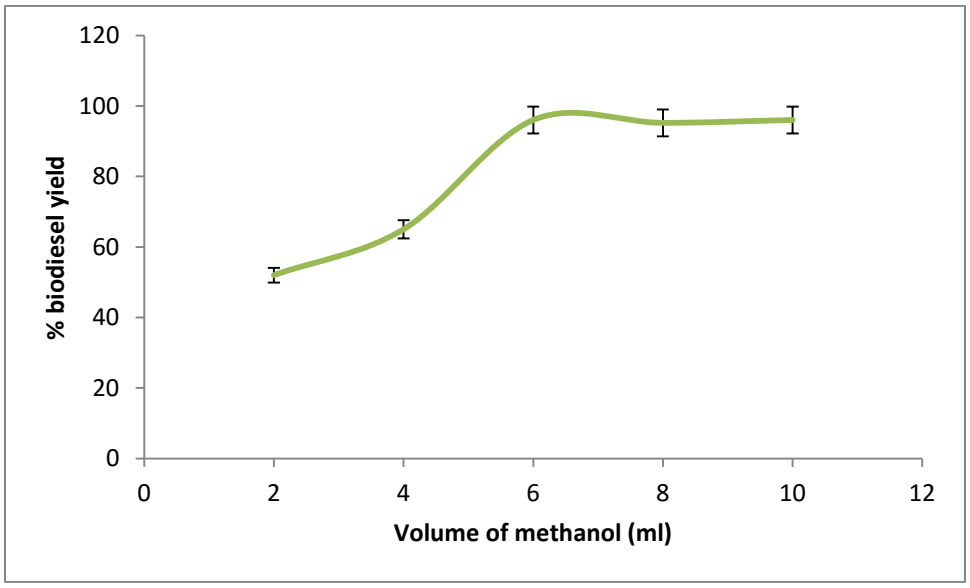


Figure 4.8 Effect of methanol volume on percentage yield of biodiesel in lipase catalysed transesterification

4.3.3 Effect of time and temperature on lipase catalysis

The percentage yield of biodiesel initially increased as the reaction temperature increased. The maximum production of biodiesel was achieved at reaction temperatures of 35 ^{o}C and 40 ^{o}C. Further increase in temperature above 40 ^{o}C led to a drastic decrease in yield of biodiesel. The temperature of 35 ^{o}C was therefore selected as the optimal reaction temperature due to energy considerations. The observations can be explicated by the fact that the lipase enzymes attain their optimal catalytic effects around the physiological temperature of 37 ^{o}C. Although higher temperatures deactivate the active sites on the enzymes, the small reduction in yield of biodiesel at temperatures greater than 37 ^{o}C could be attributed to thermostability of *T. lanuginosus* [Singh *et al.*, 2003]. However, lower temperatures make the reversible reaction to take a longer time before reaching equilibrium [Qin *et al*., 2008]. The use of optimal conditions yielded the maximum yield of biodiesel within a reaction time of 1 hour. Figures 4.9 and 4.10 show the variation of percentage yield of biodiesel with temperature and time respectively.

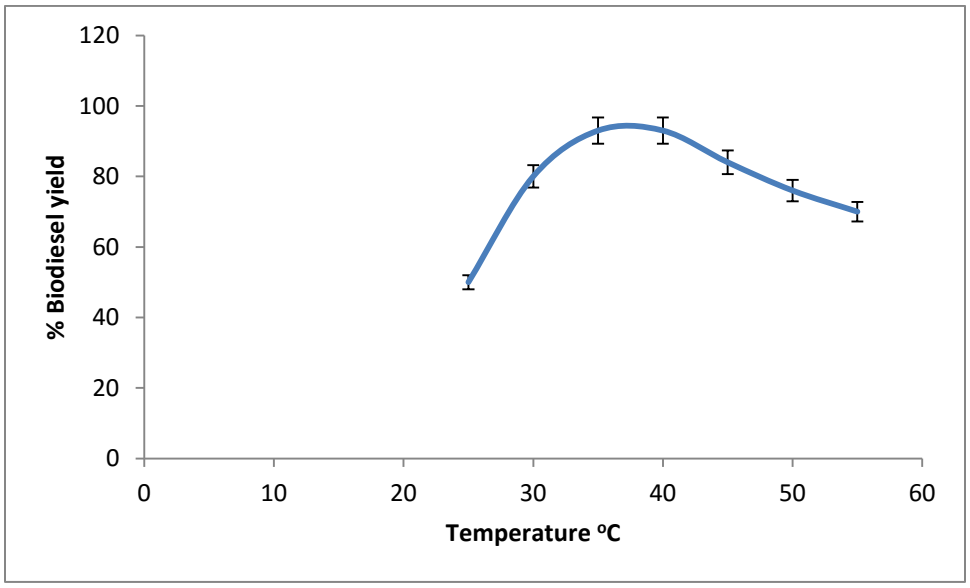


Figure 4.9 Effect of temperature on yield of Croton biodiesel

The low temperatures required for enzymatic transesterification to produce optimum yield of biodiesel could translate to savings in energy and hence reduce the overall cost of production. These observations were in agreement with those stated by Yagiz *et al.*, [2007].

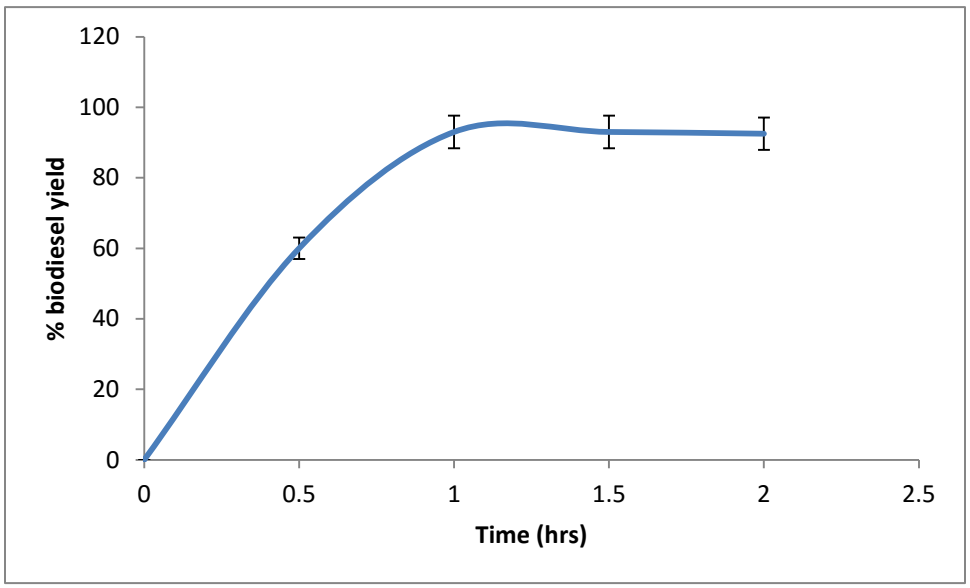


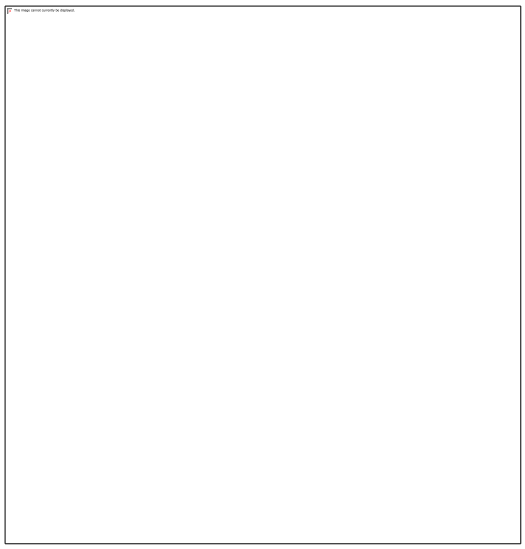
Figure 4.10 Effect of time on lipase catalysis

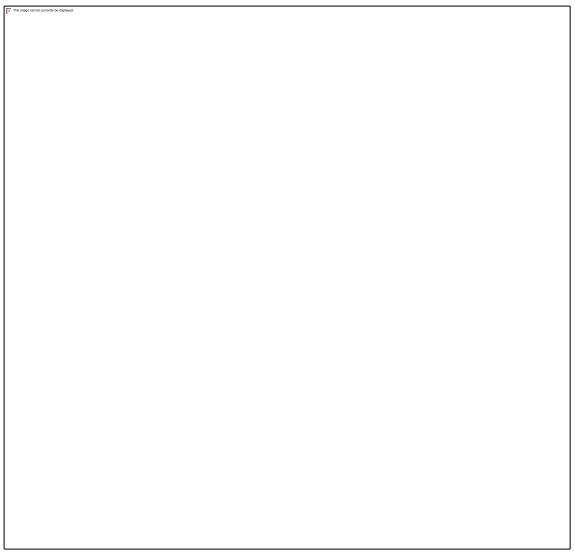
The improved yield of biodiesel in enzyme catalysed process in comparison to the chemically catalysed reaction at optimal conditions can be credited to the stability and high selective nature of the enzymes which leads to more complete reactions and formation of less side products such as alkaline salts [Krishna and Karanth, 2002, Yagiz *et al.*, 2007]. Salis *et al.*, [2005], stated a yield of 100% on transesterification of triolein with butanol using *Pseudomonas cepacia* catalyst in the absence of a solvent. Linko *et al.*, [1998] obtained a yield of 97% during transesterification of rapeseed oil with 2-ethyl-1-hexanol in the presence of *Candida rugosa* powder lipase as catalyst. In addition to the higher yield, the enzymatic process of biodiesel preparation required a shorter time of only 1 hour for the reaction to reach endpoint and did not involve an intermediate step since it is not affected by presence of high FFAs or presence of minimal quantities of water in oil feedstock [Kumar and Kanwar, 2011]. The glycerol produced during enzymatic transesterifications could be readily separated and purified since it was not mixed with alkaline salts as was the case with the chemically catalysed reactions.

4.4 Isolation and activity of extracellular lipase producing microorganisms

4.4.1 Isolation, screening and identification of lipase producing microorganisms

The enriched microbes readily grew on the agar plate with minimum salt media and Croton oil as the main carbon source in an incubator after 12 hours. The microorganisms also produced distinct orange halo around the colonies of microorganisms on the Agar/Rhodamine B plates. The extensive growth of the microorganisms and formation of orange halos indicated that they were able to digest Croton oil and produce lipase enzymes. Figure 4.11a shows growth of the microorganism on the plate while Figure 4.11b shows formation of orange halo around the microorganism colonies on Agar/Rhodamine B plate respectively.



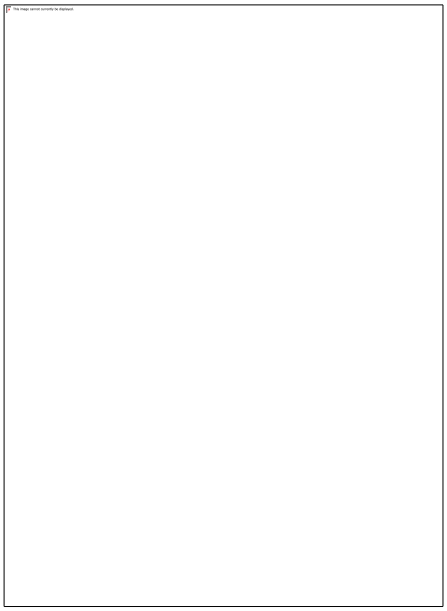


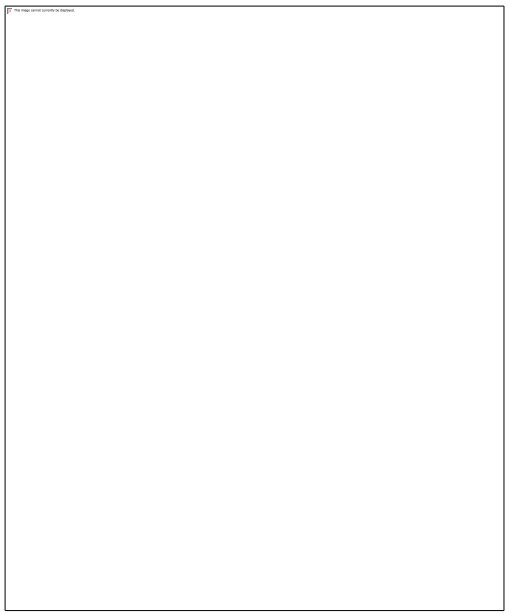
4.11a **4.11b**

Figure 4.11(a) Microorganism on agar plate, (**b)** Halo on Agar/Rhodamine B plate

4.4.2 Identification of lipase producing microorganisms

The gram stained slides of the isolated lipase producing microorganism showed that they were gram positive rod-shaped bacilli. Thakur, [2012], reported that some of the most frequently used microbial lipases include *Bacillus subtilis*, *Bacillus pumilus, Bacillus licheniformis*, *Bacillus coagulans, Bacillus stearothermophilus,* and *Bacillus alcalophilus*. Appendix 1 shows the DNA Sequence for isolated lipase producing microorganism B while Figures 4.12a, b and 4.13 shows the photo of gram stained microscope slide view of a lipase producing microorganism, Gel electrophoresis view of DNA under UV and a phylogenetic tree correspondingly.





4.12a **4.12b**

Figure 4.12(a) Microscope slide view of gram stained 3B, lipase producing microorganisms, **(b)** Gel electrophoresis view of DNA under UV

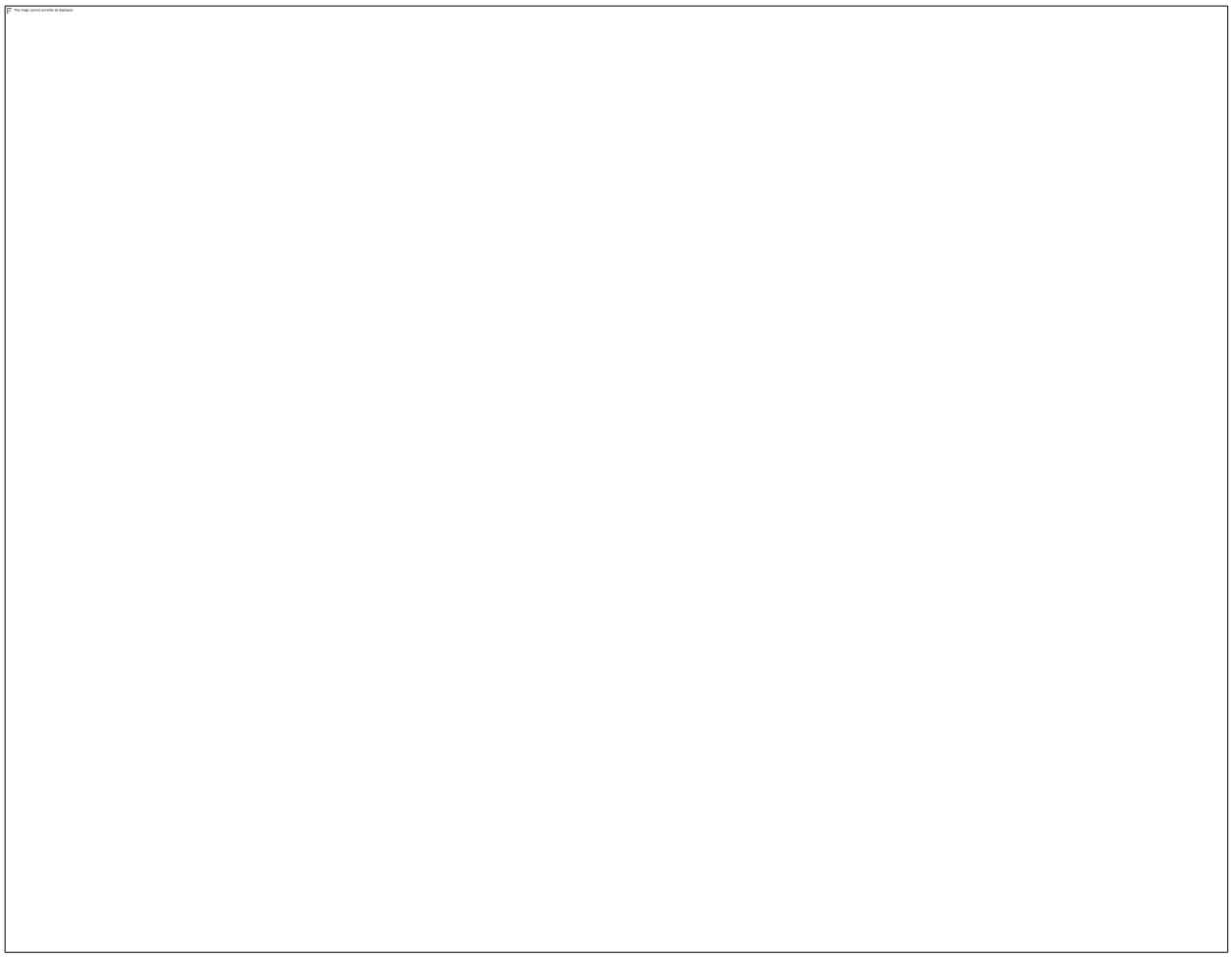


Figure 4.13 Phylogenetic tree for isolated lipase producing microorganisms

4.4.3 Activity of lipase extracted from microorganisms

Biochemical tests such as extensive growth on minimum salt media with Croton oil as the main carbon source and production of halos on Agar/Rhodamine plates showed that the enriched microorganism produced lipases. However, hydrolysis experiments showed that the rate of hydrolysis of croton oil was much slower as compared to that of the commercial enzyme. Both the cells and supernatant solution of the cultured microorganism also poorly catalysed transesterification of Croton oil in the presence of methanol. This observation could be attributed to the slow rate of hydrolysis of the isolated enzymes and the negative effects of methanol on the extracted lipase. Du *et al.*, [2004] reported that methanol has severe harmful effects on the ability

of enzymes to catalyse transesterification of oils and methanol: oil ratio above 1:1 results in serious deactivation of the enzyme. Salis *et al.*, [2005] reported that methanol and 2-butanol produced lower yields as compared to butanol in enzyme catalysed transesterification of triolein. They elucidated that the lower yields were probably due to immiscibility of methanol with triolein and the regio-specificity of enzymes towards primary alcohols.

4.5 TLC and GC - MS Analysis of Croton biodiesel

Both the GC-MS spectra and TLC chromatograms of Croton biodiesel were quite distinct from that of Croton oil. Although the GC-MS spectra of Croton biodiesel obtained from transesterification using commercial enzyme produced very well defined peaks at specific retardation times, that of Croton oil or biodiesel obtained using extracted lipase did not give appreciable pronounced peaks under the same conditions. Appendices 2, 3 and 4 show the GC-MS spectra for Croton biodiesels obtained using commercial and extracted lipase and Croton oil respectively. Figure 4.14 shows TLC chromatogram for Croton methyl esters and Croton oil while Table 4.2 shows results for the GC-MS analysis of Croton biodiesel.

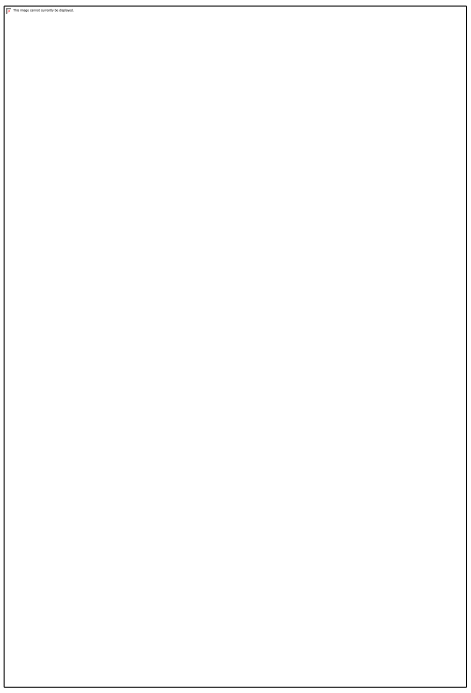


Figure 4.14 TLC chromatogram of biodiesel and Croton oil

A: Chromatogram for Croton biodiesel obtained using commercial lipase, **B**: Chromatogram for Croton oil, **C**: Chromatogram for Croton biodiesel obtained using extracted lipase.

Table 4.2 GC-MS analysis of Croton propyl and butyl esters

Compound	**Formula**	**Retention time (mins)**
Octanoic acid methyl ester	$C_9H_{18}O_2$	1.9
Nonanoic acid, 9-oxo-, methyl ester	$C_{10}H_{18}O_3$	3.2
7-Nonenoic acid methyl ester	$C_{10}H_{18}O_2$	4.6
7-Hexadecenoic acid, methyl ester.	$C_{17}H_{32}O_2$	8.0
Hexadecanoic acid, methyl ester.	$C_{20}H_{34}O_2$	8.2
13-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	13.3
11-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	13.5
Tridecanoic acid, methyl ester	$C_{14}H_{28}O_2$	13.7
Methyl stearate	$C_{19}H_{38}O_2$	13.9
Decanoic acid methyl ester	$C_{11}H_{22}O_2$	23.6

GC–MS analysis showed that the Croton biodiesel was composed of both saturated and unsaturated methyl esters with carbon atom chain lengths ranging from C-9 to C-19. The presence of unsaturated methyl esters in Croton biodiesel implied that it can readily undergo oxidation on storage, which can lead to a deterioration of its fuel properties. The results obtained from GC-MS analysis in this study were comparable with those stated by Karavalakis *et al.,* [2010] and kivevele *et al.,* [2011a] which showed that Croton biodiesel consists of a great portion of polyunsaturated fatty acid methyl ester (about 78%). This makes croton rather vulnerable to oxidation [Osawa *et al.,* 2015]. Figures 4.15a, b and c show MS spectra of three methyl esters that were detected during the analysis of the biodiesel.

Figure 4.15a MS Spectrum for Octanoic acid methyl ester

Figure 4.15b MS Spectrum for Nonanoic acid methyl ester

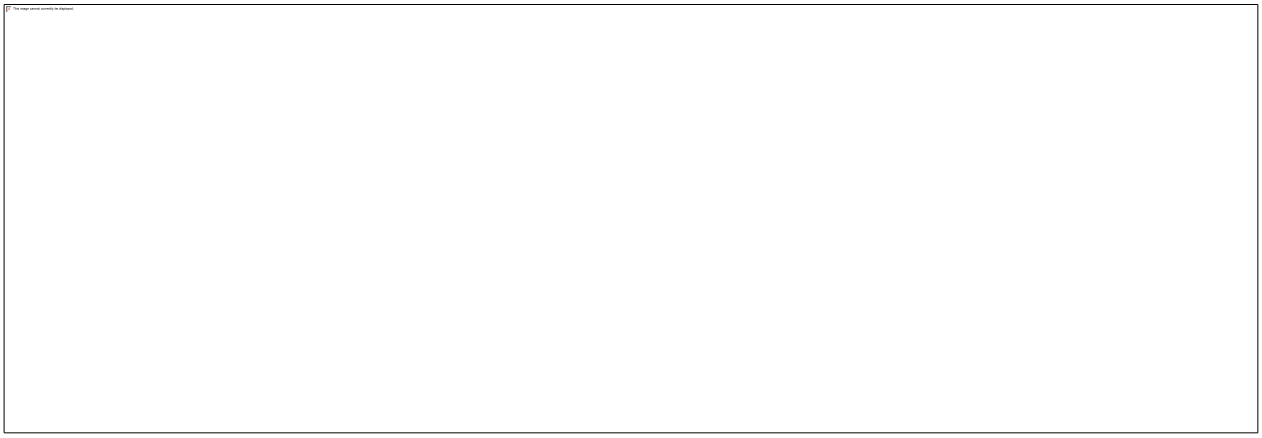


Figure 4.15c MS Spectrum for Octadecenoic acid methyl ester

4.6 Engine performance and emission characteristics of Croton methyl ester blends

The performances of biodiesel blends were tested by comparing the impact of the blends on various engine variables like brake thermal efficiency, brake specific energy consumption, exhaust gas temperature and fuel flow rate with those of petrodiesel. The nature and levels of exhaust gases and smoke emissions for petrodiesel and biodiesel blends were determined using exhaust gas and smoke analysers respectively. The effects of load and amount of biodiesel in the blends on the engine performance variables and emission attributes are described in the following sections.

4.6.1 Exhaust gas temperature

The temperature of engine exhaust emissions are directly proportional to engine temperature, and can, therefore, be used as an estimate of heat liberated during combustion in an engine. The temperature of exhaust gases increased steadily with rise in load applied to the engine due to increased fuel flow. In certain cases, a slight increase followed by decrease in temperature was recorded at particular engine loads with rising amount of biodiesel in the blends. These observations could be partly attributed to increased engine pressure resulting from improved combustion as a result of availability of oxygen in the biodiesel molecules. Figure 4.16 shows the variation of exhaust emission temperature with load for biodiesel blends and petrodiesel.

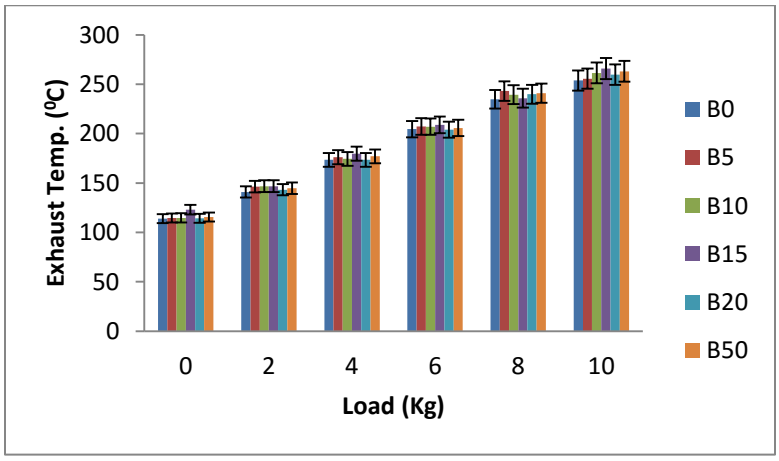


Figure 4.16 Variation in engine exhaust emission temperature with load.

These observations were comparable to those reported by Savariraj *et al.*, [2011], who found out that the temperature of exhaust gases for diesel and all blends of Mahua biodiesel rose with increase in engine load.

4.6.2 Fuel flow rate

The fuel flow rate for both petrodiesel and biodiesel blends rose steadily with increase in load due to extra energy required to overcome the increased load. At maximum load, the fuel flow rate for the B50 blend was 9.8% higher than that of petrodiesel. Figure 4.17 shows the variation in engine fuel flow rate with increasing engine load.

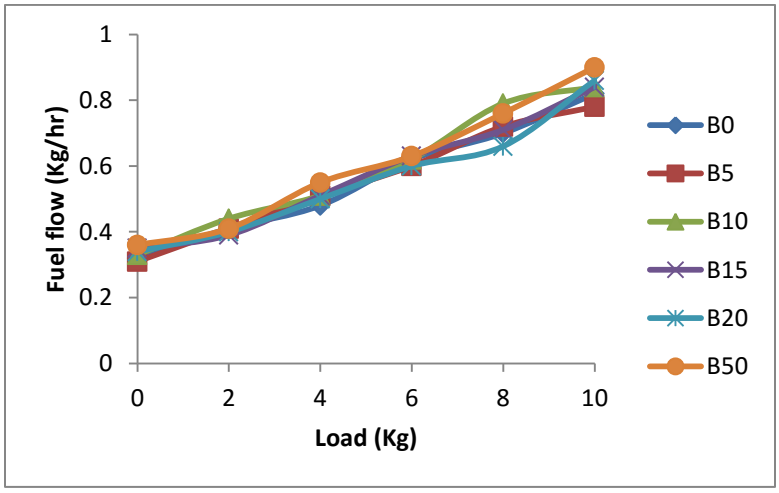


Figure 4.17 Variation in engine fuel flow rate with load.

These results were comparable to those stated by Shah *et al.*, [2009a], who found out that the specific fuel consumption of engine increased with upsurge in load for both diesel and commercial biodiesel blends. They also noted that the engine became more economical when fuelled with biodiesel.

4.6.3 Brake thermal efficiency

The brake thermal efficiency (BTE) shows the extent to which an engine transforms heat energy from burnt fuel to mechanical energy of motion. A general increase was observed in the BTE with an upsurge in load for both petrodiesel and biodiesel blends. At intermediate load of 4 Kg, all the BTE values for biodiesel blends were greater than that of petrodiesel. However, at

maximum load, the BTE of petrodiesel was the highest while that of the blend B50 was the lowest. A maximum difference of 4.9% was recorded between the BTE of petrodiesel and the B50 blend. Figure 4.18 shows the variation in BTE for biodiesel blends and petrodiesel with increasing engine load.

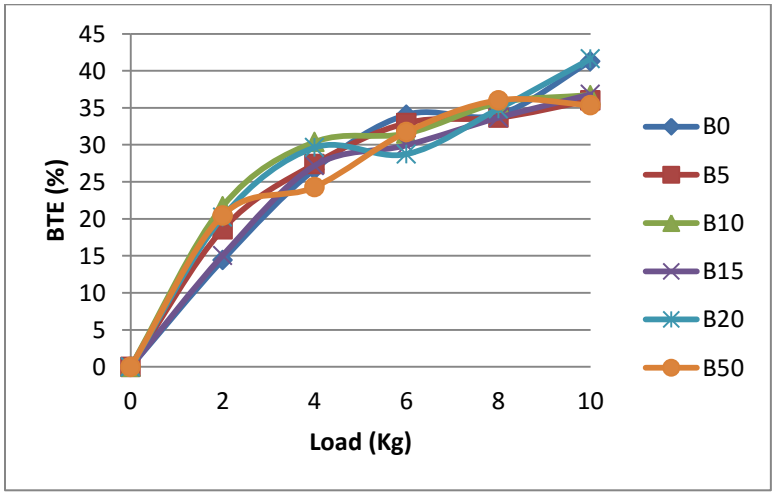


Figure 4.18 Variation in brake thermal efficiency with load.

The slightly lower values of BTE for the biodiesel blends could be explained by their lower calorific values which result in poor atomization and poor combustion. Dwivedi *et al.*, [2011], stated that the BTE of *Jatropha* methyl ester (biodiesel) blends displayed a similar trend with load and was almost the same as that of diesel at all loads.

4.6.4 Brake specific energy consumption

The Brake specific energy consumption (BSEC) refers to the mass of fuel consumed per unit power produced. The BSEC is reliant on viscosity, density and calorific value of fuel. Therefore, as the density increases and calorific value reduces, more fuel is required to provide equal power output [Dwivedi *et al.*, 2011].

The BSEC for both petrodiesel and biodiesel blends steadily dropped with increase in engine load. Deepali *et al.*, [2012] reported a similar trend in BSEC in a four stroke, single cylinder diesel engine using biodiesel blends mixed with various quantities of n-butanol. Figure 4.19 shows the variation of BSEC with load.

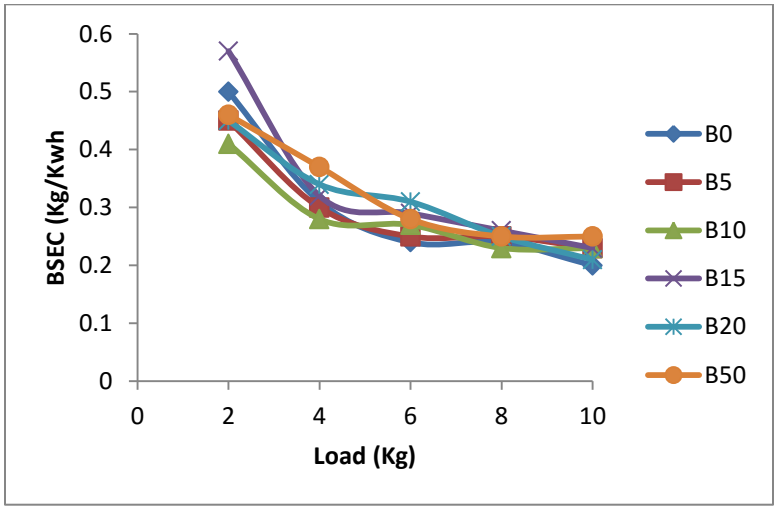


Figure 4.19 Variation in brake specific energy consumption with load.

The similar trends in variations of BSEC for Croton biodiesel blends and diesel could be explained by availability of oxygen atoms in biodiesel molecule which compensates for the inferior calorific value, greater density and viscosity by improving combustion of biodiesel.

4.6.5 Carbon monoxide (CO)

The highest levels of CO exhaust emissions were recorded at reduced engine loads but eventually declined at moderate engine loads. At higher engine loads, there was lower difference in the levels of CO exhaust emissions for petrodiesel and the corresponding biodiesel blends. The slight increase in CO levels at higher engine loads could be attributed to the rich fuel/air mixture that is injected into the engine to overcome the higher loads [Stone, 1999]. Figure 4.20 shows the variation of CO emissions with engine load.

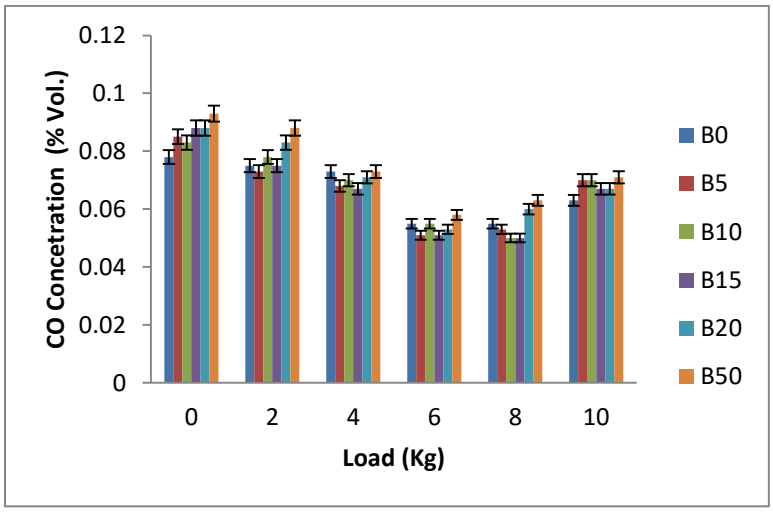


Figure 4.20 Variation in exhaust CO emission with load.

4.6.6 Nitrogen oxides (NO_x)

Nitrogen oxides are formed due to the reaction between nitrogen and oxygen at high temperature and pressure generated in the engines during combustion of fuel. A steady rise in NO_x exhaust discharge was noted with increase in load applied to the engine. A steady increase in levels of NO_x exhaust emissions was also observed for each particular engine load with increase in biodiesel concentration in the blends. These observations could be explained by increased engine temperature and pressure which led to increased reaction between nitrogen and oxygen. The increased fuel flow into the engine and the fact that biodiesel molecules contain extra oxygen atoms which took part in the combustion process could also be a contributing factor to the increased NO_x emission. These observations were consistent to the results reported by Taher *et al.*, [2011]. Figure 4.21 shows the variation of NO_x emissions with load.

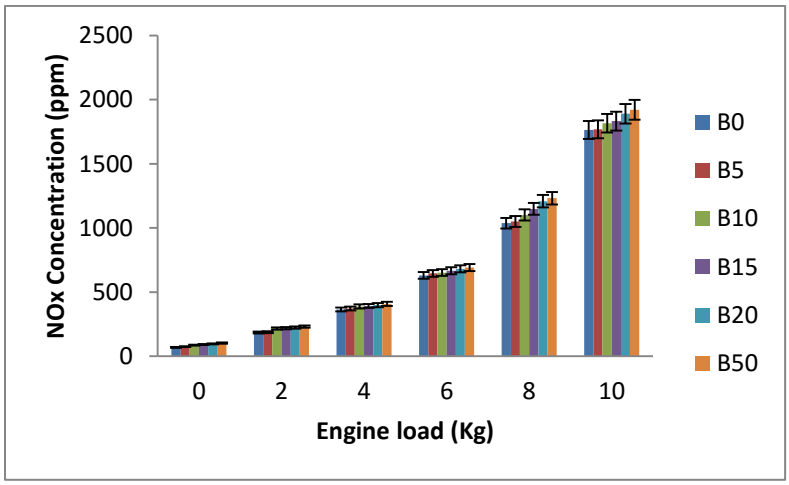


Figure 4.21 Variation in exhaust NO_x emission with load.

4.6.7 Hydrocarbons

The levels of hydrocarbon emissions initially declined with upsurge in engine load for both petrodiesel and biodiesel blends. Further rise in load (> 4 Kg) resulted to an increase in hydrocarbon emissions. It was also noted that at reduced engine loads, an increase in amount of biodiesel in blends led to an upsurge in hydrocarbon emission while at higher loads, a rise in amount of biodiesel in the blends led to a decrease in hydrocarbon emission. These observations could be attributed to the fact that at lower engine loads, lower engine temperatures resulted in poor combustion of biodiesel while at higher engine loads, increased combustion of the biodiesel took place owing to increased engine temperature and pressure. The general rise in hydrocarbon emission at increased engine load could however, be due to the rich fuel/air mixture flow into the engine. Figure 4.22 shows the variation in hydrocarbon emissions.

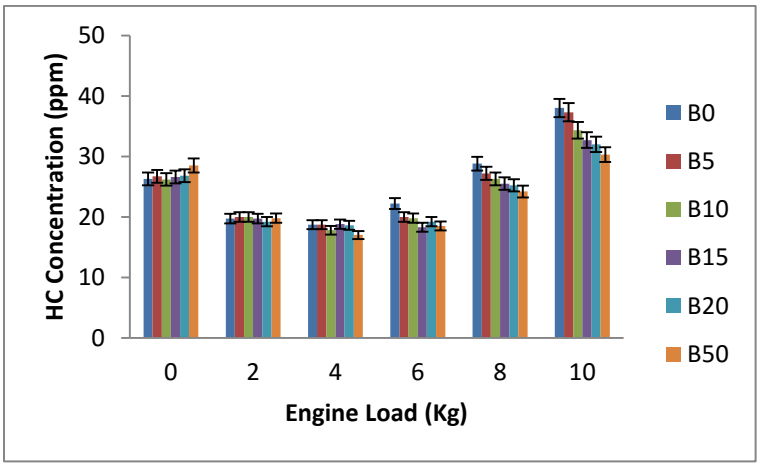


Figure 4.22 Variation in exhaust hydrocarbon emission with load.

These observations are comparable to those stated by Savariraj *et al.*, [2011] and Stone, [1999]. Kivevele *et al.*, [2011a] also observed a similar trend for petrodiesel and Croton biodiesel blends.

4.6.8 Smoke

The levels of exhaust smoke emissions declined with rise in amount of biodiesel in blends. This pattern could be explained by the presence of about 11% oxygen in the biodiesel molecules [Canakci and Van Gerpen, 2001] which results in improved combustion and hence less particulate matter (smoke). However, a general rise in smoke emissions was noted with increase in engine load due to increased fuel flow into the engine. According to Stone, [1999], the formation of smoke increases with load and fuel injected due to the increase in duration of diffusion combustion, increase in combustion temperature and low oxidation of soot during the expansion stroke due to little time after the end of diffusion combustion and also limited amount of oxygen as a result of rich fuel-air mixture.

At maximum load, a reduction of 41, 31, 26, 15 and 10% in smoke emissions were recorded for B50, B20, B15, B10 and B5 blends, correspondingly. Prabhahar *et al.*, [2012] reported a decline of 25% in smoke emissions for B20 Pongamia biodiesel blend while a similar trend was reported

for Croton biodiesel and its blend by Kivevele *et al.*, [2011a]. Schumacher *et al.*, [2001] and Dermibas, [2008] also reported significant decrease in smoke (PM) emissions for biodiesel. Figure 4.23 shows the variation of smoke emissions for different biodiesel blends and petrodiesel at various engine loads.

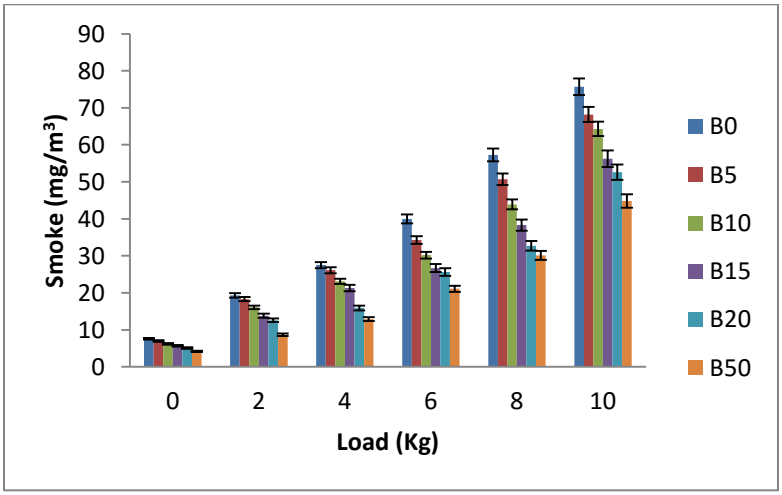


Figure 4.23 Variation in exhaust smoke emission with load.

4.6.9 Engine pressure

There was minimal difference in maximum engine pressure for both petrodiesel and biodiesel blends at engine load of 0 Kg. The maximum engine pressure improved with rise in load owing to increase in fuel flow rate and higher engine temperature on combustion. The engine pressure generally rose with upsurge in amount of biodiesel in the blends as the engine was subjected to increased load. The maximum engine pressure at maximum load of 10 Kg for the B50 Croton blend was 5.32% higher than that of petrodiesel. The increase in engine pressure with increase in concentration of biodiesel blends could be explained by the higher engine temperature which increases the rate of expansion of gases in the engine. Figures 4.24a and 4.24b show the variation of pressure with crank angle around the ignition point at 0 and 10 Kg engine loads respectively.

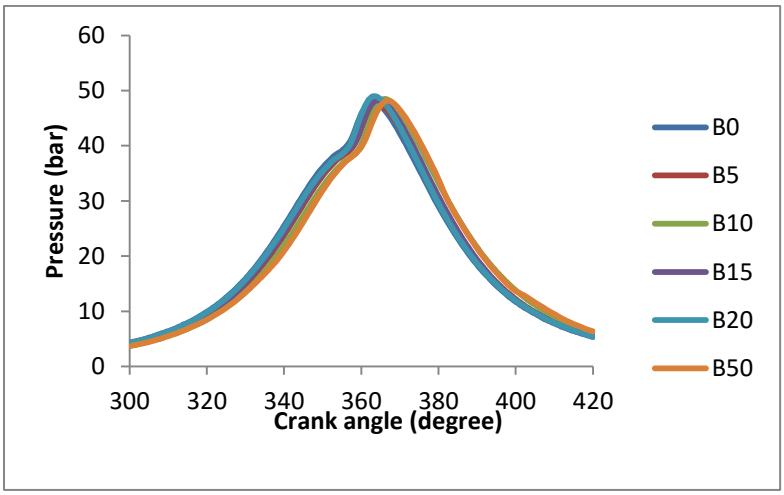


Figure 4.24a: Variation in engine pressure against crank angle at 0 Kg load.

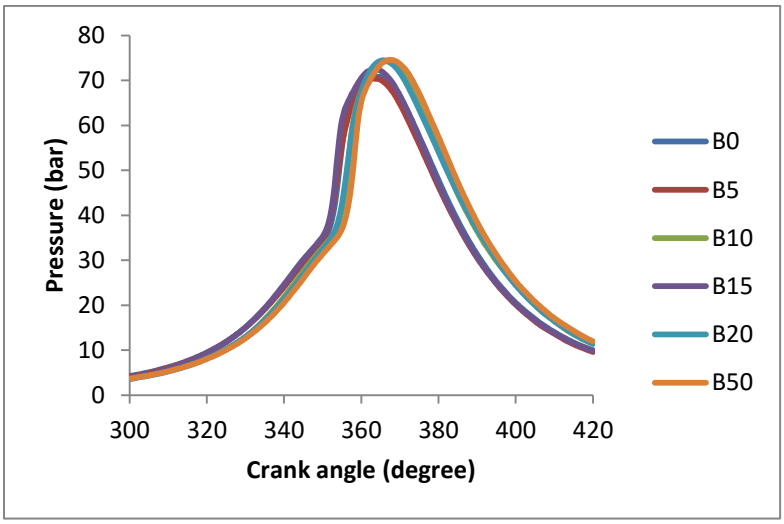


Figure 4.24b Variation in engine pressure against crank angle at 10 Kg load.

Shah *et al.*, [2009a] reported a similar trend in variation of engine pressure against crank angle for various biodiesel blends. They also reported a rise of 4.2 and 1.0% in the engine's cylinder peak pressure with B100 and B20, correspondingly, compared to diesel. They concluded that the biodiesel blends, B100 and B20 improved the ignition in the engine by boosting the highest ignition pressure of the engine.

4.6.10 Heat released by engine

A general increase in maximum heat released by the engine was noted with rise in amount of biodiesel in the blends. The peaks for maximum heat released by engine fuelled with biodiesel blends lagged behind that of petrodiesel, implying that there was delay in ignition of the biodiesel blends as compared to petrodiesel. The delay in ignition of the biodiesel blends was probably due to poor atomization of the fuel. The effects of delay in ignition of the biodiesel blends could be minimized by adjusting the injection time (angle). The variation in heat released against the crank angle around the ignition point for 0 and 10 kg engine loads are shown in figures 4.25a and 4.25b respectively.

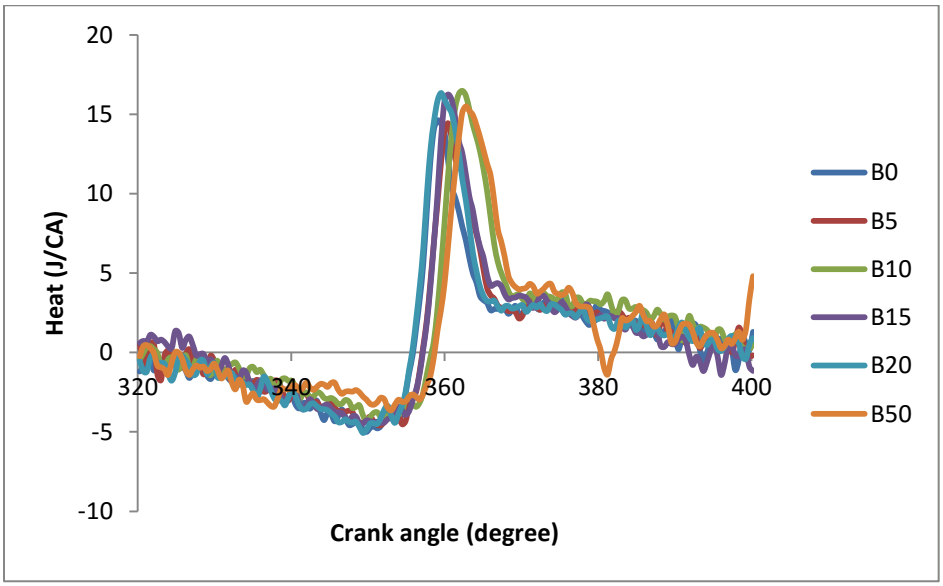


Figure 4.25a Variation in heat released against crank angle at 0 Kg load.

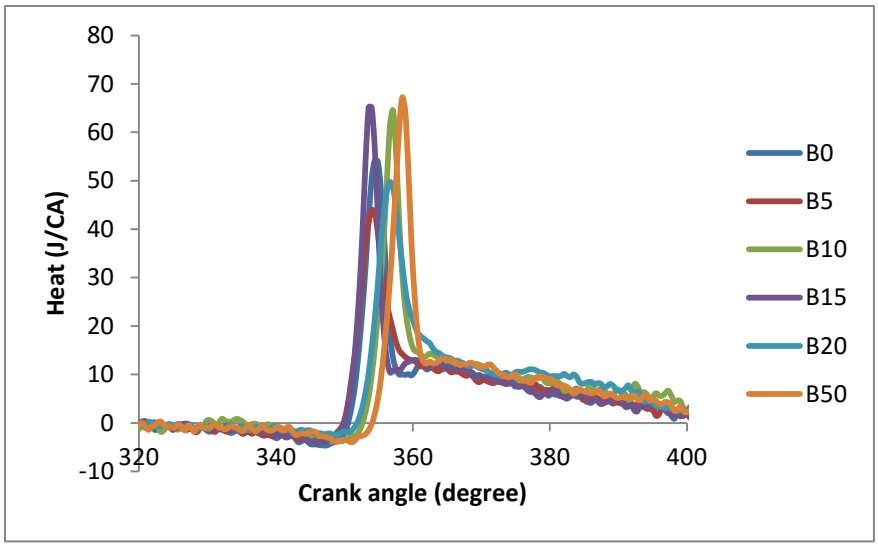


Figure 4.25b Variation in heat released against crank angle at 10 Kg load.

Zhu *et al.*, [2010] reported a similar trend in variation of heat released in an engine fuelled with methanol-biodiesel blended fuels. They also showed that the peak heat released by the engine increased with rise in load applied to engine from low to moderate, but declined at high engine load for all test fuels and occurred fairly near the top dead centre with increase in engine load.

4.7 Effect of antioxidants on oxidative and storage stability of Croton biodiesel

4.7.1 Effect of antioxidants on oxidative stability of freshly prepared neat biodiesel

Figure 4.26 shows the impacts of the five antioxidants on oxidation stability of Croton biodiesel (B100). It was observed that the oxidation stability index (OSI) of the Croton biodiesel satisfied the ASTM-6751 limit of 3 hours. However, it did not meet the EN-14112/IS-15607 standard of 6 hours. The low oxidation stability of Croton biodiesel is attributable to the high proportion of polyunsaturated fatty acid methyl ester (about 78%). Consequently, this makes it very susceptible to oxidation [Kivevele *et al.*, 2011a, Osawa *et al.*, 2015].

Overall, it was observed that all the antioxidants tested in this study enhanced the oxidation stability of the biodiesel. This was demonstrated by the higher Rancimat induction periods (IPs)

for Croton biodiesel with the antioxidants studied. Of particular interest, pyrogallol (PYG) showed the highest increase in IP followed by propyl gallate (PRG), butylated hydroxyanisol (BHA), Butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ). Karavalakis *et al.*, [2010] reported a similar trend for pyrogallol and propyl gallate antioxidants. As shown in figure 4.26, the Rancimat IPs for Croton biodiesel with either *tert*-butylhydroquinone (TBHQ) or Butylated hydroxytoluene were both lower than the European (EN-14112/IS-15607) recommended value of 6 hours for biodiesel [Osawa *et al.*, 2015].

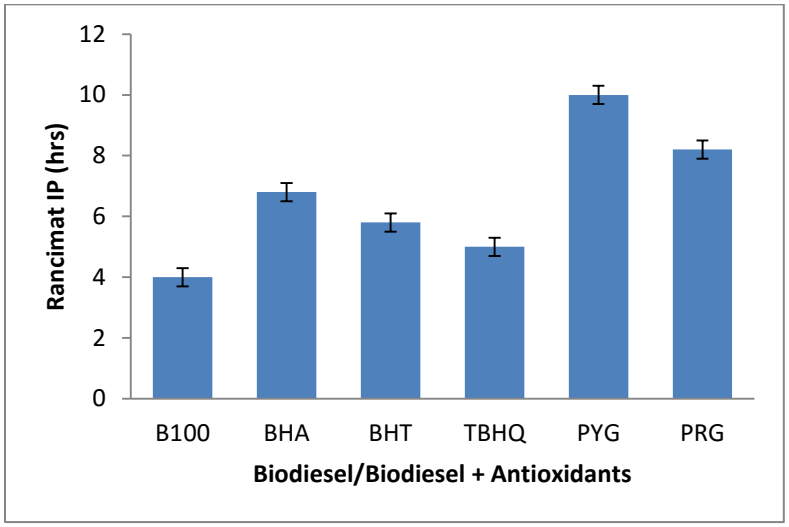


Figure 4.26 Rancimat induction periods for Croton biodiesel with various antioxidants

Liang *et al.*, [2006] reported that the antioxidant abilities of phenolic antioxidants depends on the number of -OH groups occupying positions 1, 2 or 1, 4 in the aromatic ring. In addition, the capacity and electronic properties of the ring substituent are important factors to consider. They stated that the functional -OH groups give protons which prevents accumulation of free radicals or interferes with proliferation of free radical and hence interrupt the speed of oxidation.

The lower antioxidant ability of BHA as compared to PYR and PRG could be due to the fact that BHA has only one hydroxyl group directly bonded to the aromatic ring. However, both PYR and PRG each have three –OH groups, two of which are on positions 1 and 2 in the aromatic ring. The presence of more -OH groups directly bonded to the aromatic ring in PYG and PRG provides more sites for reaction with free radical. Consequently, this prevents the oxidation of the methyl ester chain of biodiesel [DeGuzman *et al.,* 2009, Osawa *et al.*, 2015].

Although TBHQ also has –OH groups bonded to the aromatic ring on the 1, 4 positions, its lower antioxidant activity as compared to BHA could be linked to undesirable prooxidant interaction with biodiesel and blends, which can result to a decline in the oxidation stability of biodiesel [Kivevele *et al*., 2011b]. The Rancimat IPs for Croton biodiesel with either *tert*-butylhydroquinone (TBHQ) or Butylated hydroxytoluene were both lower than the European (EN-14112/IS-15607) recommended value of 6 hours for biodiesel [Osawa *et al.,* 2015].

4.7.2 Effect of 100 and 1000 ppm antioxidants on oxidative and storage stability of biodiesel.

The initial Rancimat IPs for biodiesel with 100 ppm antioxidants were 5.6, 6.8 and 7.8 hours for BHA, PRG and PYG respectively while those for the biodiesel with 1000 ppm antioxidants were 6.8, 8.2 and 10 hours for PYG, PRG and BHA respectively. Thus an increase in concentration of antioxidants led to improved oxidation stability.

The oxidation stabilities of all the samples decreased with increase in storage period. The greatest percentage decrease in Rancimat IP of 45% during the eight weeks storage period was recorded on the biodiesel without antioxidants. The Rancimat IPs for the biodiesel with 1000 ppm antioxidants depreciated by 16.0, 12.2 and 20.6% while those with 100 ppm antioxidants depreciated by 18.0, 14.3 and 25.0%, for PYG, PRG and BHA correspondingly. Thus a more

rapid decline in the oxidation stability was observed in the biodiesel without antioxidants than that with antioxidants. Figure 4.27 shows the effects of storage and addition of 100 and 1000 ppm antioxidants on storage stabilities of neat biodiesel, B100 [Osawa *et al.*, 2015].

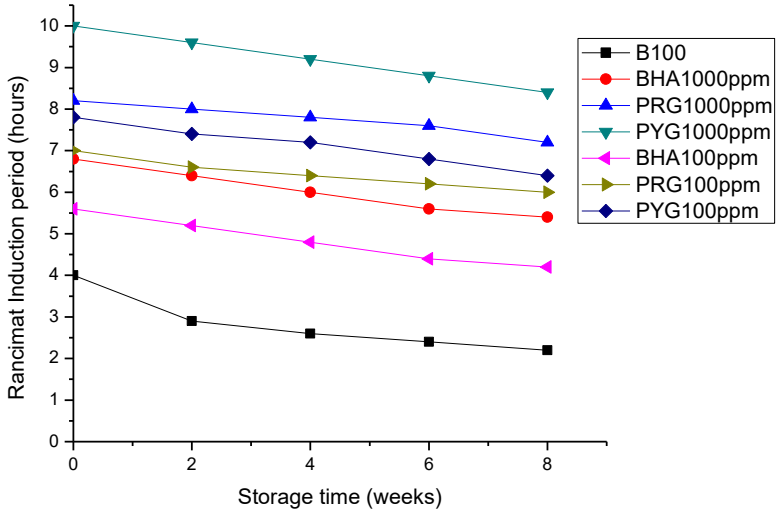


Figure 4.27: Effect of 100 and 1000 ppm antioxidants on storage stability of Croton biodiesel

4.7.3 Effect of 100 and 1000 ppm antioxidants on storage stability of B50 blend.

The B50 blend which originally had a Rancimat IP of 6 hours depreciated by 23.33% to a Rancimat IP of 4.6 hours during the eight weeks storage period. The Rancimat IPs for the B50 blends with 100 ppm antioxidants varied between 9.6 – 8, 13.8 – 13 and 16 – 13.8 hours for BHA, PRG and PYG correspondingly while the blends with 1000 ppm antioxidants varied between 17 – 14, 23.4 – 20.2 and 25.6 – 19.6 hours for BHA, PRG and PYG respectively during the eight weeks storage period. Both the 100 and 1000 ppm antioxidants solutions provided effective stability for the B50 blend since the final Oxidation Stability Index (OSI) was greater than the minimum recommended value of 6 hours at the end of the eight weeks storage period.

Figure 4.28 shows the effects of storage and addition of 100 ppm and 1000 ppm antioxidants on storage stability of biodiesel blend, B50 [Osawa *et al.*, 2015].

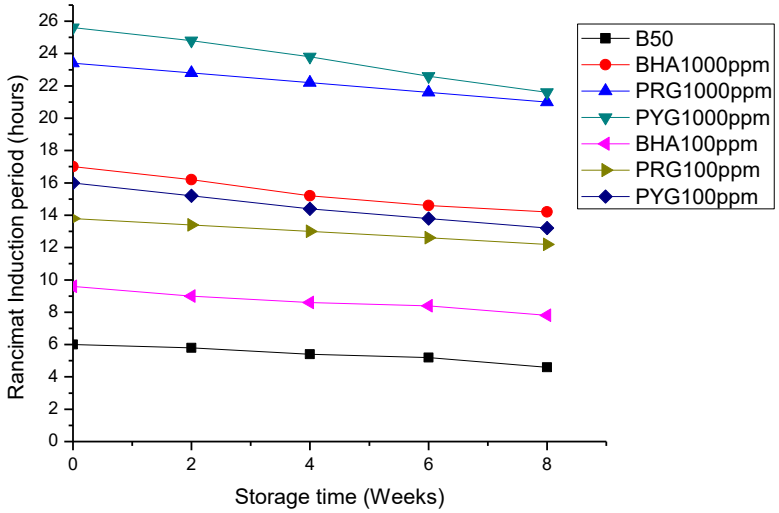


Figure 4.28 Effect of 100 and 1000 ppm antioxidants on storage stability of B50 Croton biodiesel blend

4.7.4 Effect of 100 ppm antioxidants on storage stability of B20, B15 and B10 blends.

The oxidation stability of all biodiesel blends declined with increase in storage period. The Rancimat IP for the biodiesel blends B20, B15 and B10 without antioxidants depreciated by 17.2, 15.5 and 14.4% respectively, during the 8 weeks storage period. The percentage decrease in oxidative stability dropped with decrease in concentration of biodiesel in the blends due to increased stability of petrodiesel. A more rapid drop in oxidative stability was observed in biodiesel blends without antioxidants than those with antioxidants [Osawa *et al.*, 2015]. These results were consistent with those reported by Karavalakis *et al.*, [2010]

Although pyrogallol provided the greatest initial improvement in oxidation stability, the rate of depreciation of biodiesel blends with pyrogallol was slightly faster than those with propyl gallate.

It was also observed that the biodiesel blends with pyrogallol turned dark on storage. Figures 4.29, 4.30 and 4.31 show the impacts of antioxidants and storage on oxidative stabilities of B20, B15 and B10 blends respectively. The petrodiesel used for blending in this study had Rancimat IP of 37.6 hours. Thus both 100 ppm pyrogallol and propyl gallate were able to effectively maintain the oxidation stability of the B10 blend above that of petrodiesel during the eight weeks storage period [Osawa *et al.*, 2015].

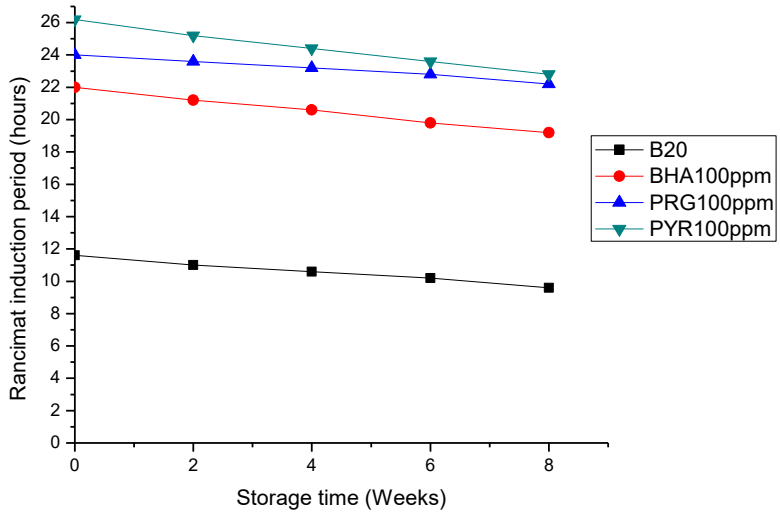


Figure 4.29 Effect of 100 ppm antioxidants on storage stability of B20 Croton biodiesel blend

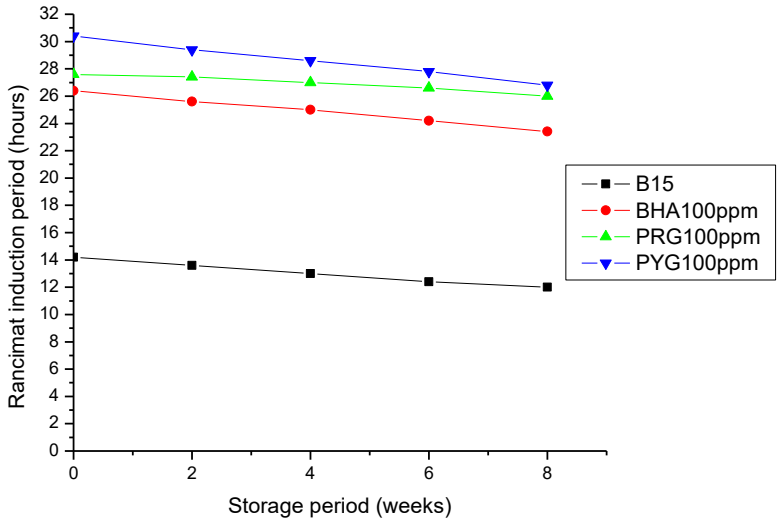


Figure 4.30 Effect of 100 ppm antioxidants on storage stability of B15 Croton biodiesel blend

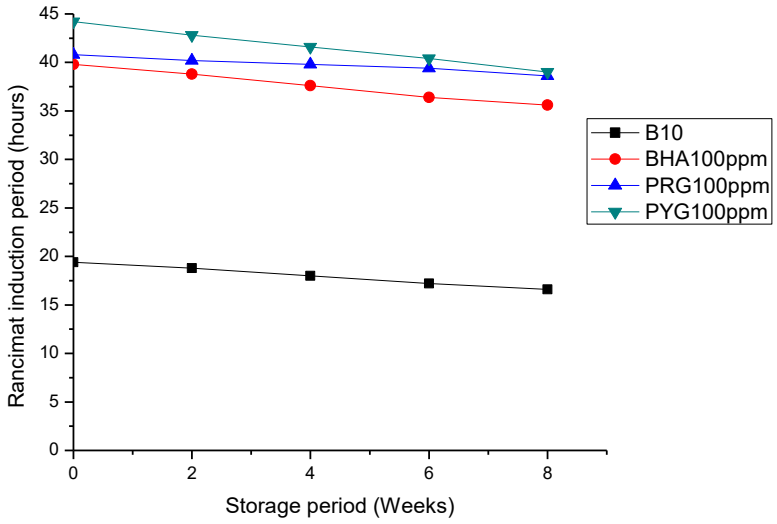


Figure 4.31 Effect of 100 ppm antioxidants on storage stability of B10 Croton biodiesel blend

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

The two-stage chemical procedure for preparation of biodiesel using croton oil resulted in a high yield of 88% (v/v) at optimum conditions. In addition to the higher yield, the biodiesel obtained separated readily from the glycerol layer and required little washing due to minimal soap and emulsion formation. However, at non-optimal conditions, a lower yield of biodiesel and increased formation of soap and emulsion which required extra washing was obtained. The enzymatic process produced a higher yield (95% v/v) of biodiesel than the two stage process due to the specificity of enzymes and non-formation of alkaline salts. However, both the two-stage chemical and enzymatic process of biodiesel preparation greatly improved the fuel properties of Croton oil in terms of physicochemical properties such as acid value, density and viscosity.

The pour and cloud points of Croton biodiesel were both lower than those of petrodiesel thus making the biodiesel and its blends more suitable for lower temperature operations. The acid value of 0.336 mg KOH/g for the biodiesel obtained from the two-stage chemical process was much lower than both the recommended ASTM and EN values of 0.8 and 0.50 mg KOH/g respectively, implying that the biodiesel has relatively minimal corrosive effects on the engine. The higher flash point of biodiesel and blends showed that they were much safer to store and transport than petrodiesel. All the physicochemical properties of biodiesel investigated in this study were within recommended ASTM and EN levels. The results from this study also demonstrated that lipases from *T. lanuginosus* were more effective in catalyzing transesterification of Croton oil than the conventional alkali catalyst. The use of a lipase resulted in a higher yield of biodiesel within a shorter duration than the conventional alkali and acid

catalysts. Despite their high costs, immobilized lipases could be used repeatedly and hence lower the overall production cost of biodiesel.

The levels of exhaust smoke emissions declined with rise in amount of biodiesel in the blends owing to enhanced ignition. The slight differences observed in the performance parameters such as Brake thermal efficiency, Brake specific energy consumption and fuel flow rate showed that the engine performance characteristics of Croton biodiesel blends were similar to those of petrodiesel at all engine loads. A significant decrease in exhaust smoke emissions ranging from 10 to 41% was recorded while a small rise was observed in the NO_x emissions with increase in amount of biodiesel in the blends. A general rise in engine pressure and heat released was noted with upsurge in amount of biodiesel in the blends. The results from this investigation indicated that high blends of Croton biodiesel of up to B50 displayed similar engine performance as petrodiesel and could therefore effectively act as an alternative to petrodiesel. Since *Croton megalocarpus* readily grows in many parts of Kenya, the non-edible plant seeds can be readily used for commercial preparation of biodiesel.

Higher exhaust emissions of HC and CO that were observed with biodiesel blends could be minimized by operating with lean fuel mixtures, fitting the vehicles with exhaust gas catalytic reactors to complete the oxidation process or admitting extra air into the cylinders. The higher levels of NO_x productions from engines fuelled with biodiesel blends could be mitigated by reducing combustion temperature or duration of combustion. The higher peak pressure and temperature, which contributes to higher NO_x emission could be reduced by retarding ignition while the amount of residual gas in the cylinder may be increased by exhaust gas recirculation

(EGR) which reduces the speed and flame temperature but results in significant reductions in levels of NO_x. Catalytic converters could also be employed to reduce the levels of NO_x emission.

The oxidation stability of Croton biodiesel satisfied the commonly used American standard, ASTM D-6751 of 3 hours, however, it did not satisfy the European standard, EN-14214 Rancimat induction period standard of 6 hours. The oxidation stability of the freshly prepared neat biodiesel also reduced at a very fast rate of 45% during the eight weeks storage period to an oxidation stability index of 2.2 hours. The lower oxidation stability and the rapid deterioration in its oxidation stability with storage showed that it requires some improvement through the addition of adequate concentration of antioxidants. The B50 and lower blends of the biodiesel had Rancimat IP values equal to or greater than the minimum recommended value of 6 hours. However, the Rancimat IP of the B50 blend reduced from 6.0 to 4.6 hours over the 8 weeks storage period [Osawa *et al.*, 2015].

The oxidation stability of Croton biodiesel and blends with no antioxidants depreciated more rapidly than those with antioxidants. The addition of antioxidants greatly enhanced the stability of the biodiesel and its blends. Among the three antioxidants investigated in this study, pyrogallol was the most effective in enhancing the oxidation stability of the biodiesel and blends since it resulted in the highest increase in oxidation stability followed by propyl gallate and butyl hydroxyanisole. However, the rates of depreciation of oxidation stability of the biodiesel and blends with pyrogallol were slightly faster than those with propyl gallate during the eight week storage period, implying that propyl gallate provided the best storage stability as shown by the lowest percentage decrease in induction periods during the eight weeks storage period. The B10 blend could effectively be protected by either 100 ppm PYG or PRG since the Rancimat IPs of

the blend with either antioxidant by the end of the 8 weeks storage duration were both greater than that of petrodiesel used for blending [Osawa *et al.*, 2015].

The investigations on oxidation and storage stability showed that use of appropriate concentrations of suitable antioxidants can greatly increase the stability of Croton biodiesel and its blends under ordinary storage situations. Addition of suitable antioxidants may, therefore, be employed to enable Croton biodiesel and blends to be stored over a longer duration of time before use without undergoing extensive and deleterious oxidative deterioration. Since croton biodiesel blends with petrodiesel can act as an effective alternative to petrodiesel, the identification of suitable antioxidants can tremendously contribute to enhancing large scale production and use of the Croton biodiesel blends. Apart from improving the economies of rural areas, commercial production and use of Croton biodiesel blends can immensely assist in reducing the pollution effects associated with use of fossil fuels by providing readily available renewable fuel and hence reduce the high demand on existing petroleum reserves [Osawa *et al.*, 2015].

5.2 RECOMMENDATIONS

Despite the good performance characteristics of Croton biodiesel that can enable its blends to act effectively as a potential alternative to petrodiesel, no concerted efforts have been made to cultivate the plant or produce its biodiesel on a large scale. In view of the findings obtained from this study, the following recommendations are proposed:

1. Farmers and the general public should be encouraged to plant more Croton trees since it grows readily in many parts of Kenya and requires minimal care.

2. Investigations should be done to identify any long term effects of using higher blends of Croton biodiesel in a CI engine and ways of mitigating them.
3. Due to the advantages of biodiesel over petrodiesel, the government should provide incentives to encourage large scale production and use of biodiesel blends.
4. More effort should be directed at isolation and use of microorganisms or extracellular microbial enzymes with high levels of tolerance towards methanol that can effectively catalyse transesterification of various non-edible oils from commonly available sources such as the alkaline Rift Valley lakes, dairy and cooking oil processing plants.
5. Whole cell microorganisms or lipases immobilized on inert biomass support which can provide a cheaper alternative and environment-friendly way of producing biodiesel from Croton and other non-edible oils should be employed in large scale preparation of biodiesel to lower the overall cost. The microorganisms may be immobilized during their production by submerged fermentation.
6. Investigations should be undertaken to identify the most appropriate concentrations of effective synthetic and natural antioxidants that can provide long-term storage stability of Croton biodiesel and blends under different conditions. The identification and use of suitable low-cost antioxidants can tremendously boost saleable production and use of the Croton biodiesel blends.

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APPENDICES

Appendix 1: DNA Sequence for isolated lipase producing microorganism B

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ATCAGATGAGCCTAGGTCGGATAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGA

CGATCCGTAACTGGTCTGAGAGGATGACAGTCACACTGGAACTGAGACACGGTC

CAGACTCCTACGGGAGGCAGCAGTGGGGATATTGGACAATGGGCGAAAGCCTGATC

CAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGTTGTAAAGCACTTTAAGTTGGGAG

GAAGGGCAGTAAGTTAATACCTTGCTGTTTGACGTTACCAACAGAATAAGACCGGCT

AACTTCGTGCCACAGCCGCGTATACAAAGGGTGCAAGCGTTAATCGGAATTACTGGG

CGAAAGCGCGCGAGTGGTTCGTAATTGGTGTGAAAGCCCCGGGCTCACCTGGGAACT

GCATCCAAACTGGGAGCTAGAGTATGGAGAGGTGGGGATTTCCTGTGTAGCGTGAA

ATCGTAATAAGGAAGAACACAGTGGCGTAGGGACCACCTGGGTAATACTGACCTGA

GGTGCAAACGTGGGGAGCAACCGGATAAATACCCTGGTATCCCGCCGAACAAGTCA

CTACCTTGGATCTTGAATTTAGGGGCACTACGCTTAATCACCGCCGGGGAGACGGCC

CAGGTTAAACTCAATGATTGACGGGGCCCAAACGGGGAGCTGTGGTTTATTCGAACA

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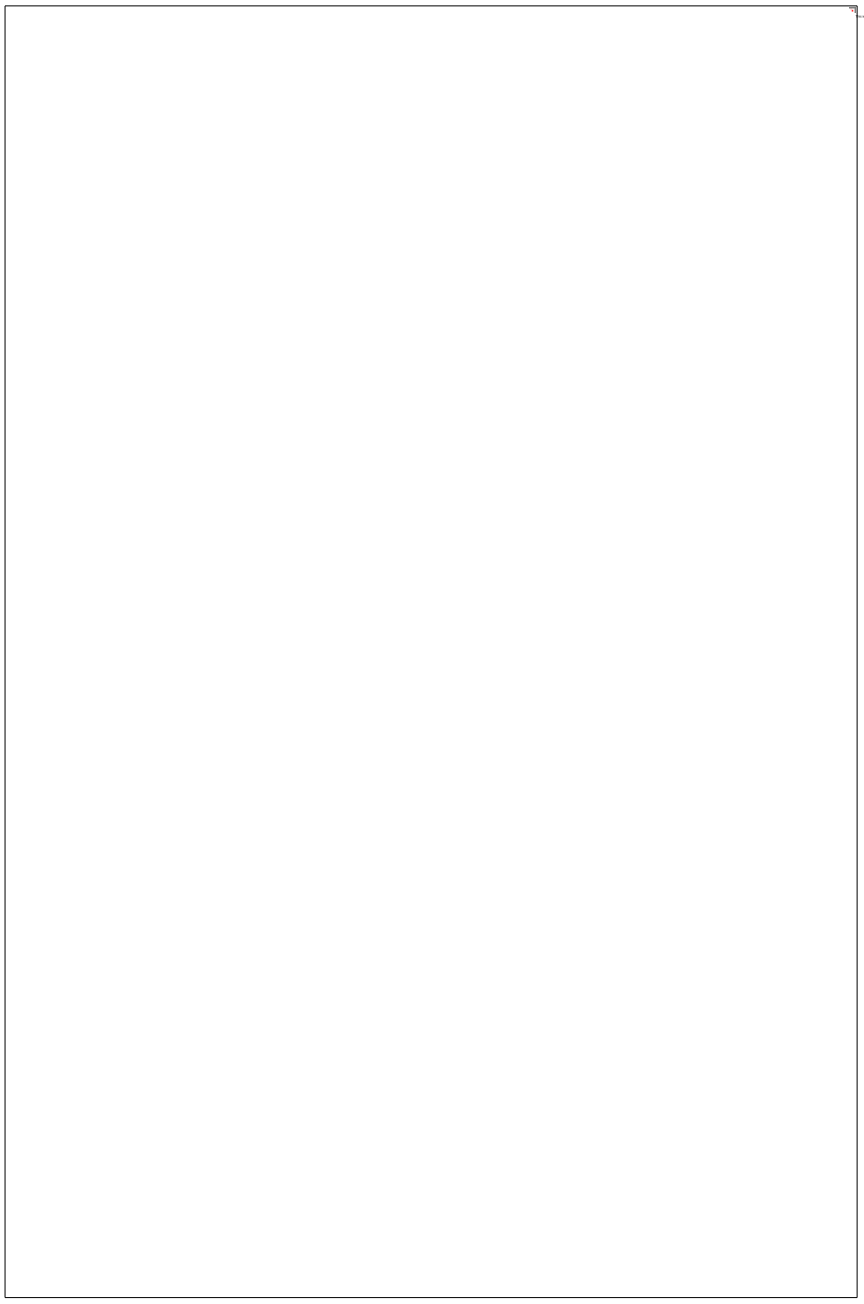
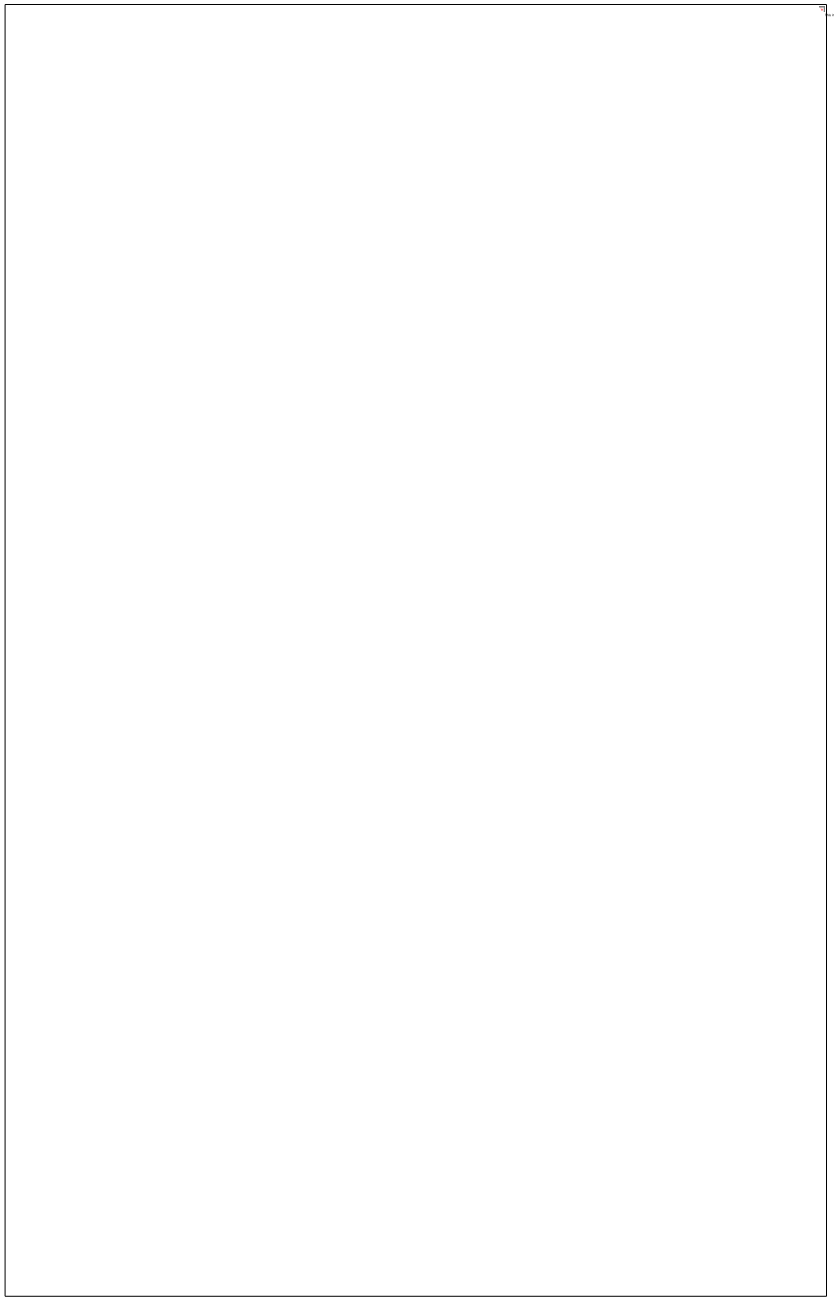
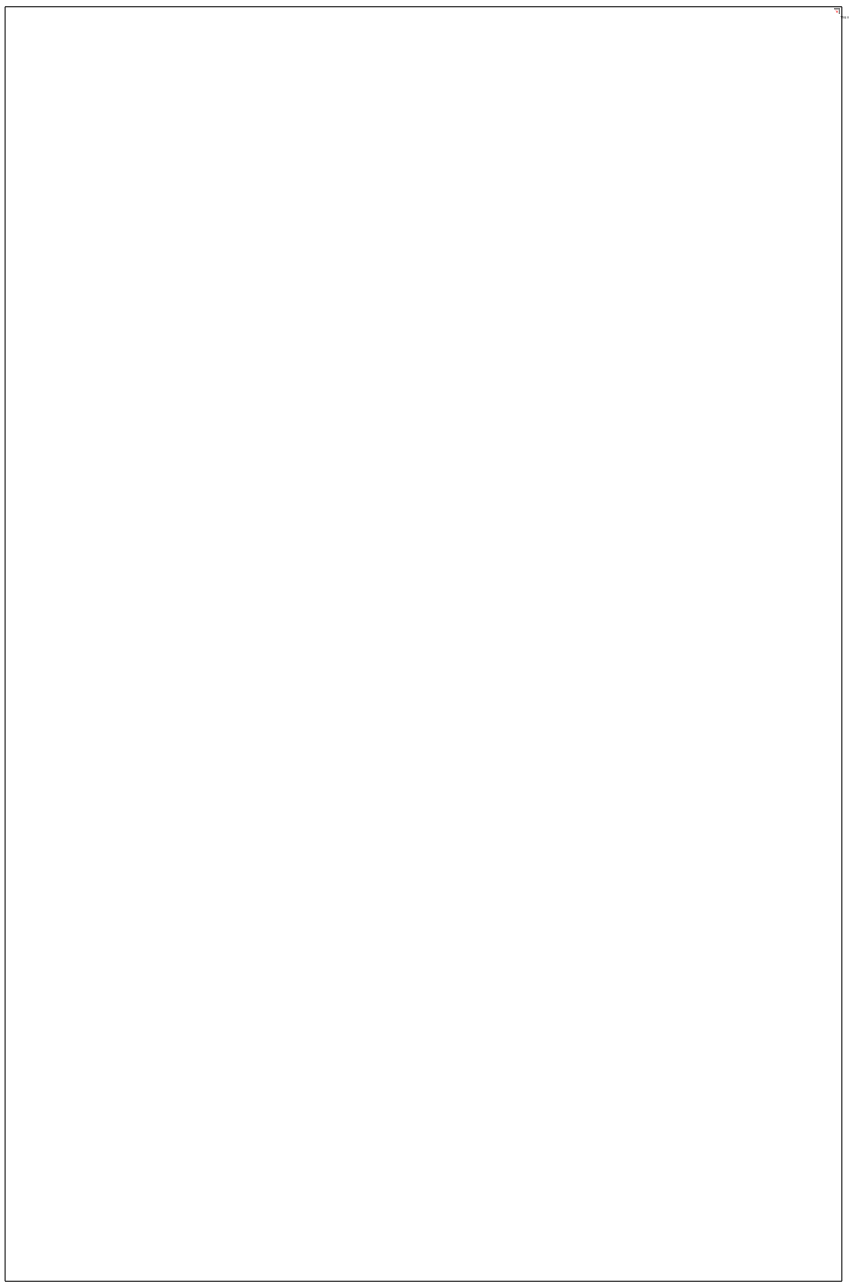
Appendix 2: GC-MS spectrum for Croton biodiesel prepared using commercial lipase



Appendix 3: GC-MS spectrum for Croton biodiesel prepared using extracted lipase



Appendix 4: GC-MS spectrum for Croton oil



Appendix 5: Paper I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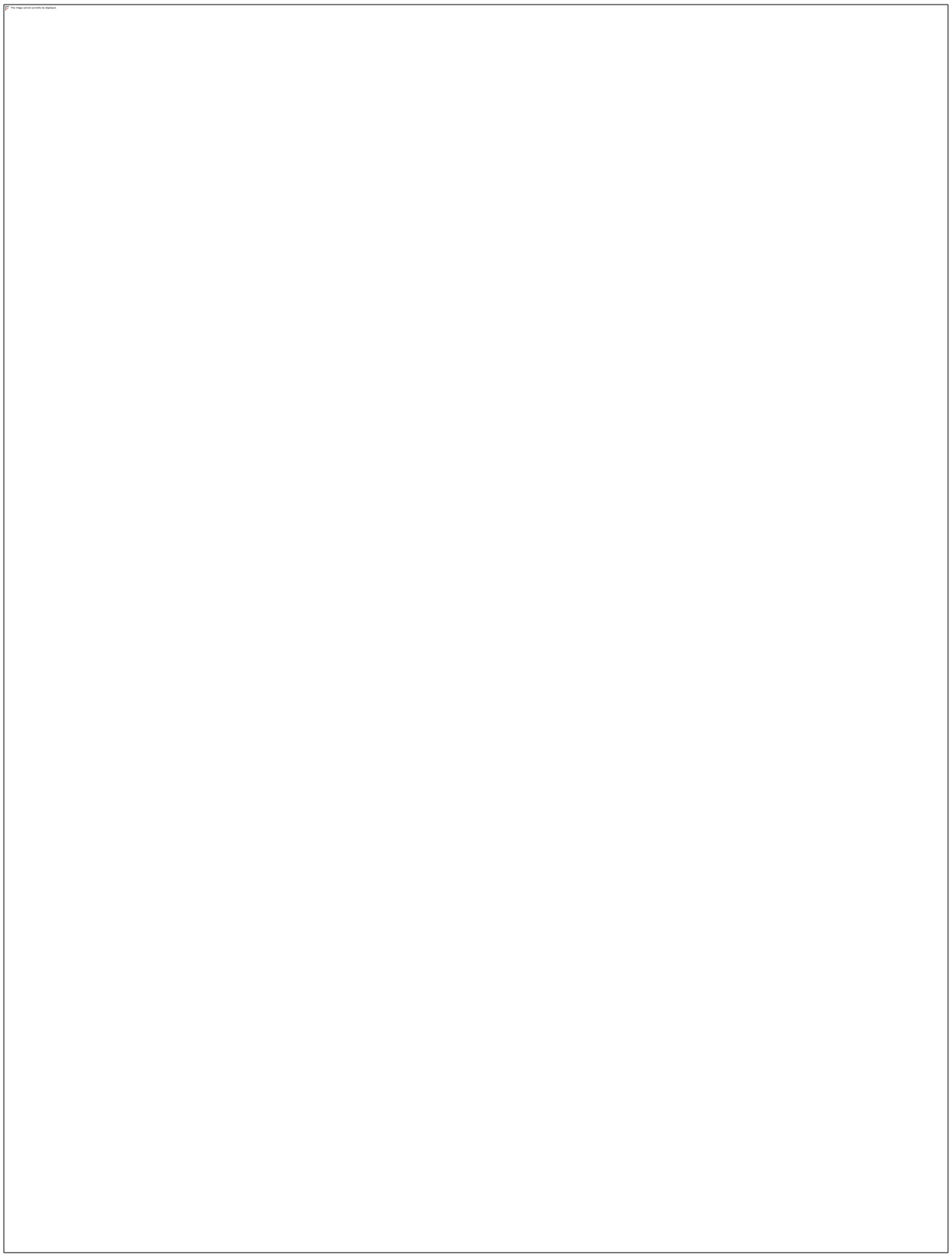
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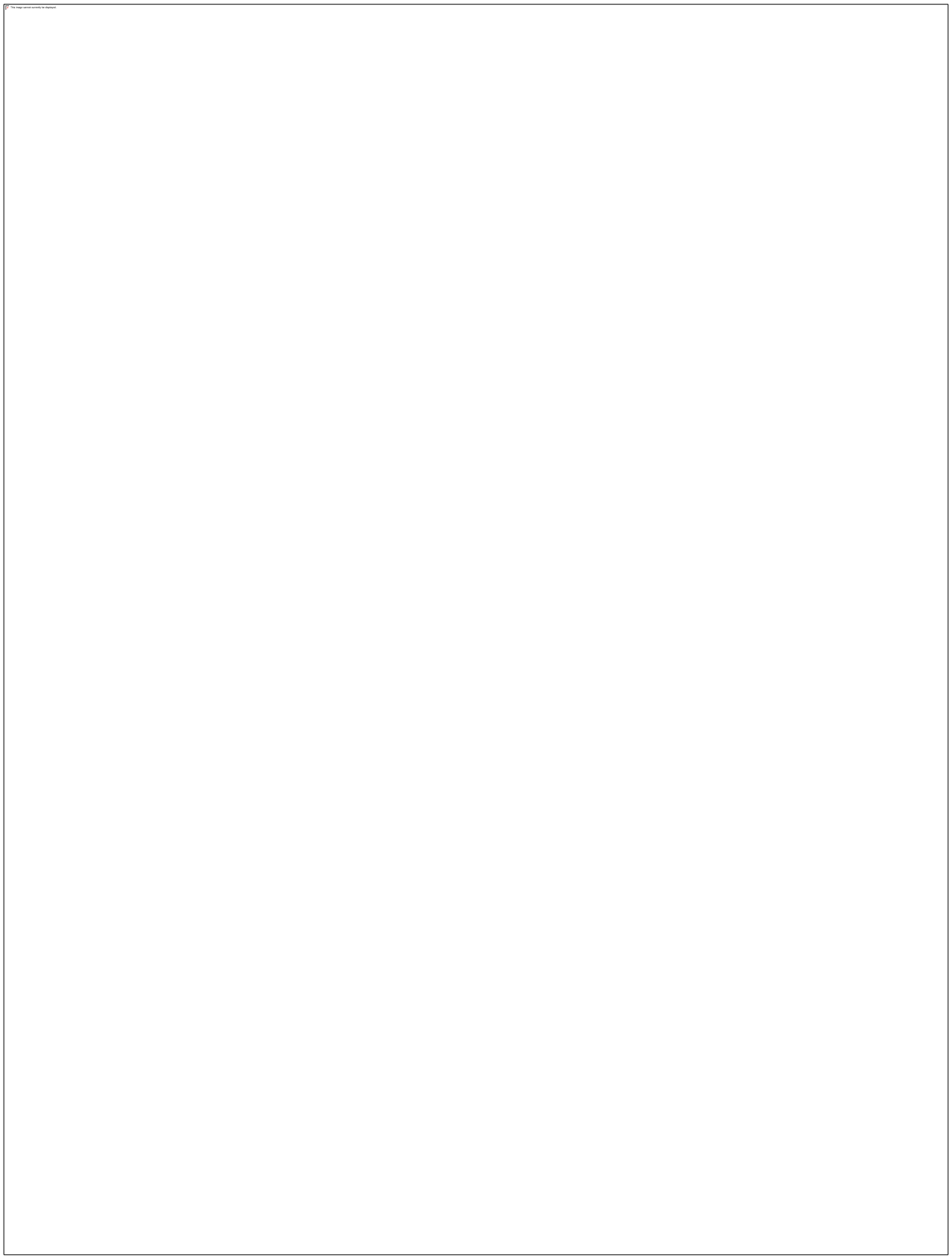

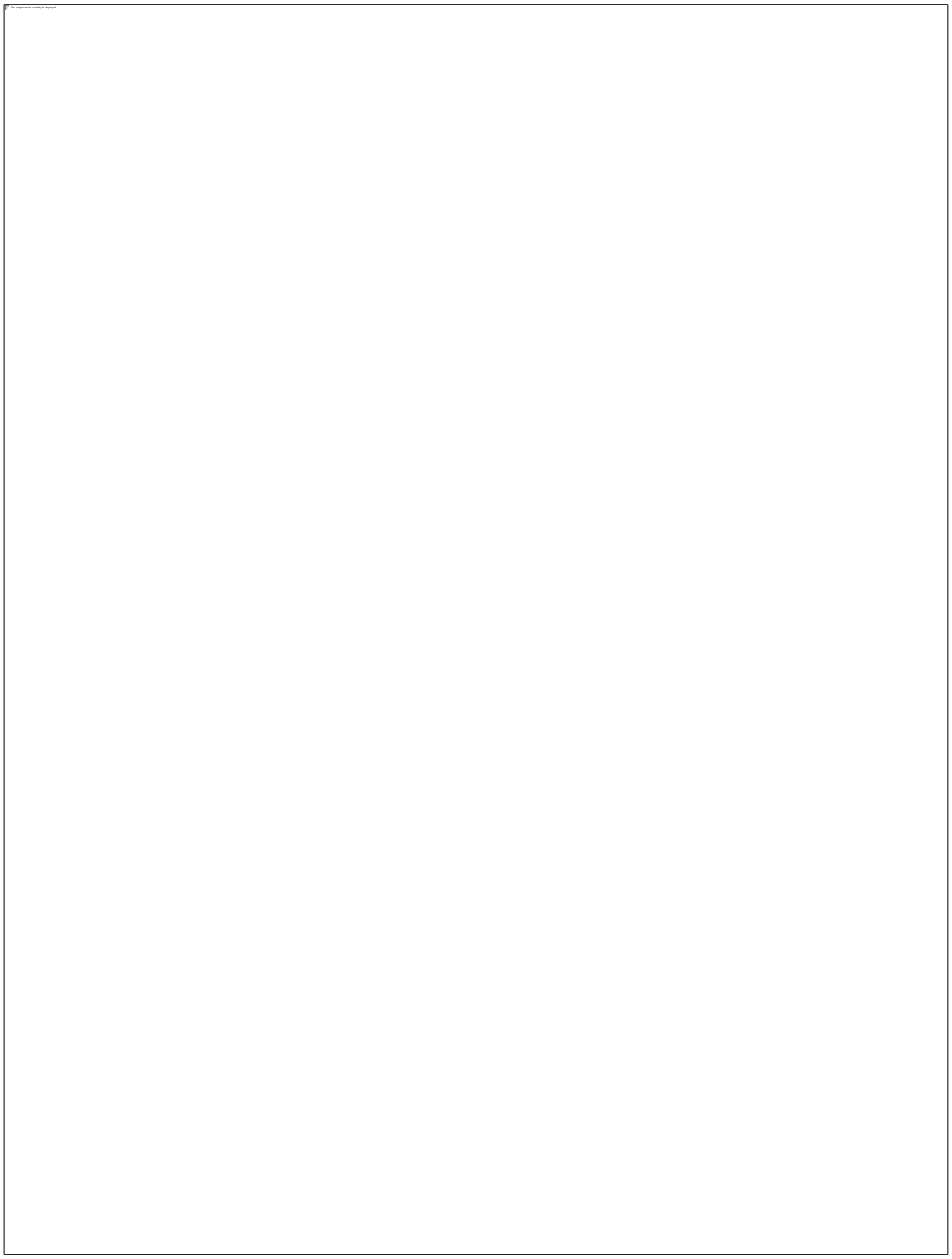

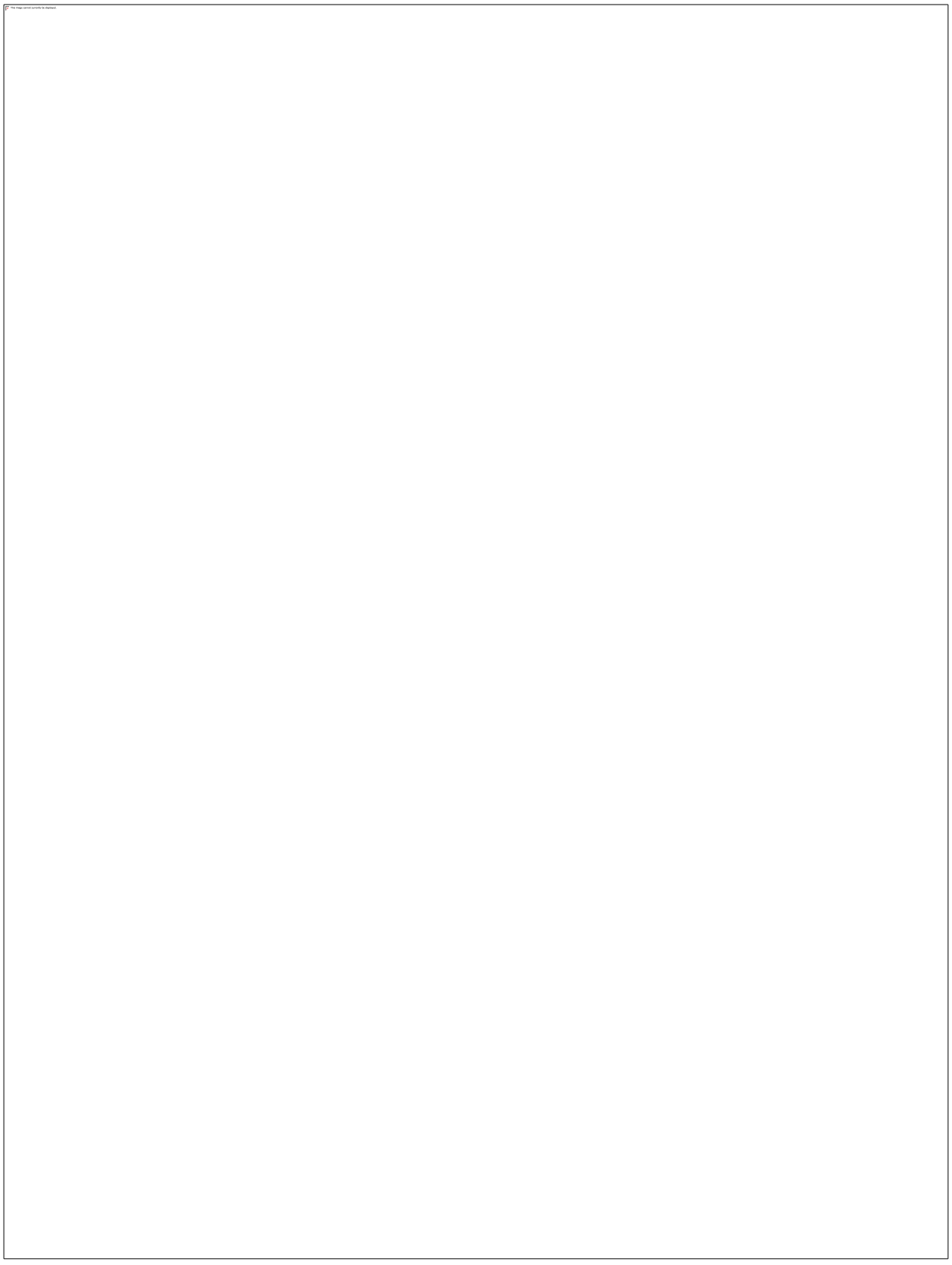
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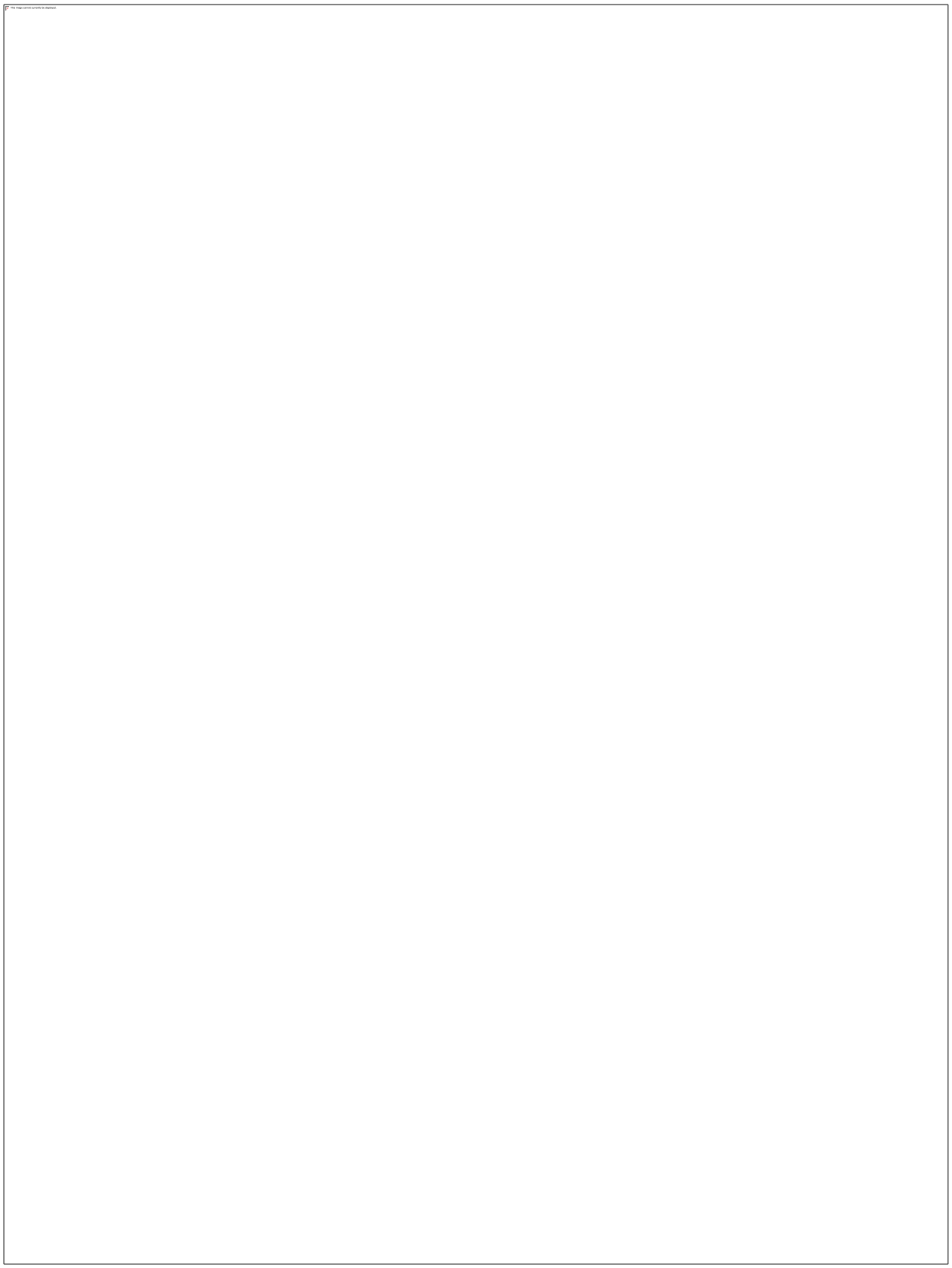
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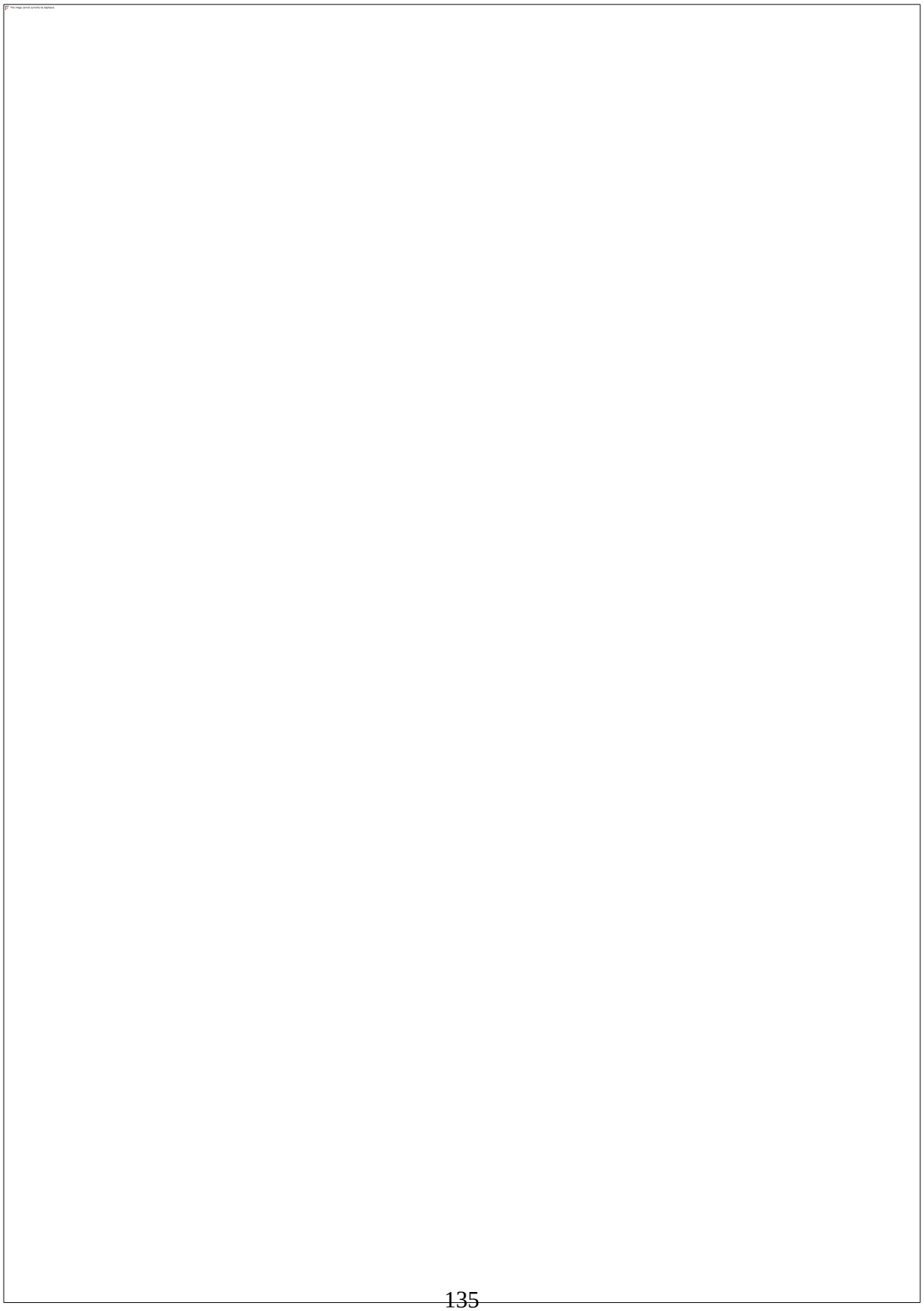
Appendix 6: Paper II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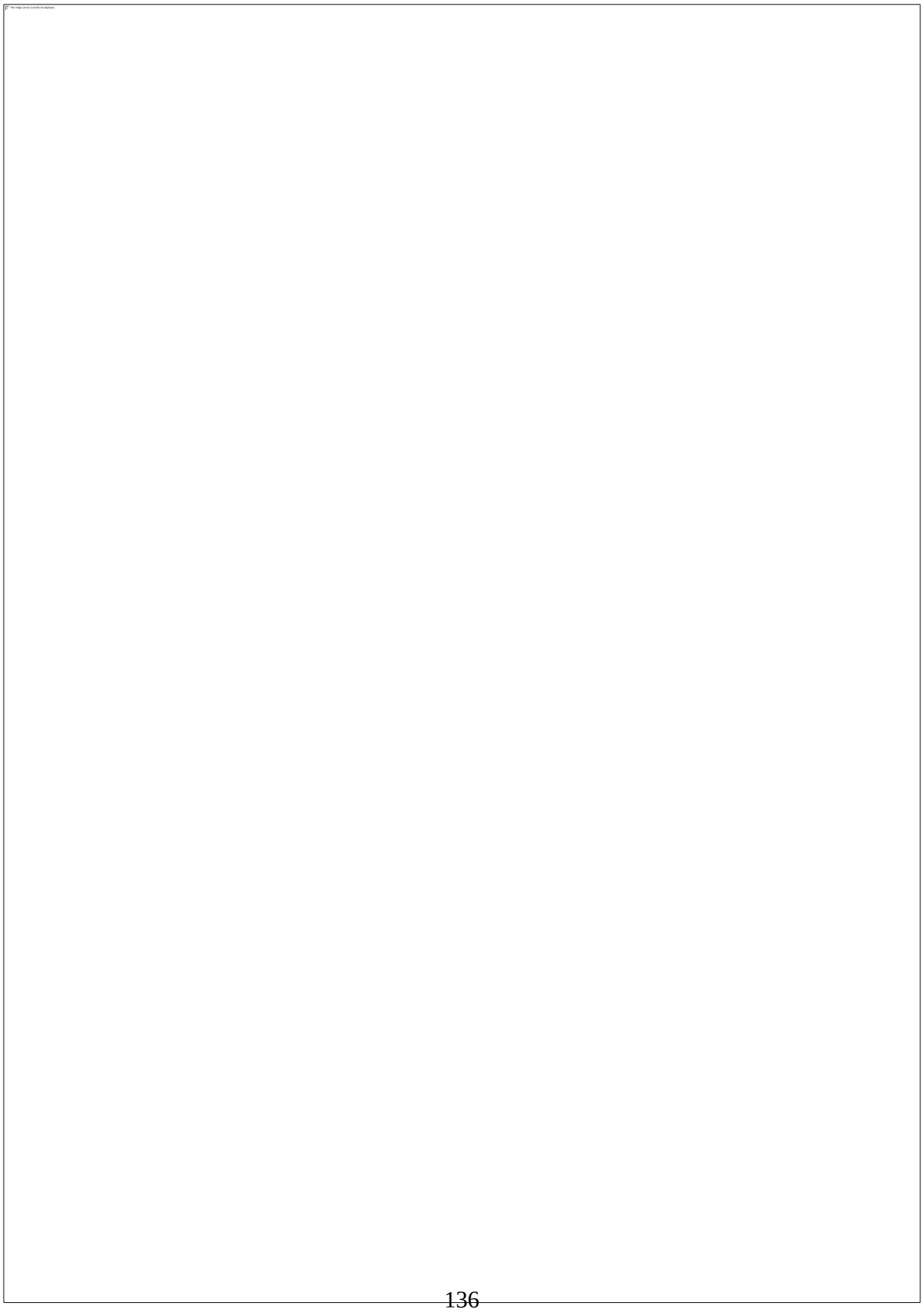
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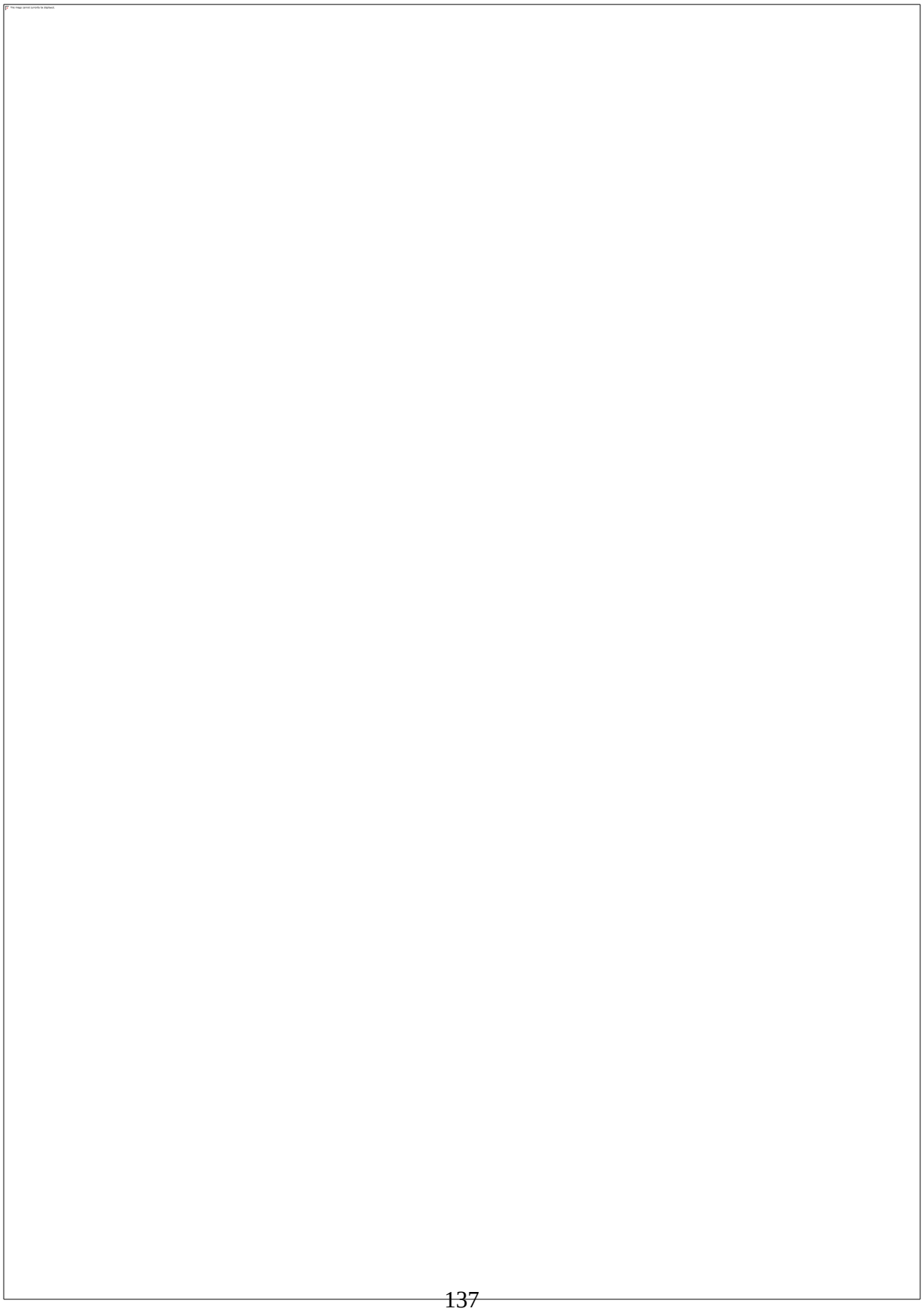
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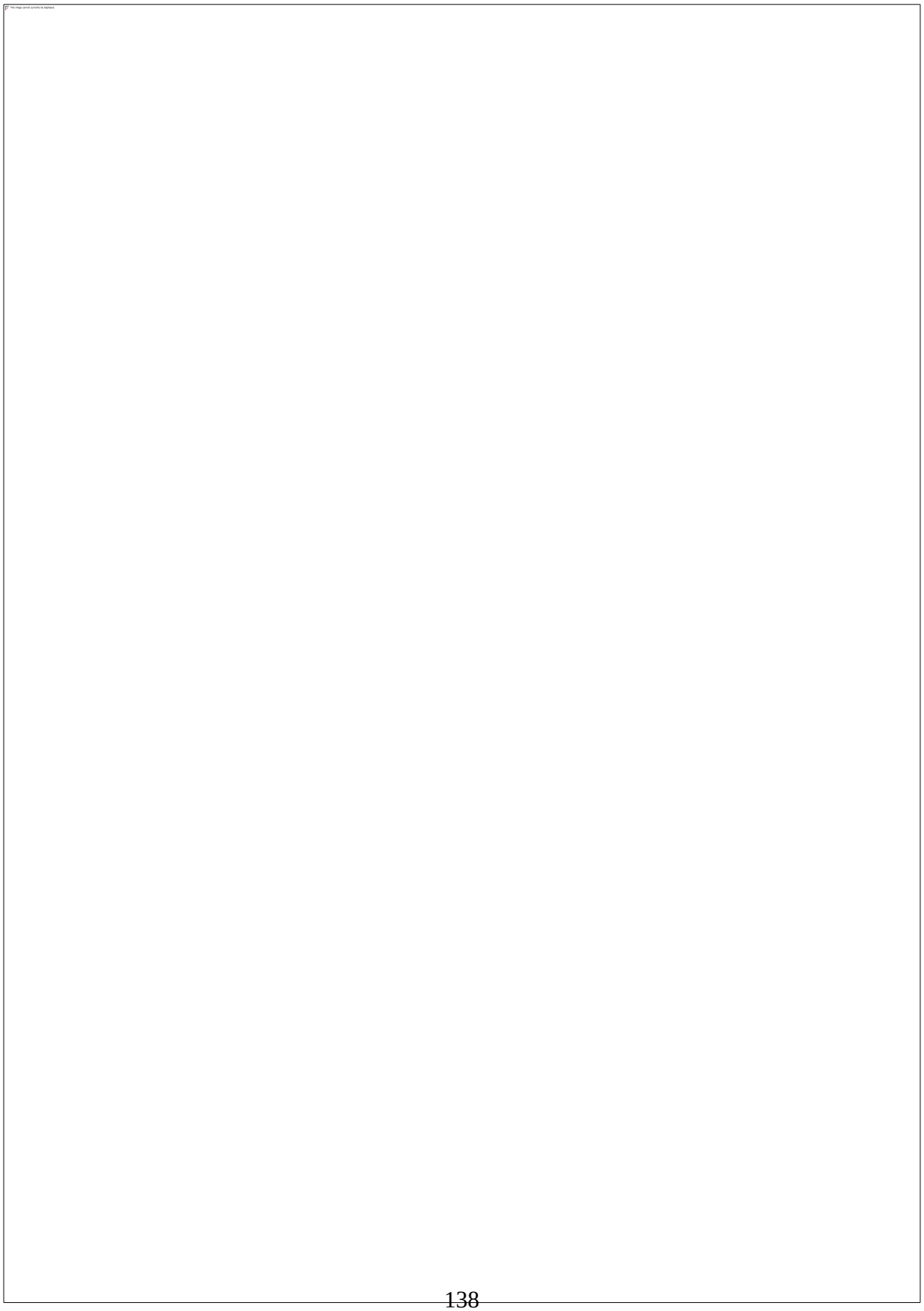
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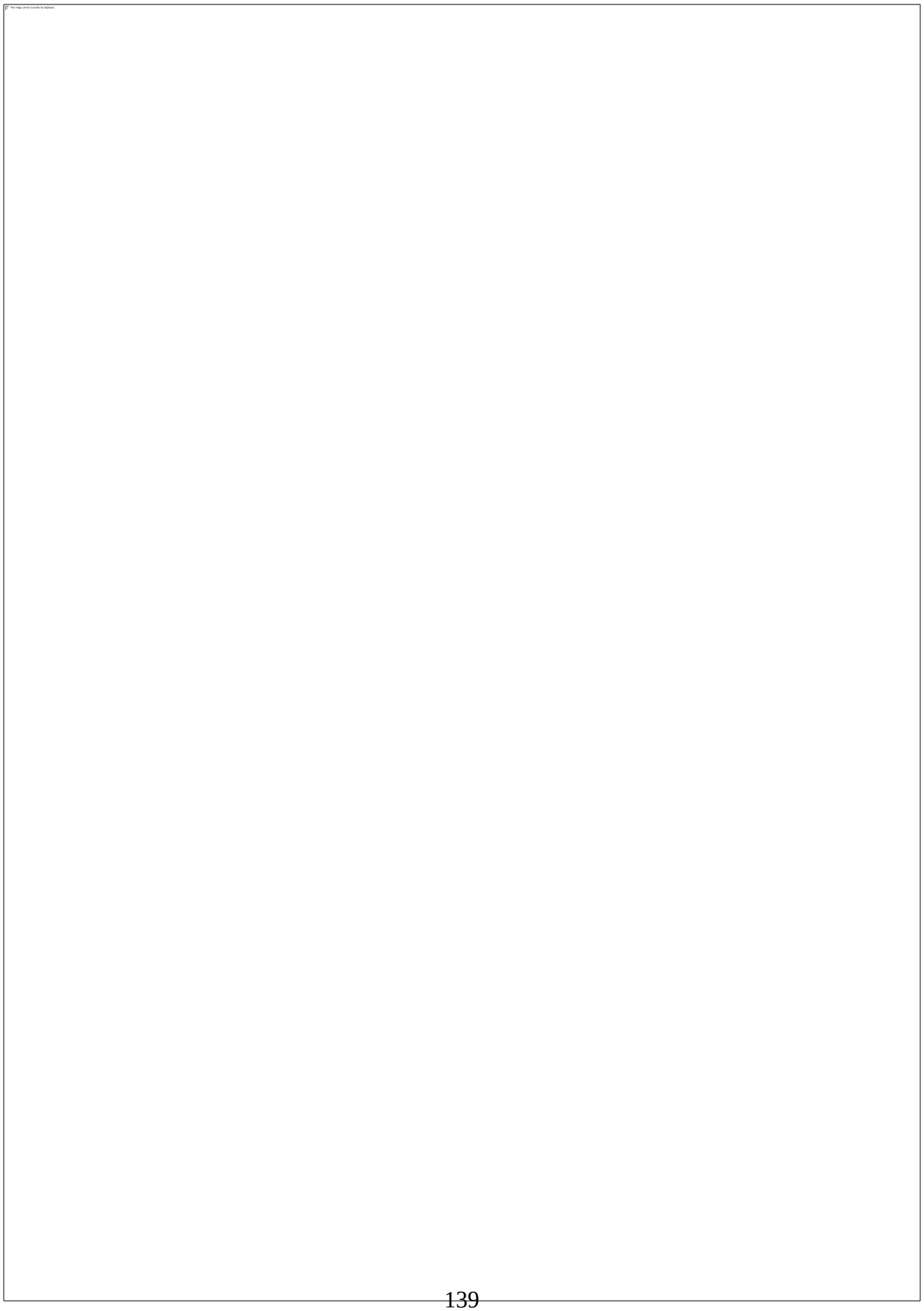
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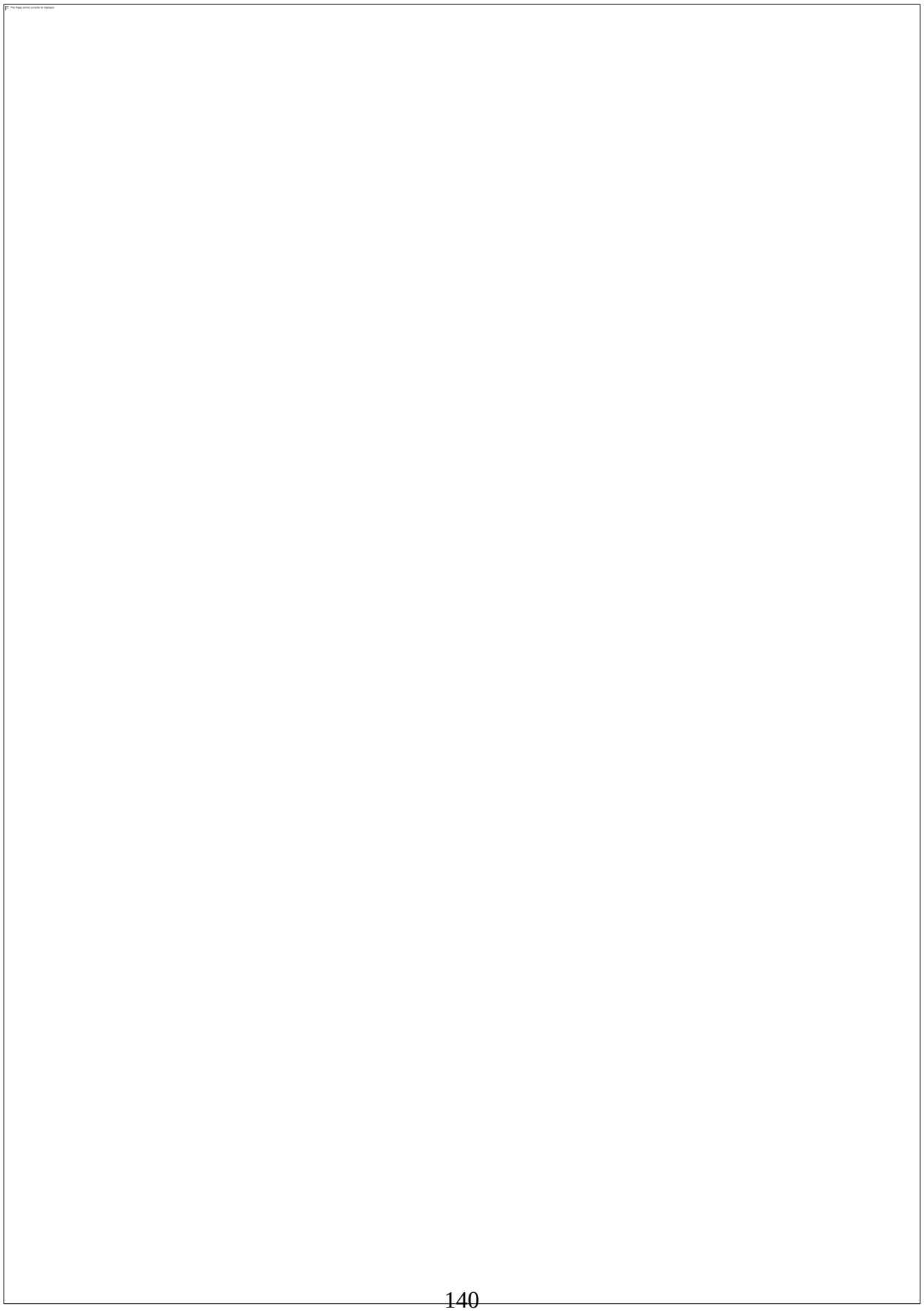
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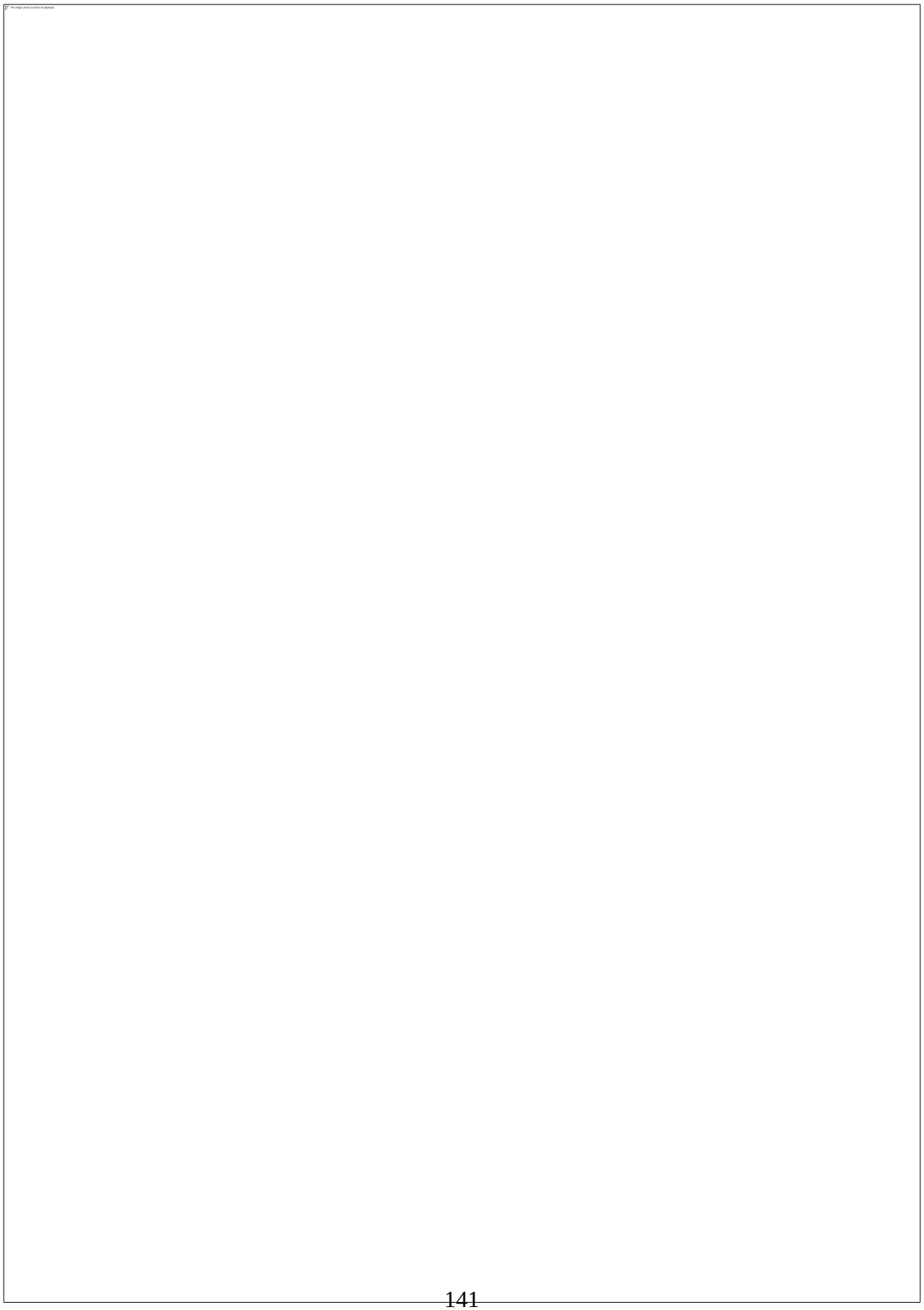
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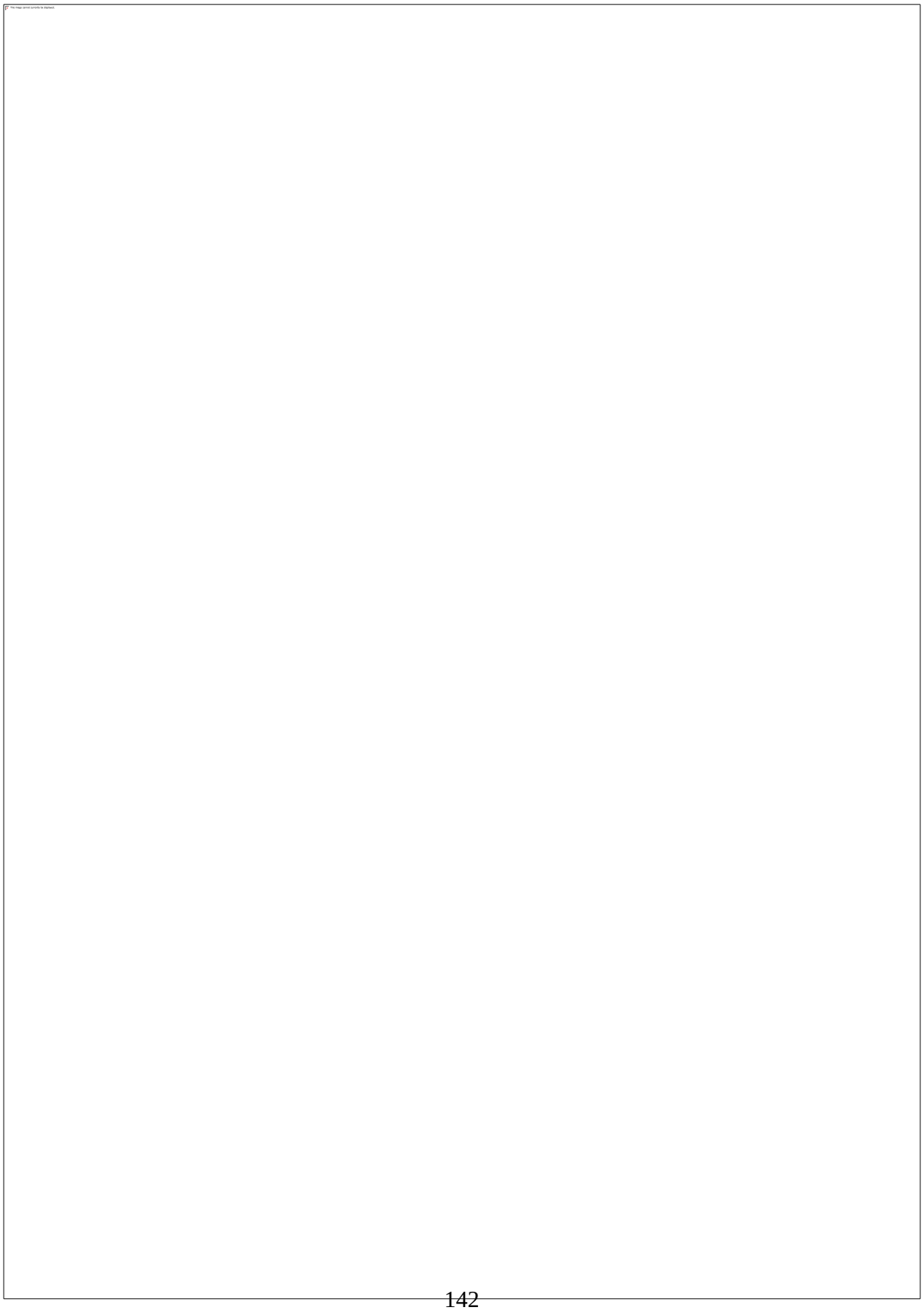
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Appendix 7: Paper III


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