Faculty of Science

Department of Biological Sciences and Biotechnology

BAOBAB (*ADANSONIA DIGITATA*) SEED AS A SOURCE OF BIODIESEL AND PROTEIN HYDROLYSATES: OPTIMIZATION OF PRODUCTION PROCESSES AND CHARACTERIZATION OF THE PRODUCTS

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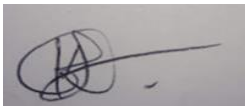
Signature:  Date: **14 March 2022**

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I, Amare Gessesse hereby confirm that I have inspected the above titled thesis, and, to the best of my knowledge, it is based on the original work of the candidate.

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DEDICATION

This thesis is dedicated to myself for never giving up and trusting in my potential of becoming the best version of myself.

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List of abbreviations

LB - Luria-Bertani

GAE – Gallic Acid Equivalent

BC – Bacterial Culture

SDS-PAGE - Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

ATCC – American Type Culture Collection

FAME – Fatty Acid Methyl Esters

FFA – Free Fatty Acids

TG – Triglycerides

BPA – Baobab Protein Agar

BPH – Baobab Protein Hydrolysate

BPB – Baobab Peptone Broth

BPI – Baobab Protein Isolate

BSPI – Baobab Seed Protein Isolate

BSPH – Baobab Seed Protein Hydrolysate

BSP – Baobab Seed Powder

AOAC – Association of Official Analytical Chemists

IOS – International Organization for Standardization

ASTM – American Society for Testing and Materials

BSA – Bovine Serum Albumin

DPBS – Dulbecco's Phosphate Buffered Saline

EWP – Egg White Protein

OD – Optical Density

kDa – kilo Daltons

Abstract

Increase in global population places a demand on fuel and protein sources therefore putting a strain on their supply. The demand for fuel products is currently met by increased usage of fossil fuel, a nonrenewable energy source. However, burning of fossil fuel has challenges such as the release of greenhouse gases associated with negative environmental impacts. To overcome these challenges, attention has been given to renewable energy sources as biodiesel, a biofuel that has good potential to replace petroleum diesel. Despite the attractive potential, its production is hindered by different challenges such as the limited supply of feedstocks. Therefore, identification of hitherto unexploited natural resources such as oil bearing plants could overcome this challenge. *Adansonia digitata* is an under used plant that can be exploited and used as a source of oil for biodiesel production. In this study, *A. digitata* seed oil was used to produce biodiesel. The resulting biodiesel showed a density of 878 kg/m^3, a viscosity of 4.276 mm^2/s, a cloud point of 5°C, our point of -11°C and an iodine number of 57.9. These values fall within the international standards indicating its usefulness for fuel application. In addition to using the seed oil for biodiesel production, the oil meal was used for the isolation of protein. The functional properties of the seed protein exhibited properties that make it suitable for application in the food industry. The protein has a water holding capacity of 1.29 g/g, water solubility index of 3.25 %, oil holding capacity of 1.14%, foam capacity of 41% and a foam stability of 67%. This indicates the potential usefulness of the seed protein. The protein was also enzymatically hydrolyzed and used for microbiological growth as a peptone. The peptone was able to support the growth of the test microorganisms the same as or better than commercial peptone. Utilization of the protein for food and nonfood application could lower the production cost of biodiesel and help to generate

economic benefits to local communities. Therefore, further research leading to the production of new products from *A. digitata* to better understand the biology of the plant is recommended.

Keywords: *A. digitata*, Energy, Biodiesel, Protein and Peptone

CHAPTER 1

INTRODUCTION

1.1. Modern energy sources and challenges associated with its utilization

Access to energy is vital for human survival and most aspects of human activity require energy. Prior to the discovery of modern energy resources, mankind used animal and human power for most activities and firewood was mostly used to meet the energy requirements for cooking and heating. However, with the discovery of fossil fuel, most of mankind's energy requirement is obtained from burning of fossil fuels (EESI, 2021). Today access to modern energy resources is considered as an indicator of development. The more a country uses modern energy in its economy the more is that country's economic development (Zhang *et al.,* 2019). Therefore, at present a handful of economically advanced nations account for a large proportion of the world's fossil fuel consumption.

Massive burning of fossil fuels, although a pillar of the modern economy, leads to the release of greenhouse gases and is responsible for climate change that the world currently faces. The environmental pollution due to the burning of fossil fuels leads to severe public health problems and causes ecological problems (Ahmed *et al*., 2010). Although only few economically advanced countries burn the greatest amount of fossil fuels, climate change and other environmental pollutions caused from fossil fuel burning affect all of mankind. Therefore, it is in everyone's interest to reduce burning of fossil fuels through various solutions.

In recent years a lot of attention has been given to the search of alternative energy sources, collectively known as renewable energy sources. These include wind, solar, tidal power and

biomass. Among the biomass derived energy sources more attention has been given to biogas and liquid biofuel. The liquid biofuels, which include bioethanol and biodiesel, attracted huge attention because of their use in the transport sector replacing fossil fuel derived gasoline and diesel fuel (Mulugetta, 2009).

1.2. Biodiesel production for energy security and poverty alleviation

In recent years biofuels received increased attention from researchers and governments. This is because first, biofuels are renewable energy sources where increased production and use can lead to alleviation of environmental pollution and help to curb climate change associated with the burning of fossil fuels (Haas *et al*., 2006). Secondly, production of biofuels locally could lead to energy security and diversification that can help to reduce dependency on petroleum.

Biodiesel is one of the main liquid biofuels that received increased attention. One attractive feature of biodiesel is that it can be produced from locally available feedstocks such as waste cooking oils, seed oils and animal fats therefore allowing flexibility based on local availability (Haas *et al*., 2006). The use of locally available feedstocks for biofuel production could in turn play a role in poverty alleviation because it enables creation of jobs locally and a market for local producers. This in turn help to improve the standard of living of local communities (Amigun *et al*., 2011; Yang *et al*., 2014). Examples of countries that used the biofuel industry to meet their energy demand and address rural poverty include Brazil and South Africa. In Brazil the biofuel industry was given significant government attention which encouraged the establishment of local businesses specialized in biofuel production. This has in turn led to the creation of jobs in the rural areas which helped to tackle rural poverty. Similarly, South Africa revised its regulation on biofuel production where small-scale farmers and residents were encouraged to contribute for up to 25%

of the feedstock used for biofuel production. This alone was expected to lead to the creation of about 125,000 direct jobs (Pradhan & Mbohwa, 2014). The establishments of local biodiesel production industries, setting up an encouraging regulatory framework, and capacity development for research on biofuel production could be expected to drive the industry forward (Pradhan & Mbohwa, 2014).

Therefore, establishment of local biodiesel production using locally available feedstock could on the one hand lead to energy security, and on the other hand play a role in poverty alleviation. One of the most important prerequisites in the development of a biodiesel industry in any country is availability of the right feedstock in sufficient quantity and quality (Amigun *et al*., 2008).

1.3. The potential of *A. digitata* seed as a source of oil and protein

At present the most widely used sources of plant oils for the production of biodiesel include *Jatropha curcas* oil, cotton seed oil, sunflower oil, soybean oil and rapeseed oil (Schinas *et al*., 2009). However, there are several challenges associated with the use of these oil sources. Firstly, most of these plant oils are edible which leads to direct competition with food. Second, these crops do not grow equally well in all regions of the world causing complications with the availability of the feedstock. This is especially true in arid and semi-arid environments where most crops cannot grow due to high heat and/or moisture stress. Therefore, oil from plants that grow naturally in arid and semi-arid environment which does not directly or indirectly compete with food supply could offer huge potential as feedstocks for biodiesel production. In this regard *A. digitata,* a tree that grows in arid and semi-arid regions offers an attractive potential as a source of feedstock for biodiesel production.

A. digitata (Figure 1), commonly known as baobab (or 'mowana' in Botswana), produces seeds containing appreciable amount of oil (Chilabade *et al*., 2021). It grows in arid and semi-arid areas where there is moisture stress (Osman, 2004). To date, eight species of baobab have been identified in different parts of the world. Of these *A. digitata* from the Southern Africa region is the most common species which widely grows in Botswana, South Africa, Zimbabwe, Malawi and Zambia (Kamatou *et al*., 2011).

In different countries different parts of *A. digitata* plant are used for a variety of uses by local communities. These include preparation of cosmetics for skin and hair from the seed oil, preparation of food and beverage from the fruit pulp, production of animal feed from leaves and fruits, and as a source of traditional medicine from different parts of the plant (Kamatou *et al*., 2011). In some areas the seeds of *A. digitata* serve as food which are eaten after being roasted (Komane *et al*., 2017).

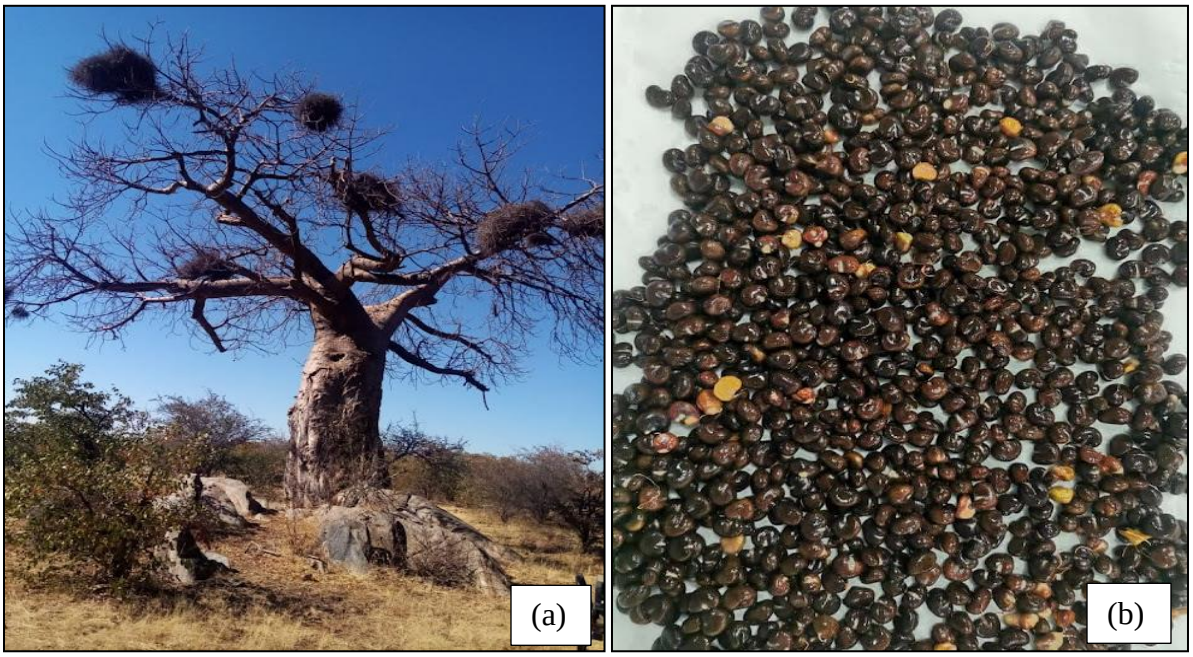

Figure 1. (a). *A. digitata* tree growing in a semi-arid environment (Tuli block, Botswana) (Photo by: Lefang Leo Chobolo) and (b). *A. digitata* seeds

Previous studies reported that *A. digitata* seed oil contains up to 33% oil. In terms of fatty acid composition 33% is saturated fatty acids and the remaining is accounted for by mono- and polyunsaturated fatty acids (36% and 31%, respectively) (Birnin-Yauri and Garba, 2011; Wapwera & Egila, 2017). Palmitic, stearic, oleic and linoleic acid are the main fatty acids of *A. digitata* oil, a similar composition to most conventional feedstocks such as sunflower, rapeseed and soybean oils (Chilabade *et al*., 2021). This shows that *A. digitata* seed oil is potentially useful as a feedstock for the production of biodiesel (Yang *et al*., 2014).

In addition to its high oil content, *A. digitata* seed has also high protein content, high energy and high mineral content and can be a good option as a protein source (Osman, 2004). Therefore, after extraction of the oil, the meal can be used as a source of protein to be used in the food industry or hydrolyzed enzymatically to produce protein hydrolysates that are very useful in the formulation of microbiological media or for other applications. It is important to note that the utilization of the protein isolated from the oil meal could help to substantially reduce the production cost of biodiesel while at the same time offering an alternative protein source to meet the growing global demand for proteins. Since *A. digitata* grows in arid and semi-arid environments where most other crops cannot grow, production of biodiesel and value-added protein products could play an important role in improving the socioeconomic conditions of these areas by helping to create new jobs and new markets for local producers. Therefore, this study aims at using *A. digitata* seed as a source of oil for biodiesel production and use the protein from the seed meal for food and non-food applications.

1.4. Statement of the problem

The global population growth is causing a demand on petroleum fuel products which are the most used transportation fuels. These fuels, however, have major disadvantages including contribution to environmental pollution and unreliable supply since they are non-renewable (Mumtaz *et al*., 2012). The unreliable supply of the fuels also influences their pricing which often leads to constantly increasing prices. Studies indicate that in Africa, road transport uses 85% of fossil fuels of which over 55% is accounted for by diesel fuel consumption (Mulugetta, 2009). Although this high dependency on fossil diesel fuels can be alleviated through partial replacement by biofuel, lack of feedstocks is a major limiting factor in its production. Feedstocks for biodiesel production appears as the limiting factor for a successful production mainly due to lack of feedstock diversification (dos Santos Alves *et al*., 2017). Another setback associated with the production of biodiesels from seed oils is the accumulation of seed meals. Although seed meals could be highly nutritious in terms of their protein content, some of them like in Jatropha and castor bean seeds, contain highly toxic substances forcing producers to discard it after oil production (Santibáñez & Varnero, 2014). The discarding of oil meals, instead of assisting in lowering biodiesel production cost through the production of other value added products, it creates a disposal challenge which increases the overall production cost. If it is not properly disposed, it could create environmental pollution. Therefore, feedstocks that allow utilization of the protein from the seed meal as a source of protein could be expected to lead to significant reduction in biodiesel production and offer many other economic benefits.

1.5. Objectives of the study

1.5.1. General objective

The main objective of this study was to evaluate the potential of *A. digitata* seeds as a source of oil used to produce biodiesel and develop methods for the utilization of the protein from the oil meal and test its potential applications.

1.5.2. Specific objectives

The specific objectives of the study were to:

1.5.2.1. Evaluate the potential of *A. digitata* seed as a source of oil for biodiesel production and characterize the biodiesel produced

1.5.2.2. Isolate proteins from *A. digitata* seed meal and characterize its functional properties to determine its potential application in the food industry.

1.5.2.3. Produce protein hydrolysates (peptone) from proteins isolated from *A. digitata* seed meal and test its potential as a component of microbial culture media.

1.5.3. Hypothesis

The study was designed to assess the hypothesis that *A. digitata* seed is an appreciable source of oil that can be used to produce biodiesel and its oil meal a source of protein with food and non-food applications.

1.6. Justification of the study

With increasing global population, the demand for energy correspondingly increases. At present the main source of energy is burning of fossil fuels. However, excessive use of fossil fuels, besides being a nonrenewable resource, poses several environmental challenges and cause climate change (Mumtaz *et al*., 2012). The transport sector is one of the major users of fossil fuel. Since in Africa up to 85% of the fossil fuel consumption is used for road transport and 55% is accounted for by diesel fuel (Mulugetta, 2009), replacing all or part of Africa's diesel consumption by biodiesel could pay huge economic and environmental dividends. However, lack of feedstocks in sufficient quantity, quality, and with an affordable cost puts a limit on Africa's biodiesel development (Yang *et al*, 2014). Similarly in other parts of the world, lack of feedstocks has also been reported as a major factor limiting biodiesel production (dos Santos Alves *et al*., 2017)

In the production of biodiesel, the oil accounts a significant proportion of the production cost. One way to lower production cost is to find use for the oil meal byproduct. Although the meal could be a valuable source of protein and other nutrients, some of the important oil seeds contain highly toxic substances leading to the disposal of the meal (Santibáñez & Varnero, 2014). Therefore, in these cases the meal, instead of helping to reduce the production cost, it is discarded therefore causing an additional cost of disposal.

The use of *A. digitata* seeds as a source of oil for biodiesel is expected to offer several advantages. *A. digitata* is a multipurpose tree that grows in arid and semi-arid environments mostly in areas having severe moisture stress. While the seeds serve as sources of oil and proteins, the other parts of the tree also find several other uses. These include the use of the leaves as animal feeds, the fruit pulp for food application, and the pod for charcoal production. Development of appropriate

technology for the utilization of the different parts of the plant and scale up of these technologies could be expected to economically benefit local communities. For example, establishment of biodiesel processing factories could encourage local communities to collect seeds and supply to the processors while at the same time selling the fruit pulp for food application. After oil extraction from the seed, the meal can be used for protein isolation for food and nonfood applications. All these in turn create new jobs and bring about economic benefit to the local community. Therefore, development of technology for the utilization of *A. digitata* seed oil and meal could ultimately improve the livelihood of rural communities.

CHAPTER 2

LITERATURE REVIEW

2.1. The potential of biodiesel as a renewable energy source

At present up to 80% of the world's energy demand is obtained from fossil fuels where a significant portion of it is used in the transport sector (EESI, 2021). However, such an overdependence on fossil fuels poses several challenges. First, fossil fuels are non-renewable and finite energy resources which can be depleted at any time in the foreseeable future. Second, burning of fossil fuels leads to the release of greenhouses gases (GHG), such as carbon dioxide and nitrous oxide (Uzeh *et al*., 2006) which are associated with climate change, global warming, air pollution and increased incidences of respiratory diseases (Rathore *et al*., 2016). In addition, air pollution caused by pollutants released during the burning of fossil fuels is responsible for acid rain that affect soil and water (Gamalero *et al*., 2003). Third, the high cost of imported fossil fuels greatly affects the economies of many developing countries slowing down or stalling their development efforts.

To overcome these challenges and to ensure sustainable development, in recent years, emphasis is given to the development of renewable energy sources. Liquid biofuels (mainly biodiesel and bioethanol) are two of the renewable energy sources that have attracted attention because of their potential to replace petroleum derived fuels used in the transport sector. Different studies showed that burning of biofuels reduces smoke, carbon dioxide and carbon monoxide density by 22.5%, 14% and 17.5%, respectively (Ribeiro *et al*., 2011) making them more environmentally friendly than fossil fuels. Therefore, by 2050 biofuels are expected to contribute up to 27% of the world's transport fuel (Pradhan & Mbohwa, 2014).

Of the liquid biofuels, biodiesel attracted a lot of attention from researchers and governments because of its potential to replace petroleum derived diesel fuel used in the transport sector. Biodiesel is produced through transesterification of triglycerides with short chain alcohols using chemical or enzyme catalysts (Lin *et al*., 2011). Apart from being a renewable energy source, one other important feature of biodiesel is that it is highly biodegradable posing little or no risk of environmental pollution. Some studies showed that over 90% of biodiesel can be biodegraded within 21 days compared to only 18% for diesel fuel (Leung *et al*., 2010). The high biodegradability of biodiesel is attributed to the presence of an ester functional group which can be easily hydrolyzed by microbial esterases to its constituent fatty acid and alcohol followed by further oxidation of the hydrolysis products as growth substrates by microorganisms (Demirbaş, 2009).

2.1.1. The process of biodiesel production

The main raw materials used for the production of biodiesel are fats and oils (triglycerides) obtained from animal, plant and microbial sources. Triglycerides react with short chain alcohols, such as methanol or ethanol, in the presence of appropriate catalysts through a reaction known as transesterification (Knothe & Razon, 2017). Figure 2 shows the transesterification of a triglyceride with methanol in the presence of a catalyst to produce biodiesel (fatty acid methyl esters) and glycerol. Catalysts used in the reaction include acid, base, or enzyme catalysts. In acid catalyzed reactions mineral acids, such as sulfuric acid and hydrochloric acid, are used to catalyze transesterification reactions. In general acid catalyzed transesterification reactions take longer reaction times and require a high reaction temperature (Leung *et al*., 2010).

The most commonly used catalysts in the production of biodiesel through chemical means are bases, such as KOH and NaOH. The main advantages of using bases as catalysts include a higher biodiesel yield obtained in a shorter reaction time and at a lower temperature (Ribeiro *et al*., 2011). Thus, after completion of the transesterification, the reaction mixture is allowed to separate into a top organic phase (the biodiesel) and a bottom glycerol phase which also contains the residual base. The use of bases as catalysts also pose some challenges, the main one being accumulation of soap that could lead to emulsification and causing cleaning of the biodiesel very difficult (Atadashi *et al*., 2010). This especially poses serious challenges when the feedstocks contains higher concentration of free fatty acids (Canakci & Van Gerpen, 1999).

The third type of catalysts used in the production of biodiesel are enzymes. Enzyme based transesterification reactions involves the use of immobilized enzymes (mainly) lipases to convert triglycerides to fatty acid methyl esters and glycerol (Norjannah *et al*., 2016). Some of the advantages of using enzymatic transesterification include avoidance of soap accumulation which is common with alkaline catalyzed transesterification and is environmentally safe as it does not use chemicals (Atadashi *et al*., 2010). Although the high cost of enzymes could make the process expensive, the use of immobilized biocatalysts in the transesterification reaction allows reuse for several cycles therefore helping to reduce the cost (Ghaly *et al*., 2010). However, the temperature sensitivity of enzymes is a drawback that needs careful monitoring and optimization of the reaction conditions. In addition, compared to the chemical transesterification reactions, enzyme catalyzed reactions are also characterized by slow reaction rates (Norjannah *et al*., 2016).

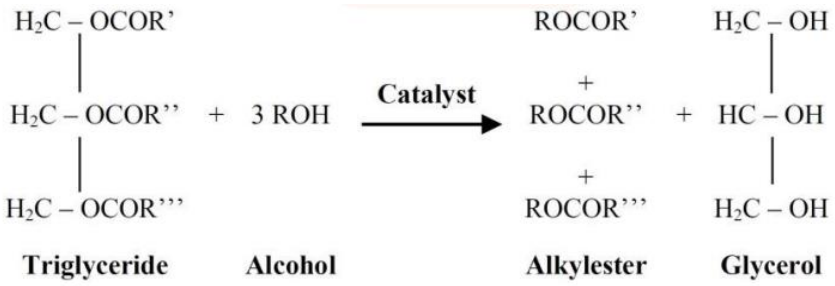

Figure 2. Transesterification process (Ribeiro *et al*., 2011)

The quality of biodiesel produced through transesterification is usually analyzed using various analytical techniques. The most commonly used analytical methods to check quality of biodiesel include gas chromatography (GC), thin layer chromatography (TLC) and Fourier transform infrared spectroscopy (FTIR) (Bansal *et al*., 2008; Wembabazi *et al*., 2015).

2.1.2. Feedstocks used for biodiesel production

Commonly used feedstocks for biodiesel production include plant oils, waste cooking oil, and animal fats (Karmakar *et al.*, 2010). The plant oils used as feedstocks for biodiesel production could include edible and non-edible oils. Edible oil feedstocks include rapeseed oil, soybean oil, sunflower oil, palm oil and peanut oil. Although edible oils are excellent feedstocks for biodiesel production, their direct competition with food use could make it economically and morally unacceptable. As a result attention has been focused on the use of non-edible plant oils such as jatropha oil, neem oil, mahua oil and jojoba oil as feedstocks for biodiesel production (Karmakar *et al*., 2010).

In some countries, such as such as the United States of America, Brazil, Germany, Philippines, Belgium and Spain animal fats contribute up to 20% of feedstocks used for biodiesel production.

Examples of animal fats used for biodiesel production include pork lard, chicken fat, beef and mutton tallow. The use of animal fats as feedstocks for biodiesel production is useful for the meat industries as it allows them to channel the fat released as byproduct to biodiesel production and help them avoid the cost of disposal. As a result, compared to plant oils, waste animal fats are considered much cheaper feedstocks for biodiesel production (Banković-Ilić *et al*., 2014). On the other hand, when plant oils are used as feedstocks up to 75% of the production cost is accounted for by the raw material (Phan & Phan, 2008).

Another feedstock used for biodiesel production is waste cooking oil from domestic or industrial origin. When waste cooking oil is used as a feedstock for biodiesel production the production cost is reduced because it is up to three times cheaper than edible plant oil. However, the use of waste cooking oils requires extensive refining before being used as feedstocks (Phan & Phan, 2008). For example, waste cooking oils can have high free fatty acid (FFA) content which can hinder the transesterification process. Although a pre-transesterification step with sulfuric acid is usually performed which reduces the FFA content in the oil before continuing with an alkaline catalyst, the process can cause problems because of the water released when the FFA reacts with the alcohol (Karmakar *et al*., 2010). This indicates a need for waste oils with low FFA content or more optimized pretreatments of the oils before they are used as feedstocks for biodiesel production.

Another feedstock for biodiesel production that is gaining popularity in recent years is algal oil obtained from oil producing micro and macro algae (Ahmed *et al*., 2010; Karmakar *et al*., 2010). In some of these algae, up to 55% of cell dry weight is accounted for by the oil (Carvalho *et al*., 2011). Examples of oil bearing algae include *Botryococcus braunii* and *Chlorella* sp *Gracilaria dura* and *Acanthophora specifera* (Carvalho *et al*., 2011). Compared to other feedstocks the use of algal oil is very attractive for biodiesel production because, unlike plant oils, it does not directly

or indirectly compete with food supply. Moreover, the oil is of very good quality and does not require expensive purification steps.

2.1.3. Challenges associated with feedstocks used for biodiesel production

As stated earlier the use of edible plant oils directly competes with food consumption while many of the nonedible oils such as jatropha oil indirectly compete with food production through the use of arable land or use of water resources (Kgathi *et al*., 2017). For the other feedstocks, availability in sufficient quantity and/or quality is a challenge. Since the feedstock account for up to 75% of the cost of production of biodiesel, finding cheaper and easy to access feedstocks with an acceptable quality has been receiving a lot of attention (Lin *et al*., 2011). In recent years the use of algal oil has been receiving a lot of attention because of its ability to produce high oil quantities of oil per acre. However, cultivation of algae requires strict and highly optimized culture conditions (Rajkumar *et al*., 2014). Therefore, search for new plant oil sources that cannot directly and indirectly compete with food production or consumption still offers an attractive potential. In this respect *A. digitata* offers an interesting potential for biodiesel production (Yang *et al*., 2014). The plant grows in arid and semi-arid regions and does not compete with food production. Unlike other plants where the seed meal contains toxic substances and cannot be used for food and feed application, there has not been reports on *A. digitata* seed meal having toxic substances making it suited for applications such as food or animal feed. Therefore, in the case of *A. digitata* the potential use of the seed meal for different applications could further help to reduce the biodiesel production cost (Chilabade *et al*., 2021; Castro & Freire, 2021).

2.2. Other applications of biodiesel

In addition to potentially being used as a fuel for transportation, biodiesel could also find other uses such as being used in lubricants, plasticizers and as a solvent during oil spill remediation. At present lubricants and plasticizers are widely used in the process of manufacturing and handling of materials (Cataldo *et al*., 2013). However, almost all lubricants and plasticizers currently in use are of fossil origin and thus raise serious environmental concerns. In this regard the use of lubricants and plasticizers derived from biodiesel is considered environmentally friendly (Cavalcanti da Silva *et al*., 2013). One example where biodiesel could be used as a lubricant and plasticizer is in the process of rubber and plastic manufacturing. Thus, when used as a plasticizer, biodiesel behaves like the common mineral oil plasticizers showing that it can be used as an alternative (Cataldo *et al*., 2013).

Another area of application for biodiesel is its use as a solvent due to the presence of methyl esters. For example, biodiesel can be used for clearing of oil spills and oil blockages in pipes caused by asphaltenes (molecular substances found in crude oil responsible for blockages in oil pipes). Normally the standard method for asphaltene deposition involve the use of such solvents as benzene, xylene and toluene. However, these solvents are derived from fossil fuels making them expensive and hazardous. Biodiesel can replace the above solvents for the deposition of asphaltene therefore offering a cheap and environmentally friendly alternative (Ramdass & Chakrabati, 2017).

2.3. Proteins from oil meals: extraction and application

In the production of biodiesel, the oil accounts for a significant proportion of the production cost, up to 75% according to some estimates (Phan & Phan, 2008). In the process of oil extraction, a

significant amount of oil meal is released as byproduct. As reported by Santibáñez & Varnero (2014), for every ton of biodiesel produced, about 2.5 to 3.0 tons of seed cake could be generated. Because some oil seeds, such as Jatropha and castor bean seeds, contain toxic components, the oil meal cannot be used for any application. Rather, disposal of the meal could add to the production cost.

In cases where the seed contains no toxic substances, the protein rich oil meal could find several applications and help to reduce the overall production cost of biodiesel. The avoidance of discarding the seed meal after oil extraction and biodiesel production can lead to a more economically stable production. For example, countries such as Côte d'Ivoire, Kenya and Sudan are profiting from the sales of oil seed cakes to be used for different applications (Chilabade *et al*., 2021). Some of the applications for the oil meal include direct use of the meal as animal feed or utilization of the protein after isolation in pure form. The isolated protein can then be used as a food (or feed) supplement or used to generate protein hydrolysates for different applications (Sánchez-Arreola *et al*., 2015) (Kim *et al*., 2019).

Apart from reducing the production cost of biodiesel, utilization of the protein from the oil meal could help to alleviate the protein deficiency the world currently faces. As the world population grows exponentially the demand for protein is also increasing exponentially. For any protein to be used in the food industry, it must possess some functional properties. Therefore, understanding the functional properties of a given protein could allow to predict its potential food applications.

2.3.1. Functional properties of proteins

In addition to their nutritional value plant proteins used in the food industry serve as emulsifiers, foaming agents or used to enhance water and oil holding capacity of food products (Khalid *et al*.,

2003). Therefore, knowledge about the functional properties of proteins is important to determine their functional properties which is defined as the physiochemical properties of proteins that influence protein behavior in food systems with effect on the quality and sensory nature of the food systems (Yalçın *et al*., 2008). Addition of the right proteins to food systems could, therefore, help to enhance the quality of the food (Were *et al*., 1997). The properties of the food system that are altered upon addition of the protein include foaming, water holding capacity, oil holding and emulsification properties.

2.3.1.1. Foaming

Foaming is the ability of a protein to form a foam in the liquid mixture upon whipping by introduction of air bubbles at the air-water interface. It is caused by the interaction of the hydrophobic parts of the protein with the air and the hydrophilic parts with the water molecules in the solution leading to foam formation in both liquid and solid mediums (Stone *et al*., 2015). Foaming stability on the other hand, is the ability of the foam to hold without collapsing and is also attributed to the hydrophobic and hydrophilic interactions of the protein, air, water and other constituents in the food system (Adenekan *et al.,* 2017). Foams in food products are responsible for texture and volume. Therefore, foaming agents are applicable in the food industry for products such as ice cream, cakes, whipping cream, marshmallows, mousses, etc. (Ladjal Ettoumi & Chibane, 2015). In food systems proteins contribute to the majority of foaming agents and the most common culinary foaming agent are animal proteins such as egg white protein (EWP) and casein (Murray, 2020). However, due to high demand for animal proteins and the associated high cost, currently there is a growing interest to use plant proteins as alternative foaming agents over animal proteins (Murray, 2020). Examples of plant proteins currently used as foam formation and stabilizer agents are soy, lentil and pea proteins (Ladjal Ettoumi & Chibane, 2015).

2.*3.1.2. Water holding capacity and water solubility index*

Water holding capacity (WHC) is defined as the ability of the protein to retain water molecules in its three dimensional structure (Zayaz, 1997) and this property is considered essential in food systems because of its effect on food texture. WHC can also affect moisture content of a food and affect storage conditions. WHC of a protein can be affected by different factors such as the protein structure, presence of carbohydrates and non-protein components in the food system (Nguyen *et al*., 2015).

Water solubility index (WSI) is another equally important physiochemical property of proteins relating to water. WSI is defined as the solubility of biomolecules such as starches, fibers, proteins and sugars in the supernatant after or before the application of excess water (Sharma *et al*., 2017). Therefore, this property is also investigated with WHC when proteins are applied in food systems as both can affect the quality of the food products.

2.3.1.3. Oil holding capacity and emulsification properties

Oil holding capacity (OHC) of a protein represents the amount of oil absorbed per gram of the protein after elimination of excess oil. Addition of proteins with high OHC, therefore, enhances food flavor because fats and oils act as flavor retainers and improve the mouth feel of the food (Ladjal Ettoumi & Chibane, 2015). Oil binding capacity of a protein is related to its hydrophobic surface property and is influenced by its flexibility and surface properties (Nguyen *et al*., 2015). In other words, the hydrophobic parts of the protein are responsible for the oil binding and for the emulsification property of the protein.

When proteins are used as emulsifying agents in food systems, the hydrophobic parts bind to the oil while the hydrophilic parts bind water and this helps to maintain the oil and water without

separation leading to what is called emulsification (Stone *et al*., 2015). Therefore, proteins with good oil binding property are considered important for application in such foods as meat products, cakes and pastes and help to increase the food succulence, texture and flavor. Similarly, proteins exhibiting good emulsification properties are considered useful for application in food products such as ice creams, pastes, and sauces. Such proteins can also be added to food products such as doughnuts and pancakes to avoid excessive oil binding when the food is fried (Arntfield, 2018). Therefore, for plant proteins to be used in the food industry they must possess desirable physiochemical characteristics (Khalid *et al*., 2003).

2.3.1.4. Other constituents affecting functional properties of plant proteins

The functional properties of plant proteins can also be affected by the presence of other molecules that attach to proteins. For example, the presence of phenolic compounds, metabolites found covalently attached to proteins, are known to affect protein functionality and nutritional quality (Rawel *et al*., 2005). Other functional properties of proteins that is affected by phenolic compounds include protein hydrophobicity and protein structure. This is due to the ability of the phenolic compounds to bind to amino acid residues of the protein therefore affecting how the protein interacts with other molecules (Seczyk *et al*., 2019).

2.3.2. Preparation of peptones from plant proteins

Most microorganisms require the presence of organic nitrogen sources for growth. Although proteins are the most abundant organic nitrogen sources for microbial growth, being a large molecule, it cannot be directly absorbed into the cell and thus require proteolytic degradation in the extracellular environment into peptides and amino acids which can be readily taken up by the cell (Vasileva-Tonkova *et al*., 2007). However, most of the microorganisms lack the enzymes to hydrolyze the protein polymer in the extracellular environment before absorption. Therefore, most

microbiological media contain peptone as an organic nitrogen source (Andualem & Gessesse, 2013). Peptones are protein hydrolysates widely used as components of microbiological media serving as a source of nitrogen (Uzeh *et al*., 2006). Examples of commercially available microbiological media used containing peptones include nutrient agar (NA), Luria-Bertani (LB) medium, mannitol salt agar (MSA) and MacConkey agar.

Protein hydrolysates could be produced through enzymatic, acidic, or alkaline hydrolysis. Protein hydrolysis involving acid and base could lead to the production of modified amino acids affecting the nutritional value of the hydrolysate (Tavano, 2013). On the other hand, enzymatic hydrolysis is carried out under mild reaction conditions and result in the production of protein hydrolysates with intact amino acids with their original nutritional content. As a result enzymatic hydrolysis is the preferred and widely used method for the production of protein hydrolysates (dos Santos Aguilar & Sato, 2018)

In most cases animal proteins such as casein and meat are widely used in the production of peptones for microbiological media and protein hydrolysates for other applications. However, in recent years a rise in the demand for animal proteins led to a price increase thus forcing the search for alternative protein sources for the production of peptones used in microbial culture media (Shareef, 2019). Among the different alternatives, plant seed proteins provide an attractive potential for the preparation of peptones used in the preparation of microbial culture media (Andualem & Gessesse, 2013).

CHAPTER 3

MATERIALS AND METHODS

3.1. Sample collection and preparation

A. digitata fruits were collected in Bobonong, Botswana and transported to the laboratory at Botswana International University of Science and Technology (BIUST) in Palapye. The fruit was cracked open manually and the seeds containing the fruit pulp were hand removed using a spatula, collected in a bucket, and washed five times with tap water and once with distilled water until the fruit pulp was completely removed. The seeds were then blotted dry using a paper towel, transferred to an industrial blender (Waring Products, USA), then grinded to a fine powder using a pestle and mortar (Figure 3).

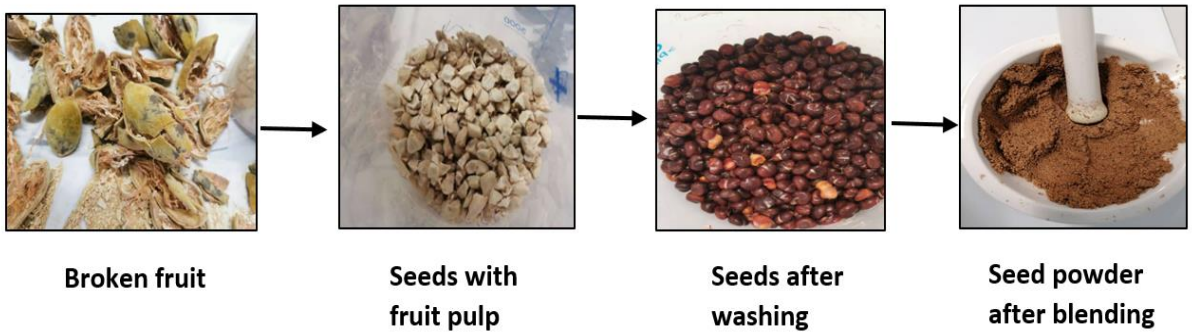

Figure 3. The process of seed removal from *A. digitata* fruit, pulp removal and blending of the seeds.

3.2. Proximate analysis of *A. digitata* seeds

3.2.1. Oil content

To extract the oil, 250g of seed was crushed, and the oil was extracted using hexane through maceration (Figure 5). The weight of the oil extracted was then measured after being refined using 96% ethanol (further elaborated in 3.3.1). The weight of the cake was then also recorded. Oil content was expressed as a percentage of the initial seed and was determined using the equation below.

$$\%\ Oil\ Content = \frac{Initial\ Seed\ Weight - Dried\ Seed\ Cake\ Weight}{Initial\ Seed\ Weight} * 100$$

3.2.2. Ash content

To determine the ash content, 10 g of seeds was placed in 5 crucibles and heated in a furnace (Ashing Furnace, AAF 1100, MRC Laboratory Instruments, Germany) at 600°C for 2 h. The resulting ash was weighed, and percentage ash content was obtained as shown below.

(a) $Weight\ of\ Ash = Weight\ of\ Crucible\ and\ Ash - Weight\ of Crucible$

(b) $\%\ Ash\ Content = \frac{Weight\ of\ Ash}{10\ g} * 100$

3.2.3. Moisture content

To determine moisture content 10 g of seeds was placed in pre-weighed crucibles and then dried in an oven at 80°C until it reached constant weight. Percentage moisture content was calculated as shown below.

$$\%\ Moisture\ Content$$

$$= \frac{Initial\ Weight\ of\ Crucible\ and\ Sample - Final\ Weight\ of\ Crucible\ and\ Sample}{10\ g} * 100$$

3.2.4. Protein content

Protein content was determined using the biuret method (Janairo *et al*., 2011) with modifications using bovine serum albumin (BSA) (Sigma-Aldrich) as a standard. The seed powder (2.1 mg/ml) in Dulbecco's phosphate buffered saline (DPBS) (Sigma-Aldrich) was thoroughly mixed using a vortex mixer. Then to 1 ml of the sample, 1.5 ml of Biuret reagent was added followed by incubation at room temperature for 30 minutes. Absorbance was measured at 550 nm using Spec200E UV-Vis spectrophotometer (ThermoFisher Scientific, USA) and the concentration of the sample and protein content of the seed were determined from the BSA standard curve (Figure 4).

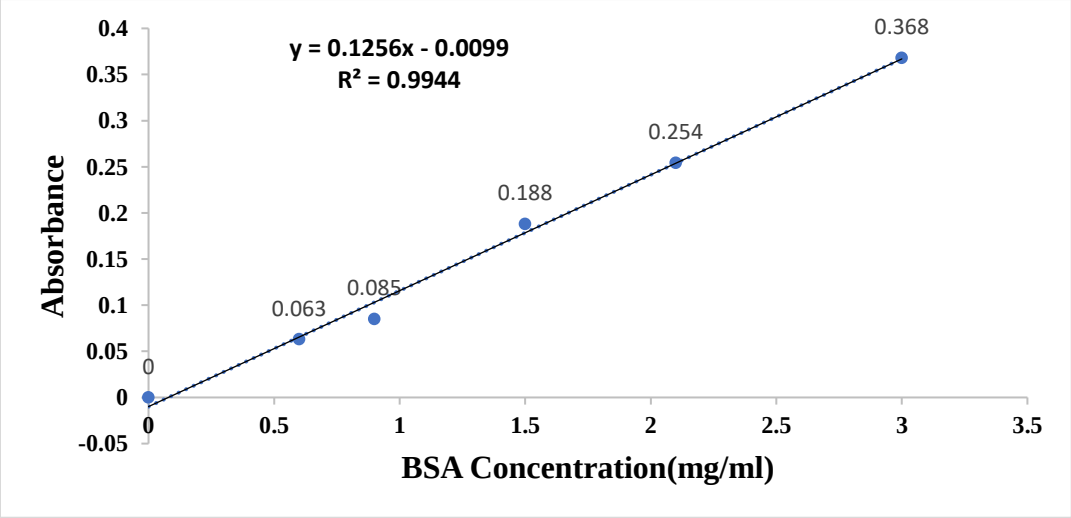

Figure 4. BSA standard curve.

3.2.5 Carbohydrate/starch content by difference

The carbohydrate/starch content was obtained by difference. The percentage of the other seed constituents was subtracted from hundred percent as shown below.

$$\%\ Carbohydrate\ or\ Starch\ Content = 100\% - Sum\ of\ Seed\ Content\ (\%)$$

3.3. Biodiesel production

3.3.1. Oil extraction and refining

To extract the seed oil, 242 g seed powder was suspended in hexane (Rochelle Chemicals) and incubated at room temperature for two days with occasional agitation (Figure 5.a). The liquid phase was then separated, and the solvent removed using a rotary evaporator at 40°C and 110 rpm. The oil obtained after solvent evaporation appeared cloudy probably indicating the presence of other components. Therefore, the oil was further refined using 96% ethanol (Rochelle Chemicals) at an oil to ethanol ratio of 2:1. The mixture was added to a separating funnel and allowed to separate overnight (Figure 5.b). The top layer containing the refined oil was collected and the bottom phase was discarded. The top layer was centrifuged at 2500 rpm for 8 minutes to remove any remaining suspended particulate matter and the supernatant was collected. Any excess ethanol remaining in the oil during the refining phase was removed using a rotary evaporator. The resulting clear oil was used for biodiesel production.

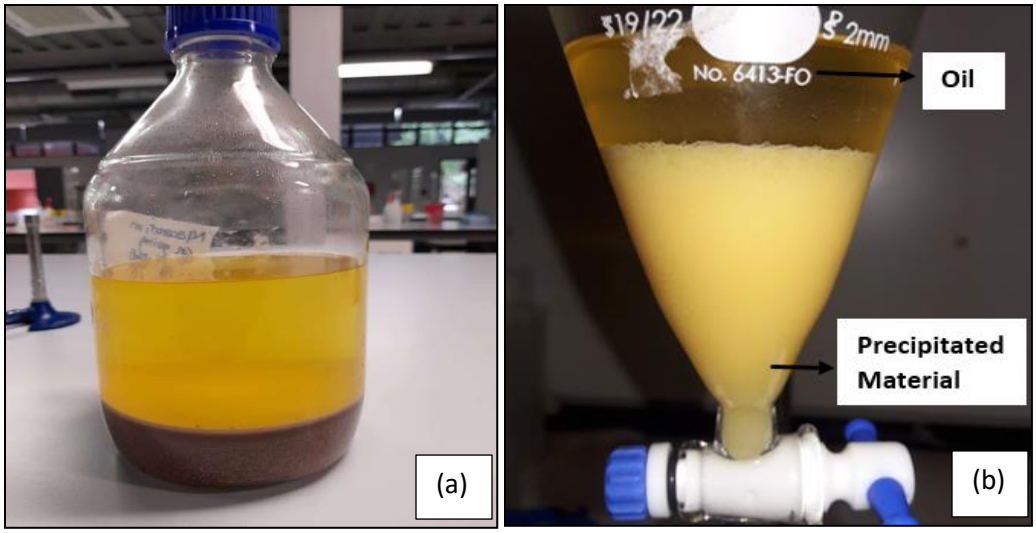

Figure 5. (a) Oil extraction through maceration using hexane and (b) oil refining using ethanol.

3.3.2. Biodiesel production

To produce biodiesel from *A. digitata* seed oil, 1.0 g of KOH was dissolved in 22.64 g of methanol through continuous stirring followed by addition of 100 g of the oil. After 2 h incubation at room temperature with continuous stirring the reaction mixture was transferred to a separating funnel and left to stand overnight (Figure 6). The bottom layer (containing glycerol and residual catalyst) was separated from the top layer containing the biodiesel. Residual methanol (Rochelle Chemicals) was removed using a rotary evaporator.

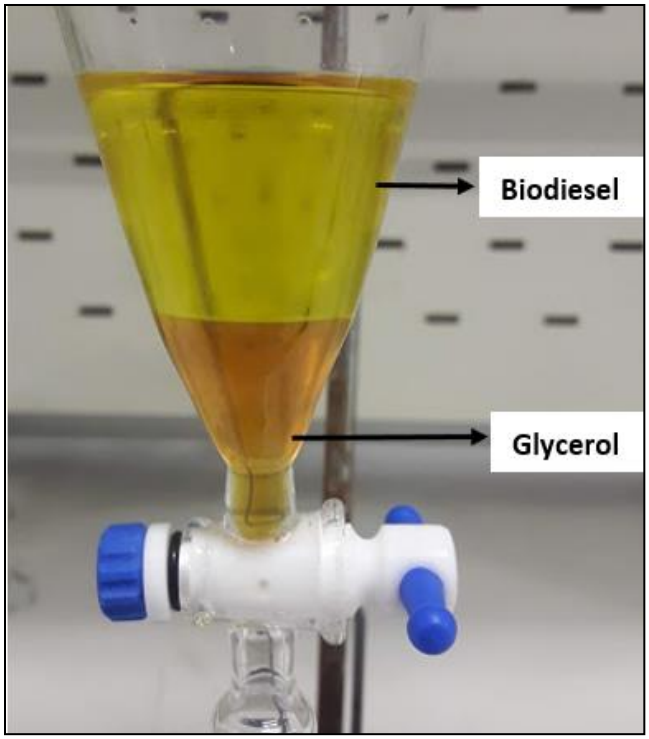

Figure 6. Separation of biodiesel and glycerol after transesterification

3.4. Biodiesel analysis

3.4.1. Biodiesel yield ratios

The transesterification efficiency was analyzed by calculating the biodiesel yield and glycerol output using the following equations.

$$BY(\%) = \frac{Biodiesel\ Produced\ (g)}{Oil\ Used\ (g)} * 100$$

$$GO(\%) = \frac{Glycerol\ Produced\ (g)}{Biodiesel\ Produced\ (g)} * 100$$

Where *BY* is biodiesel yield and *GO* is glycerol output.

The properties of the biodiesel (density, kinematic viscosity, flashpoint, cloud point, pour point and iodine number) were tested following the standard methods of the International Organization for Standardization (ISO) (3675, 3679 and 3104), American Society for Testing and Materials (ASTM) International and Association of Official Analytical Chemists (AOAC) described by (ISO, 2013; 2015; 2020), Modiba *et al*. (2014) and Kruatian & Jitmanee (2020).

3.4.2 Density

The density of the biodiesel produced was measured using Density/Specific Gravity Meter DA-640 at 15°C. This was done by passing enough sample of the biodiesel into the machine using an insert tube making sure to avoid air bubbles by fully immersing the tube into the sample. The sample was analyzed for density and then removed using an outlet tubing. The experiment was then repeated three times.

3.4.3. Kinematic viscosity

The kinematic viscosity was measured using CANNON Instrument Company Ubbelohde Viscometer following the instructions of the manufacturer. The viscometer was cleaned using acetone then dried using clean filtered air. The lower reservoir of the viscometer was charged with the biodiesel sample until the sample meniscus was between the minimum and maximum fill lines on the reservoir. The viscometer was vertically placed on a holder and placed in a bath previously adjusted to 40°C. After 1 hour of equilibration the sample was drawn using a pipette through the measuring bulb into the feeder bulb. The time it took for the meniscus to pass from the maximum

line to the minimum line of the measuring bulb was taken and used to calculate the kinematic viscosity using the equation below.

$$Kinematic\ Viscosity = Efflux\ Time(s) * Viscosity\ Constant(0.04941)$$

3.4.4. Flash point

To measure the flash point of the biodiesel, Automated Abel Closed Cup Flash Point Tester was used following the manufacturer's instructions. The cup was filled with the sample and placed into the flash point tester. The flash point was tested by increasing the temperature until the flash point temperature was detected by passing the automated flash over the biodiesel cup.

3.4.5 Cloud point

To measure the cloud point of the biodiesel, 20 ml of the sample was pipetted into a clean beaker then placed in a refrigerator constantly monitoring the temperature using a thermometer. The temperature at which the biodiesel started forming a cloudy or waxy appearance was then noted.

3.4.6. Pour point

To determine the pour point, 20 ml biodiesel sample was kept in a freezer (-20°C) for approximately 10 minutes and the temperature measured constantly. The temperature at which the biodiesel completely stops flowing was then recorded.

3.4.7. Copper corrosion test

To carry out corrosion test, copper strips were thoroughly polished with sanding paper and were placed in 30 ml sample of biodiesel then kept at 40°C in a measuring cylinder for 3 hours. At the end of the 3 hour incubation the strips were carefully blotted dry using a tissue towel and compared with a freshly polished copper strip and the ASTM copper strip corrosion standard (Figure 7).

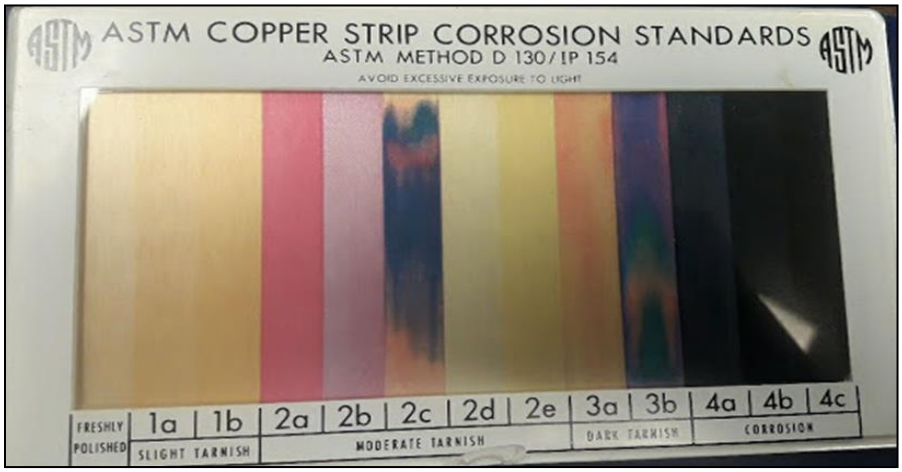

Figure 7. Copper strip corrosion levels of ASTM copper strip corrosion standards

3.4.8. Iodine number

Iodine number was determined by methods described by Kruatian & Jitmanee (2020) with modifications. To do this 0.3 g of the biodiesel was transferred into a flask and mixed with 25ml Wij's solution. A blank was prepared by mixing 0.3 g of chloroform and 25 ml of Wij's solution. After 1 hour incubation in the dark 10 ml of 10% potassium iodide solution was added and titrated against 0.1 N sodium thiosulphate until a pale-yellow color appeared. Then 1 ml of 1% starch solution was added and the titration continued until the dark blue color disappeared. The iodine value was calculated as shown below.

$$Iodine\ Value = \frac{12.69 * (V_B - V_S) * N}{W_S}$$

Where V_B is the volume of Sodium thiosulphate used on the blank, V_S is the volume of thiosulphate used on the biodiesel sample, W_S is the weight of the sample used and *12.69* is the AOAC standard constant for calculation of Iodine Value.

3.5. Thin layer chromatography for biodiesel

Thin layer chromatography was carried out using a 10 cm x 10 cm silica gel coated Merck TLC plates (Merck TLC). Before application, the sample was diluted with hexane (1:4 ratio) and mixed using a vortex mixer then 1 µL was applied on the TLC plate and developed using the solvent mixture of ethyl acetate: hexane: methanol (1:9:1 ratio). When the solvent front reached to the top, the plate was removed from the chamber and air dried at room temperature for 15 minutes followed by spraying with 10% phosphomolybdic acid in ethanol. The plate was immediately heated on a hot plate until a dark blue color appeared (Figure 11). The retention factor (Rf) values of the spots were calculated using the following formula.

$$Rf\ value = \frac{(a)}{(b)}$$

Where *(a)* is the distance travelled by sample and *(b)* is the distance travelled by mobile phase.

3.6. FTIR analysis for *A. digitata* biodiesel

Biodiesel produced from *A. digitata* seed oil was further analyzed by FTIR using Bruker Vertex FTIR machine equipped with platinum-attenuated total reflection (ATR) using the Opus software. The sample insert chamber was cleaned with 99% ethanol and a blank background was ran before the biodiesel sample. About 100 µLof the biodiesel sample was placed on the insert chamber and the sample was analyzed for transmittance at wavelength ranging from 4000-500cm^{-1}. The graph was plotted using ORIGIN-PRO software to show the spectrum for the biodiesel sample.

3.7. Isolation of *A. digitata* seed meal protein and its functional properties

3.7.1. Protein isolation from *A. digitata* seed meal

The dry seed powder (190g) which was defatted using hexane was weighed and suspended in distilled water and thoroughly mixed using a magnetic stirrer. The pH was adjusted to pH 9.5 by gradually adding 2M NaOH while stirring. Stirring continued for 5 minutes to dissolve the protein then left to settle for 1 hour. After 1 hour the top layer containing dissolved protein was collected for acid precipitation. Acid precipitation was done by gradually adding concentrated HCl while stirring until protein precipitation was visible. The mixture was then left to settle for an hour, the top liquid layer was discarded and the bottom layer with the precipitated proteins was collected and centrifuged at 3500 rpm for 10 minutes. The supernatant was further discarded, and the pellet was collected. The pellet was then resuspended in 96% ethanol and centrifuged at 3500rpm for 10 minutes then the supernatant was discarded. The pellet was dried at room temperature in an evaporating dish overnight, the dried protein was further grinded into fine powder using pestle and

mortar and stored. The process is pictorially depicted in Figure 8. The protein was analyzed for functional properties, electrophoretic profile and then hydrolyzed to be used in microbial media formulation.

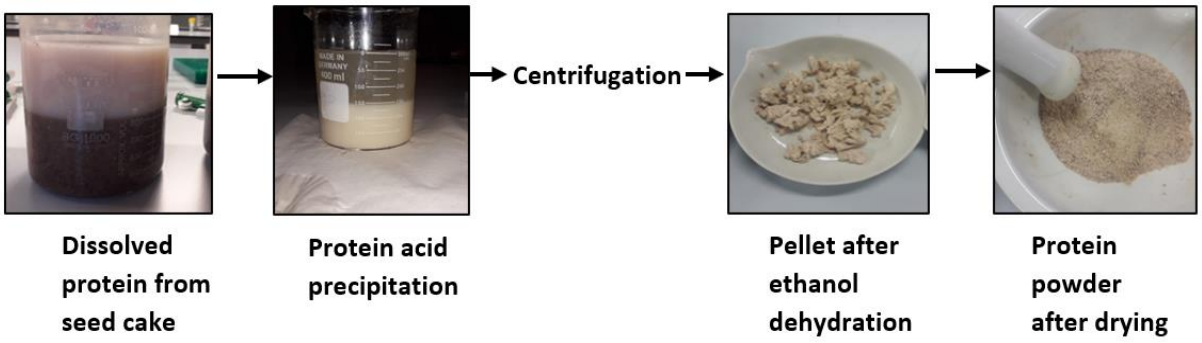

Figure 8. Protein isolation from the seed cake after oil extraction

3.7.2. Functional properties of *A. digitata* seed protein

3.7.2.1 Water holding capacity (WHC)

To 2 g of protein powder 20 ml of distilled water was added and vortexed for 30s. After being hydrated for 2 hours at room temperature the mixture was centrifuged at 2800 rpm for 10min. The supernatant was discarded, and the hydrated sample was weighed. The water holding capacity was calculated as follows:

$$Weight\ of\ hydrated\ sample = Weight\ of\ tube\ and\ hydrated\ pellet - Weight\ of\ tube$$

$$WHC\ (g/g) = \frac{Weight\ of\ hydrated\ sample - Weight\ of\ dry\ sample}{Weight\ of\ dry\ sample}$$

3.7.2.2. Water solubility index (WSI)

To 2 g of the protein in a 50 ml tube 20 ml distilled water was added and mixed for 10 minutes using a vortex mixer. The mixture was centrifuged at 2800 rpm for 10 minutes, the supernatant was transferred into an evaporating dish and dried at 80°C until a constant weight was obtained in three consecutive measurements. The water solubility index (WSI) was then calculated using the following equation.

$$\%\, WSI = \frac{Weight\ of\ dissolved\ solids\ in\ supernatant}{Weight\ of\ dry\ sample} * 100$$

3.7.2.3. Foaming capacity (FC) and foaming stability (FS)

To 50 ml of distilled water, 1.5 g of protein powder was added (3% w/v) and homogenized using a hand blender at the highest setting for 3 minutes. The mixture was immediately poured into a 100 ml measuring cylinder and the volume recorded after 30s. The foam capacity (FC) was expressed as the percentage increase in volume by the following formula:

$$\%FC = \frac{Vaw - Vbw}{Vbw} * 100$$

Where *FC* is the foaming capacity, *Vaw* is the volume after whipping and *Vbw* is the volume before whipping.

Foaming stability was calculated as:

$$\%\, Foam\ Stability = \frac{Foam\ volume\ after\ time(t)}{Initial\ foam\ volume} * 100$$

Where t = 60 minutes

3.7.2.4. Oil holding capacity (OHC)

To 3g of protein powder in a centrifuge tube 30 ml oil was added and mixed using a vortex mixer. The mixture was allowed to stand for 1 hour at room temperature and then centrifuged at 2800 rpm for 10 minutes. The supernatant was discarded, and the pellet was weighed. The oil holding capacity was calculated as follows:

$$Oil\ Holding\ Capacity\ (g/g) = \frac{Weight\ of\ pellet - Weight\ of\ dry\ sample}{Weigh\ of\ dry\ sample}$$

3.7.2.5. Solubility

The protein solubility was determined using the methods described by (Khalid et al., 2003) with modifications. This was done by preparing a 1% (w/v) protein solution in 0.01M NaCl solution, pH adjusted from pH 3 to 9 using 0.1M HCl or 0.1M NaOH. After 30 minutes of stirring with a magnetic stirrer at room temperature the pH of the solution was measured, readjusted if necessary and centrifuged at 10 000g for 20 minutes. For total soluble protein content of the control the sample was dispersed in 0.1M NaOH to solubilize the protein and centrifuged at 10 000g for 20minutes. The protein content of the supernatants was then determined using bovine serum albumin (BSA) as the standard. The protein solubility was then determined for the samples done in triplicates as follows:

$$\%\ Protein\ Solubility = \frac{Protein\ Content\ of\ Sample}{Protein\ Content\ of\ Control} * 100$$

3.7.2.6. Total phenolics

Total phenolic content was determined for *A. digitata* seed protein isolate (Table 4) using gallic acid standard (Figure 9) to obtain gallic acid equivalent (GAE) per gram of dry weight of the

sample using the methods described by Medina (2011) and Alu' datt *et al.*, (2010) with modifications. The samples were prepared by dissolving 10 mg of the sample in 1ml methanol and vortexed for 5 minutes. The mixture was then centrifuged for 2 minutes at 1000g and the supernatant was collected. To 0.5ml of the supernatant, 0.5ml of 1N Folin Ciocalteu reagent was added followed by the addition of 1ml of 20% NaOH and 8ml of deionized water. The mixture was gently swirled and allowed to stand for 45 minutes for color development. Absorbance was measured at 725 nm using Spec200E UV-Vis spectrophotometer (ThermoFisher Scientific, USA) and the total phenolic content was determined using the gallic acid standard (Figure 9).

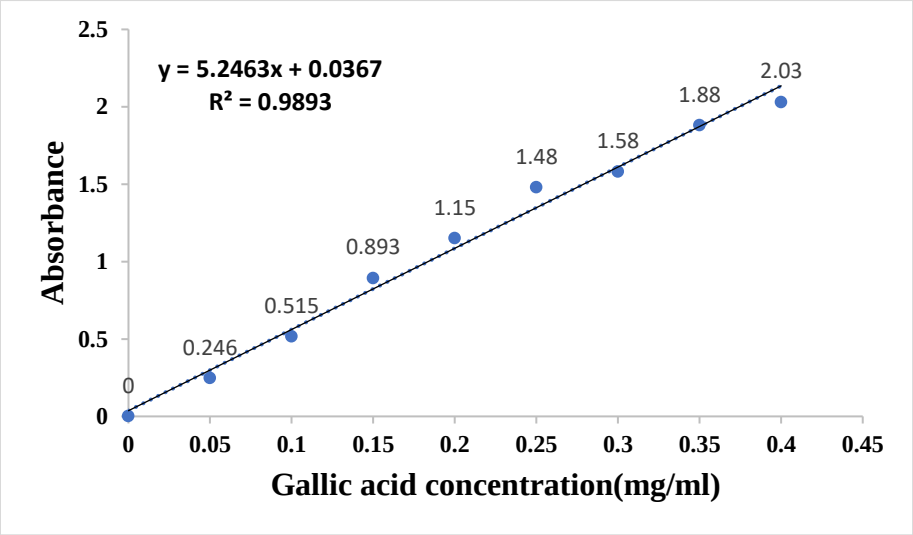

Figure 9. Gallic acid standard curve

3.8. Enzymatic hydrolysis of *A. digitata* seed meal protein and evaluation of the hydrolysate as a microbiological peptone

3.8.1. Enzyme preparation and hydrolysis of *A. digitata* seed protein

The enzymes used for the hydrolysis of the protein were extracted from two protease producing alkaliphilic bacterial strains, *Bacillus spp.* BC 022 and *Bacillus pseudofirmus* BC 026 isolated in

our laboratory using methods detailed by (Mokoba, unpublished data). This was done by cultivating both organisms in liquid culture then centrifuging to obtain a cell free supernatant. The cell free culture supernatant obtained after centrifugation was concentrated through acetone precipitation. The activity of the enzymes was assayed following the methods of Mokoba (unpublished data) using a tryrosine standard curve (Figure 10). One unit of enzyme activity was expressed as the amount of enzyme that release 1µg of amino acids equivalent to tyrosine. The concentrated enzyme preparations were used to hydrolyze *A. digitata* seed protein to obtain hydrolysates.

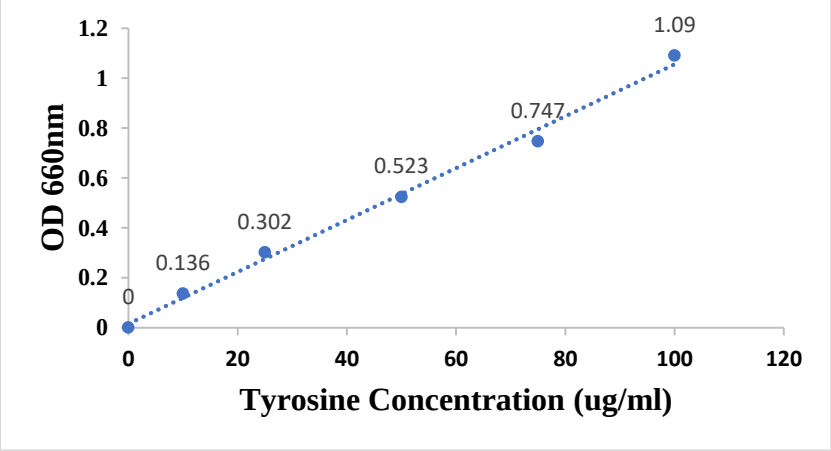

Figure 10. Tyrosine standard curve.

Protein hydrolysis was carried out enzymatically by suspending the isolated protein in 10 mM carbonate bicarbonate buffer, pH 9 to a final concentration of 10%. The hydrolysis reaction was carried out at 20% (v/v) enzyme to substrate solution ratio and incubated at 50°C with shaking. After 8 h of incubation the reaction was stopped by heating in a boiling water bath for 10 minutes followed by centrifugation at 4000 rpm for 10 minutes. The supernatant was recovered, and pH adjusted to 7.0. The resulting protein hydrolysate was dried in an oven at 80°C until a constant

weight was achieved, and the dry weight determined by taking the average triplicate determinations using the equation shown below.

$$Hydrolysate\ Dry\ Weight(g) = Weight\ of\ Crucible\ with\ Dry\ Sample - Weight\ of\ Crucible$$

$$Hydrolysate\ Concentration(g/ml) = \frac{Hydrolysate\ Dry\ Weight}{5ml}$$

Where *5ml* was the volume of the hydrolysate

The electrophoretic profile of the hydrolysate was analyzed using SDS-PAGE and then used for microbial media formulation.

3.8.2. SDS-PAGE analysis of *A. digitata* seed protein isolate and hydrolysate

The electrophoretic profile of the different samples was analyzed by loading the samples in a standard SDS-PAGE. To 100 µL of sample 100 µL of the 4X Laemmli sample buffer (BIORAD) was added and heated at 95°C for 5 minutes. Then 20 µL of the sample was loaded on each well of a 12% acrylamide gel and run at 70V until the samples passed the 4% acrylamide stacking gel followed by a run at 120V until the dye reached the bottom of the gel. The gel was then stained for 2 h in 0.2% Coomasie blue solution containing 25% isopropanol, 10% acetic acid and 65% water. The gel was destained using a destaining solution containing 5% methanol, 7% acetic acid and 88% water for 2 hours, rinsed with distilled water, and photographed using BIO RAD ChemiDoc™ MP Imaging System.

3.9. Microbial culture media formulation and test for microbial growth

Microbial media was prepared using *A. digitata* protein hydrolysate and used to grow four different bacterial test strains, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29123, *Bacillus subtillis, Pseudomonas aeruginosa* ATCC 10145. The test strains were grown in Luria-Bertani (LB) agar containing commercial peptone (0.5%), yeast extract (0.2%), sodium chloride (0.5%) and agar (3%). The baobab peptone agar (BPA) was prepared by replacing the commercial peptone in the above media with 0.5% baobab protein hydrolysate (BPH). For growth on agar plates 0.1 ml of an appropriately diluted sample from an overnight culture was spread on duplicate plates of both LB agar and BPA. After 24 h incubation at 37°C the number of colonies were counted using a colony counter and growth was expressed as colony forming units per milliliter of culture (CFU/ml).

Growth in liquid culture was determined by growing the test organisms in same media as above minus the agar (Liquid LB broth and baobab peptone broth (BPB)). First the inoculum was adjusted with McFarland standard to OD 600 nm reading of 0.3 which translated to $2x10^8$ CFU/ml. The media were inoculated with 5% (v/v) inoculum and grown at 37°C with shaking. Then OD readings were taken every two hours until constant and used to prepare the growth curves.

CHAPTER 4

RESULTS

4.1. Proximate analysis of *A. digitata* seed composition

Proximate analysis of *A. digitata* seeds showed that oil and protein account for about 60% of the total seed dry weight (21.3% oil and 39% protein) (Table 1). The remaining 40% of the seed dry weight was accounted for by mineral (ash) (3.7%), moisture (3.0%) and carbohydrate (33%).

Table 1. Proximate analysis of *A. digitata* seeds composition

Assay	Proximate Composition (%)
Oil content	21.3
Protein content	39.0
Moisture content	3.0
Carbohydrate/starch content by difference	33.0
Mineral (Ash) content	3.7

4.2. Production and analysis of biodiesel from *A. digitata* seed oil

A. digitata seed oil was converted to biodiesel through transesterification reaction with methanol using potassium hydroxide as a catalyst. As shown in Table 2 oil to biodiesel conversion ratio was 0.91 and the glycerol yield ratio was 0.15. This shows the effectiveness of the transesterification reaction in converting the oil into biodiesel.

Table 2. Biodiesel yield ratios

Reaction Parameter & yield	Ratio
Glycerol yield (glycerol: biodiesel)	0.15
Biodiesel yield (oil: biodiesel)	0.91

Analysis of the resulting biodiesel using thin layer chromatography (TLC) (Figure 11) shows the presence of one major biodiesel spot with an Rf value of 0.87 as opposed to an Rf value of 0.7 for the seed oil. As shown in Figure 11 *A. digitata* oil was almost completely converted into fatty acid methyl esters (biodiesel). This could also show that TLC analysis is a valuable tool to monitor the biodiesel production process and monitor the conversion of oils as other also previously suggested. TLC has also been recommended as a tool to detect biodiesel purity (Fontana *et al*., 2009).

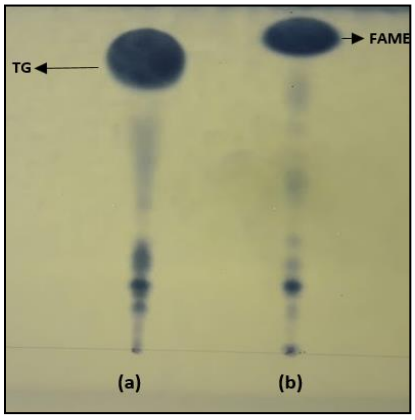

Figure 11. TLC of (a) *A. digitata* seed oil with Rf value of 0.70 cm and (b) Separation of *A. digitata* biodiesel with Rf value of 0.87 after treatment with phosphomolybdic acid.

As a further confirmatory test, the biodiesel was analyzed using FTIR. As shown in Figure 12 the biodiesel fingerprint region ranging from 500-4000cm^{-1} was visible therefore indicating the successful production of the biodiesel from *A. digitata* oil. The common biodiesel ester group and

asymmetric stretching alkanes shown in red at 1739 cm^{-1} and 2924 cm^{-1} were clearly visible. However weak peaks with bended vibrations were observed at wavelengths of 1437cm^{-1} to 723cm^{-1}.

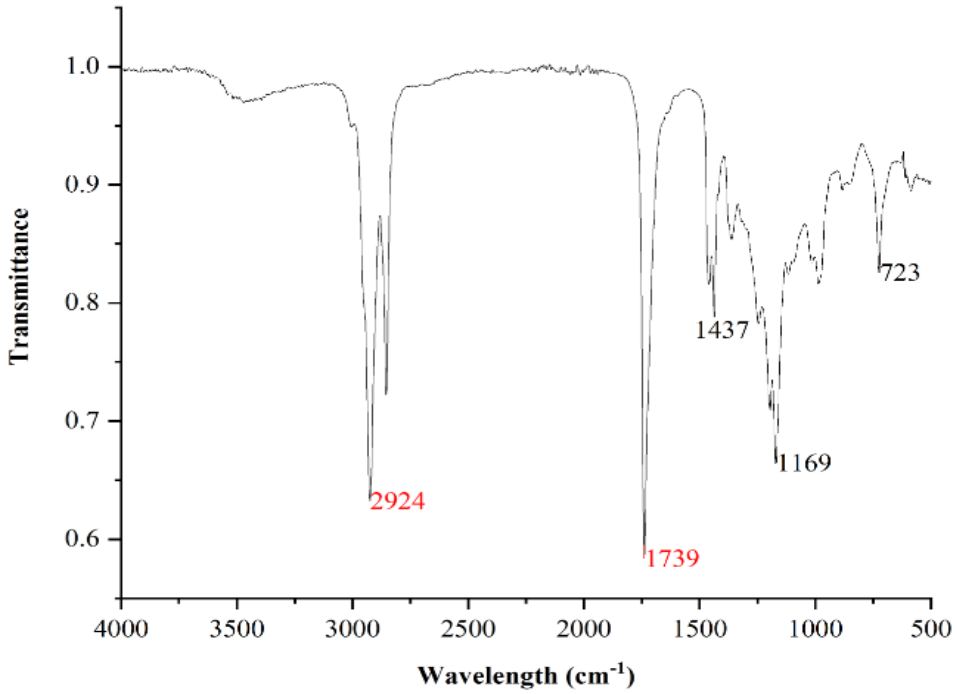

Figure 12. *A. digitata* biodiesel FTIR spectrum

4.3. Characterization of *A. digitata* biodiesel

Table 3 shows the fuel parameters used for the analysis of the biodiesel sample. The *A. digitata* oil had a deep yellow appearance and the biodiesel prepared from it also appeared yellow (Figure 6). In general, the physical appearance of the biodiesel can differ depending on the oil used and different purification systems used for the biodiesel (Atadashi *et al*., 2011).

Biodiesel is expected to have properties similar to diesel. Therefore, the properties of *A. digitata* biodiesel were tested to determine if it has fuel properties that meet different standards, such as

ISO, ASTMD and AOAC. *A. digitata* biodiesel had a density of 878 kg/m^3, a kinematic viscosity of 4.276 mm^2/s, a flash point of 118°C, a cloud point of 5°C, a pour point of -11°C and iodine number of 57.9 (Table 3). Therefore, in all the parameters tested *A. digitata* biodiesel fell within the standard range whereas the flashpoint was slightly lower than the standard (Table 3). This shows that *A. digitata* oil can be used for the production of biodiesel and the resulting biodiesel can be useful as a fuel as it meets the fuel quality standards.

Table 3. Parameters of biodiesel produced from *A. digitata* seed oil

Parameters	Unit	Measurement Value	Standard	Standard Limit
Color and odor	-	Bright yellow and pleasant	-	-
Density	kg/m^3	878.0	ISO 3675	860-900 kg/m^3
Kinematic Viscosity	mm^2/s	4.276	ISO 3104	3.5-5.0 mm^2/s
Flash point	°C	118	ISO 3679	> 120°C
Cloud point	°C	5	ASTMD 6751-02	-3°C - 12°C
Pour point	°C	-11	ASTMD 6751-02	-15°C - 10°C
Iodine number	(I_2g/100g of biodiesel)	57.9	AOAC	< 120

4.3.1. Copper corrosion test

The effects of the biodiesel on copper showed that the biodiesel exhibited class 1A of copper corrosion according to the ASTM copper strip corrosion standards. Figure 13 indicates a freshly polished copper strip (a) and the copper strip after being exposed to the biodiesel (b). The result show that the biodiesel is safe for use where copper is concerned as it does not exhibit corrosion.

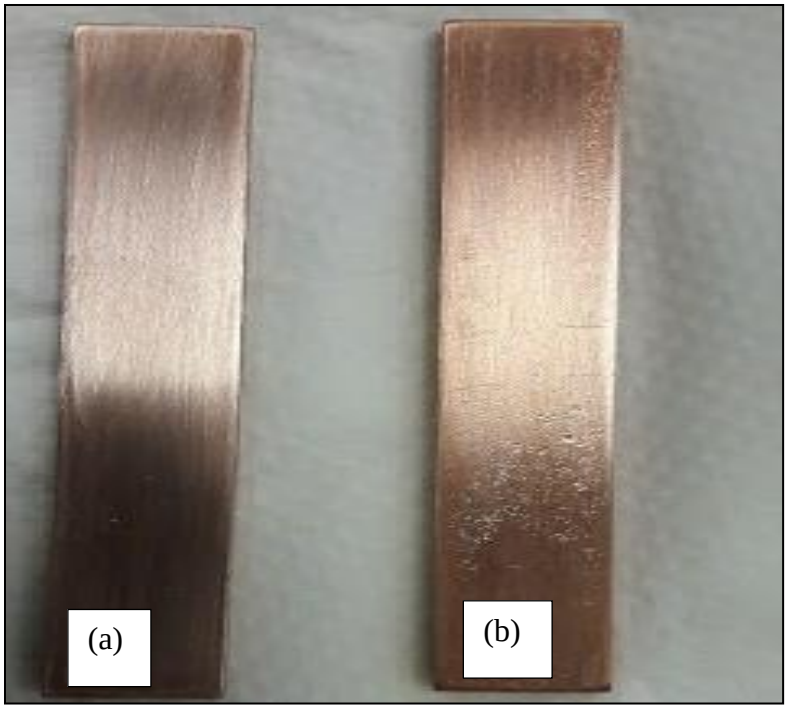

Figure 13. (a) Freshly polished copper strip and (b) copper strip exposed to biodiesel for 3 hours**.**

4.4. Protein isolation from *A. digitata* seed meal and its functional properties

4.4.1. *A. digitata* protein functional properties analysis

After oil extraction the *A. digitata* seed protein was isolated from the seed cake and its functional characteristics were determined (Table 4). One area of potential application for the isolated protein is as a component of food in the food industry. Therefore, proteins added in food must possess some properties that impact the functional quality of the food. One such property is oil holding capacity and water holding capacity which has an impact on food texture and influence the moisture content of food. *A. digitata* protein had a water holding capacity (WHC) of 1.29 g/g and a water solubility index of (WSI) of 3.25%.

Another important property of proteins is its foaming capacity and foam stability which has an impact on food texture and volume. The foaming capacity and foam stability of *A. digitata* protein were determined to be 40.66% and 67.00%, respectively.

Another important property of proteins is oil holding capacity since it has an important function in determining flavor retention and mouth feel of food. The oil holding capacity (OHC) of *A. digitata* protein was 114%. The protein also had phenolic content of 1.71mg/g, a property that can affect the functionality of the protein and showed the highest solubility at pH 9 and the lowest solubility at pH 3 (Figure 14).

Table 4. Functional properties of *A. digitata* seed protein

Property	Unit	Measurement Value
Water holding capacity (WHC)	g/g	1.29
Water solubility index (WSI)	(%)	3.25
Foaming capacity	(%)	40.66
Foam stability	(%)	67.00
Oil holding capacity	g/g	1.14
Phenolics	mg/g	1.71

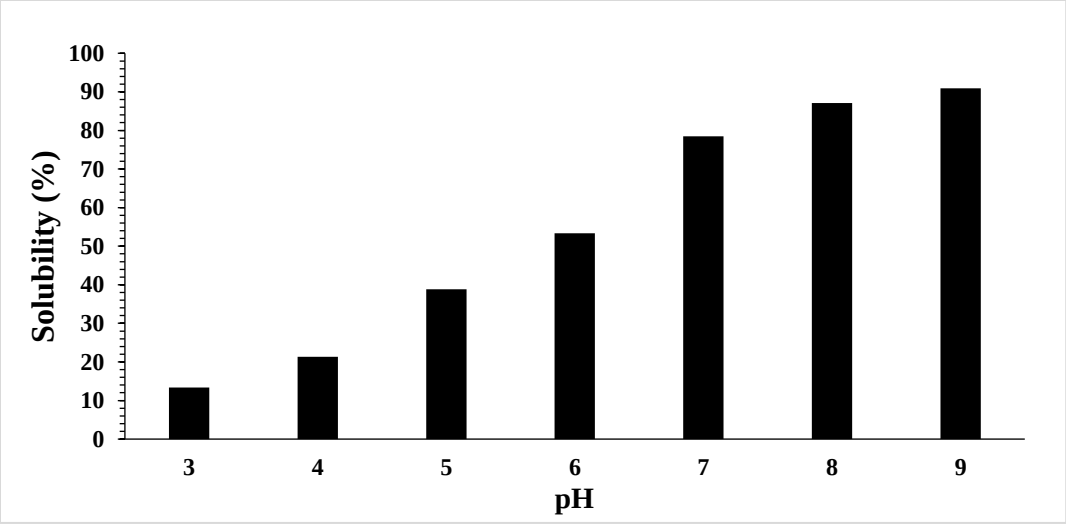

Figure 14. *A. digitata* protein solubility from pH 3-9

4.4.2. Hydrolysis of *A. digitata* protein and evaluation of the hydrolysate as a microbiological peptone

The electrophoretic profiles of the baobab seed powder (BSP), baobab seed protein isolate (BSPI) and baobab seed protein hydrolysate (BSPH) are shown in Figure 15. Three major protein bands with an approximate molecular weight of 30 kDa, 70 kDa and 75 kDa from BSP and BSPI samples are visible. This indicates that the seed contains three major proteins.

The protein was enzymatically hydrolyzed using two different proteases derived from *Bacillus spp* BCC 22 and *Bacillus pseudofirmus* BCC 26, used at 100 U/ml 120 U/ml, respectively. Analysis of the electrophoretic hydrolysate on SDS-PAGE showed disappearance of the major protein bands indicating their hydrolysis observed earlier in the protein preparation indicating complete hydrolysis of the major protein fractions (Figure 15). For the sample treated with protease BCC22, new bands with a molecular weight of 15 kD or below appeared while the high molecular weight bands in the original sample completely disappeared (Figure 15.a). This indicates partial

hydrolysis of the protein resulting in the generation of low molecular weight fragments. On the other hand, the sample treated with protease BCC 26 resulted in complete disappearance of all protein bands indicating complete hydrolysis of the protein into peptides and amino acids that cannot be detected in the SDS-PAGE (Figure 15.b). Therefore, for the microbial growth test the hydrolysate prepared using protease BCC26 was used.

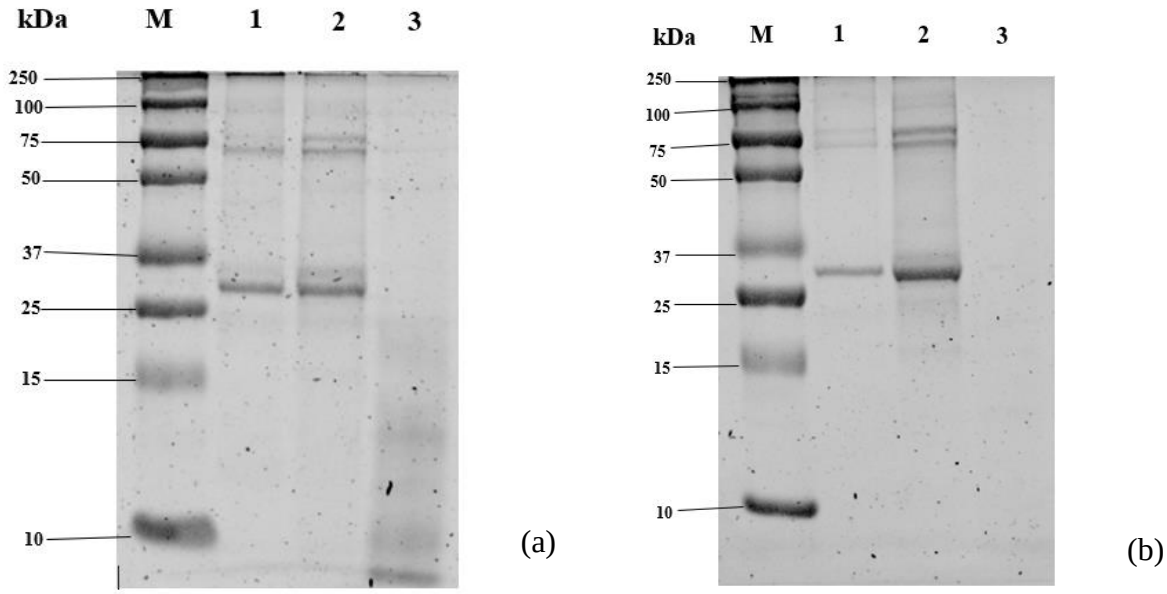

Figure 15. Electrophoretic profiles of M: marker, 1: *A. digitata* seed powder, 2: *A. digitata* seed protein isolate and 3: *A. digitata* seed protein hydrolysate (a) using *Bacillus spp* protease and (b) using *Bacillus pseudofirmus* protease.

The *A. digitata* protein hydrolysate was used as a peptone for the growth of test bacteria strains in parallel with the commercial peptone. In terms of physical appearance, the agar media prepared using *A. digitata* peptone had the same clarity as the one prepared using commercial peptone (Figure 16 .a and .b) showing that the *A. digitata* peptone did not cause any turbidity to the medium

that can cause interference in microbial growth detection and enumeration. The clarity of the media during microbial growth is indicated using *S. aureus* Figure 16 .d, showing clear visibility and enumeration of colonies.

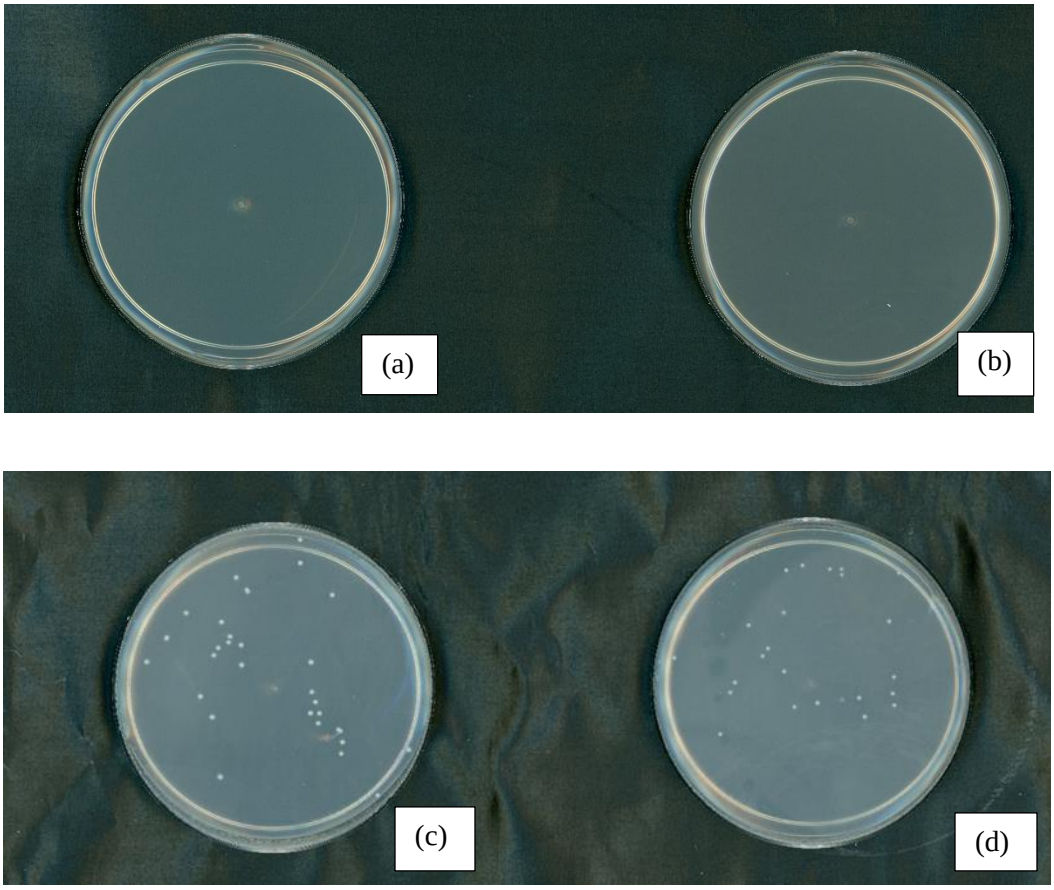

Figure 16. Media appearance of LBA (a), BPA (b), *S. aureus* colony appearance on LBA (c) and BPA (d)

Table 5 shows the CFU/ml of the four strains of bacteria grown using BPH as a peptone in microbial culture media. For three of the four test organisms used *A. digitata* peptone supported better growth than commercial peptone. Only for one strain, *B. subtillis*, growth was slightly better

in the presence commercial peptone than in *A. digitata* peptone. This shows that *A. digitata* protein hydrolysate can support many of the clinically important microorganisms same as or better than commercial peptone.

Table 5. CFU/ml of *E. coli*, *S. aureus*, *P. aeruginosa* and *B. subtillis* grown in LB agar and BPA.

	CFU/ml	
Bacterial Strain	**LB Agar**	**BP Agar**
E. coli	4.05×10^8	4.15×10^8
S. aureus	3.00×10^8	4.75×10^8
P. aeruginosa	3.65×10^8	5.20×10^8
B. subtillis	5.57×10^7	2.02×10^7

When *A. digitata* peptone was used in liquid culture for the growth of the above four test organisms very good growth was noticed (Figure 17.a-d) indicating the potential usefulness of *A. digitata* peptone for routine microbiological applications.

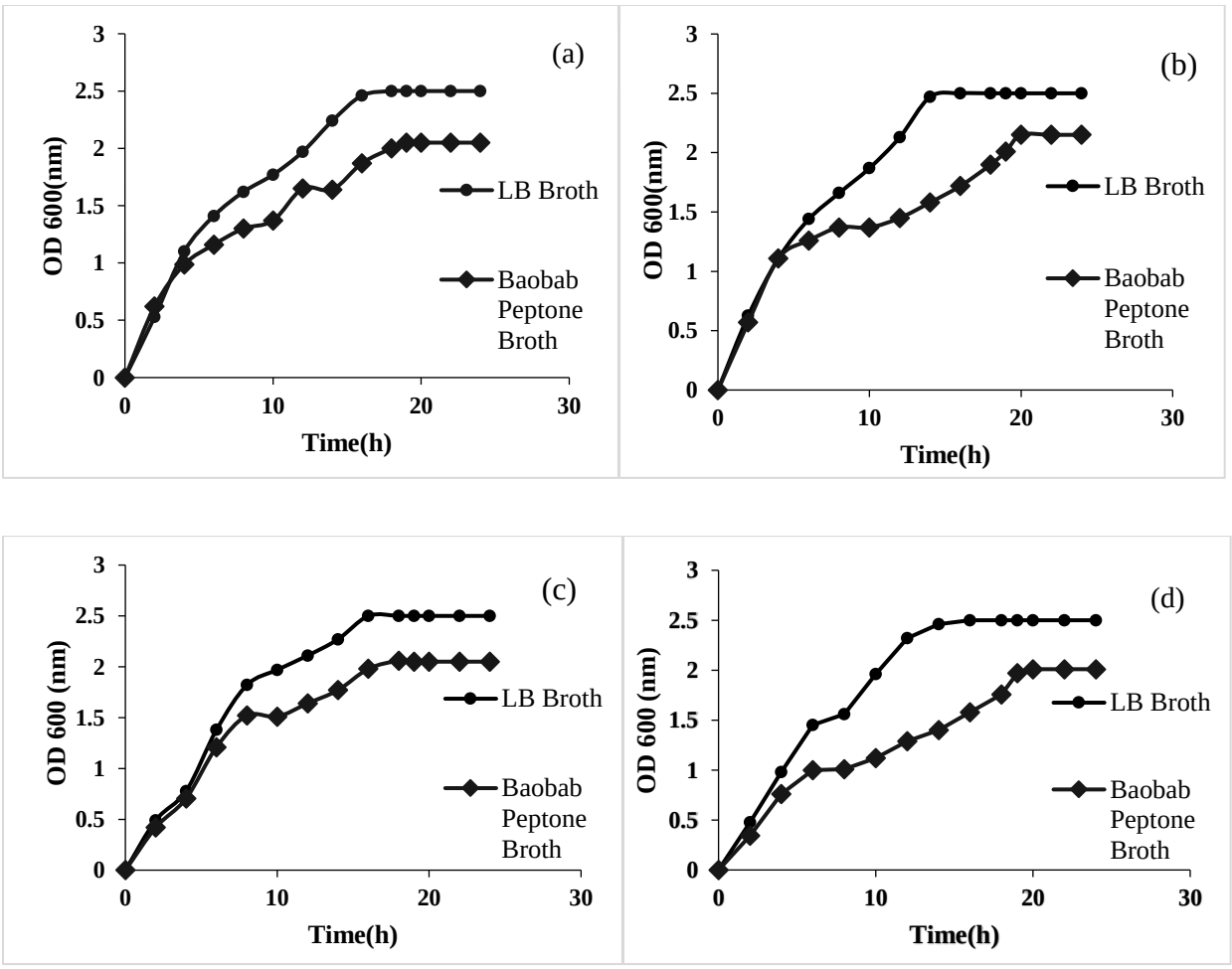

Figure 17. Growth curve of (a) *E. coli*, (b) *S. aureus*, (c) *P. aeruginosa and* (d) *B. subtillis* on LB broth with commercial peptone and BPB with *A. digitata* peptone.

CHAPTER 5

DISCUSSION

5.1. Proximate composition of *A. digitata* seed

A. digitata seed is composed of high oil and protein content, accounting for 21% and 39% of the seed dry weight, respectively. This shows that the protein content of baobab seed is comparable with commonly used leguminous seeds. For example, the protein content soybean was reported to be about 40% (Bayero *et al*., 2019). Therefore, the high oil and protein content of *A. digitata* seed indicate its potential use for food or feed applications. The *A. digitata* seed have been reported to be used for food by traditional communities (Kamatou *et al*., 2011). However, the consumption of the seed oil is a controversial issue as it was considered a health risk because it contains the carcinogenic cyclopropenoid fatty acids that pose a risk if the oil is not heated to 250°C (Msalilwa *et al*., 2020). However, the oil is used for other applications such as the cosmetic industry where it has found common use.

The *A. digitata* seed oil and protein content measured in this study is significantly different from those reported by other researchers. For example, Osman (2004) and Wapwera & Egila (2017) reported *A. digitata* seed oil content of 12.2% and 29.6%, respectively. Two other research groups reported the seed protein content of *A. digitata* to be 18.4% (Wapwera & Egila, 2017) and 25.14% (Osman, 2004) which is much lower than the protein content determined in this study. The observed variation in protein content and oil could probably be explained by variation in the plant variety where the seeds were obtained and/or a result of differences in the geographic regions the plant grows (Muthai *et al*., 2017).

Considering the potential risk in using *A. digitata* oil for food application, it is important to evaluate its application for other nonfood applications. One area of application for *A. digitata* seed oil proposed earlier was its potential application in cosmetics (Komane *et al*, 2017). Another potentially attractive area of application which is the subject of this study is its use as a feedstock in the production of biodiesel.

5.2. Application of *A. digitata* oil for biodiesel production

A. digitata seed oil was converted to biodiesel through a transesterification reaction with methanol with a 91% yield. Other studies using *A. digitata* oil for biodiesel production reported a biodiesel yield of only 80% (Modiba *et al*., 2014). This shows that the reaction conditions used for biodiesel in this study were properly optimized. This was further indicated from the TLC analysis which shows the absence or near absence of unreacted oil or partially hydrolyzed oil (diglycerides, monoglycerides, and free fatty acids). In addition to TLC analysis FTIR can also be used for analysis of biodiesel. In this study the FTIR analysis showed a strong absorption at 1745cm^{-1} indicating the presence of a C=O functional group of ester bond which is a characteristic feature of biodiesel. Such ester functional group is not found in petroleum diesel (Wembabazi *et al*., 2015). Other peaks with medium vibrations that were observed in the biodiesel FTIR spectrum were at 723, 1169, and 1437cm^{-1}, signifying -CH_3, C-C and C-H groups respectively, which are also characteristics of biodiesels. The 1437cm^{-1} vibration is an important peak for biodiesels, it is shown by an asymmetric bend and signifies the -CH_3 bond present in biodiesels. The vibrations observed in other studies for biodiesel are the strong C-H peaks from 2800-3000cm^{-1} (Wembabazi *et al*., 2015), the strong ester vibration (C=O) ranging from 1700-1800cm^{-1} and the -CH_3 vibrations between 900-1500cm^{-1} (Rabelo *et al*., 2015).

The *A. digitata* biodiesel was analyzed for different fuel parameters according to different standards. The biodiesel density falls in the acceptable range of the ISO 3675 standards. Density is one of the key fuel parameters which has direct impact on engine performance whether biodiesel is used as sole fuel or blended with other fuels (Alptekin & Canakci, 2008). According to the ISO 3675 standard the density of biodiesel should fall between 860-900kg/m^3. *A. digitata* biodiesel prepared in this study had density of 878 kg/m^3 which is within the ISO 3675 standard range. In a previous study Modiba *et al*. (2014) reported a density of 882kg/m^3 for biodiesel prepared from *A. digitata* oil.

Another important parameter of biodiesel determining its fuel property is kinematic viscosity at 40°C. Based on the ISO 3104 standard kinematic viscosity at 40°C should fall in the range of 3.5-5.0 mm^2/s. In this study the *A. digitata* biodiesel kinematic viscosity at 40°C was determined to be 4.2 mm^2/s which is within the standard. Kinematic viscosity is an important characteristic of fuels as it determines the injection flow of the fuel in engines and machinery. A higher viscosity leads to high flow resistance, and this can affect engine performance (Tüccar *et al*., 2018). Kinematic viscosities of biodiesel prepared from other seed oils such as sunflower oil and canola oil from previous studies were 4.5 mm^2/s and 4.7 mm^2/s, respectively.

The flashpoint of biodiesel, defined as the minimum temperature at which the fuel vapors ignite upon heating (Leung *et al*., 2010) is an important fuel characteristic because it determines the ease of ignition of the fuel and the safety of the fuel. A lower flashpoint shows the fuel can easily ignite and therefore less safe during transportation and storage. According to the ISO 3679 standard the

flashpoint for biodiesel should be higher than 120°C. The flashpoint of the *A. digitata* biodiesel prepared in this study was 118°C, a value slightly less than the accepted range in ISO 3679, which should be greater than 120°C. The flashpoints of biodiesel can be greatly affected due to presence of other residual molecules, for example the presence of residual methanol. The maximum allowable methanol content in biodiesel is 0.2%. Methanol concentration above this value can reduce the flash point of the biodiesel because methanol has a flashpoint of 11°C (Romano *et al*., 2009). Therefore, the lower flashpoint observed in this study might indicate the presence of residual methanol in the biodiesel above the allowable maximum limit. For example the flashpoint of *A. digitata* biodiesel reported earlier was 192°C (Modiba *et al*., 2014). Therefore, further removal of the residual methanol could increase the flashpoint of the *A. digitata* biodiesel.

Other important properties of biodiesel are cloud point and the pour point which are very crucial because both affect fuel flow in engines at lower temperatures. The cloud point is the temperature at which a cloudy or waxy appearance starts appearing in the fuel while the pour point is the temperature at which the fuel stops flowing completely (Modiba *et al*., 2014). *A. digitata* biodiesel prepared in this study had cloud point of 5°C and the pour point is -11°C. The ASTMD 6751-02 standard specifies a cloud point of between -3 - 12°C and a pour point of -15°C - 10°C. Therefore, *A. digitata* biodiesel prepared in this study falls within the standard. The pour point of *A. digitata* biodiesel was -11°C and the result falls within the specified range of -15°C - 10°C specified by the ASTMD 6751-02 standards. This indicates that the biodiesel is suitable for engine usage even at low temperatures. Previous studies on *A. digitata* biodiesel have also reported a cloud point and pour point of 10°C and 20°C (Buhari *et al*., 2014) and 2°C and -1°C (Modiba *et al*., 2014), respectively.

The iodine value is another important parameter to assess quality of biodiesel which measures the amount of double bonds in the biodiesel and serve as a good indicator of the biodiesel's stability. According to AOAC standard the iodine number should be less than 120. The iodine values of different biofuels vary in the range of 59 to 139 (Gopinath *et al*., 2009). The iodine number of *A. digitata* biodiesel prepared in this study was 57.9 which is within the standard. The presence of high amounts of double bonds in biodiesel could make the biodiesel highly susceptible to oxidative reactions during storage (Bouaid *et al*., 2007) which leads to destabilization of fatty acids ultimately resulting in the formation of gums that could clog pipes and affect fuel movement. The iodine value reported in this study shows good stability of *A. digitata* biodiesel.

The copper corrosion test carried out on *A. digitata* biodiesel showed a slight tarnish appearance which according to the ASTM D130/IP 154 standard is classified as class 1A. Biodiesel under class 1A are considered safe for engine usage to be used alone or in combination with petroleum diesel. Biodiesel prepared from soybean oil, rice bran oil and chicken fat fall under Class 1A (Popp *et al*., 2015).

5.3. *A. digitata* seed protein: functional properties and application

Assuming complete extraction of the oil, on dry weight basis, protein could account for 40% of the residual meal indicating its potential for food, animal feed, or for other specialized applications. If the process is coupled with biodiesel production, utilization of the protein from the meal could substantially reduce the production cost of the biodiesel. In this study the *A. digitata* protein was

isolated from the oil meal cake and its functional properties were investigated. SDS-PAGE analysis of the protein isolated from the *A. digitata* seed cake showed the presence of three major protein bands at molecular weights of 30 kDa, 70 kDa and 75 kDa.

The ability of proteins to retain water is defined as water holding capacity and is an important property in food systems because it enhances food texture. The property indicates the protein's water absorption ability. The WHC of *A. digitata* protein was determined to be 129% indicating its usefulness for food application. Adenekan *et al*., (2017) reported a WHC of 130% for *A. digitata* protein which is almost identical to the value obtained in this study. In another study Al-Juhaimi *et al*., (2020) reported that *A. digitata* seed protein helped to increase cooking yield because of its water holding capacity. This shows that *A. digitata* seed protein could be potentially used for food application.

Another related property of proteins is WSI which indicates the non-protein molecules such as polysaccharides dissolved in the supernatant before or after addition of water (Yousf *et al*., 2017). WSI is a useful indicator of the protein's usefulness in the manufacturing of extruded food products as it can affect the hardiness of the food (Sharma *et al*., 2017). The WSI of *A. digitata* protein was found to be 3.25 % indicating its potential application in the manufacturing of extruded food products which can potentially impart hardiness and bulkiness to the food product.

Two other useful protein properties of proteins in the food industry are foaming capacity (FC) and foam stability (FS) which are defined as the ability of the protein to form a foam and the ability of the foam to stand without collapsing, respectively. *A. digitata* protein showed a FC of 40.7% and a FS of 67% indicating its potential usefulness in the food industry. Earlier studies on *A. digitata*

protein have also reported FC of 44.86% and FS of 67.36% (Adenekan *et al.,* 2017). Foaming of proteins is believed to be a result of the interaction between its hydrophobic and hydrophilic parts with water and air which result in the formation of air bubbles in the food system resulting in the formation of a foam. The results of this study therefore shows that *A. digitata* protein may have an interesting potential to be used as a foaming agent in the production of foods such as creams.

In food systems the hydrophobic parts of proteins interact with the oil and that interaction leads to retention of the oil which defines the property known as oil holding capacity (OHC) of proteins (Ladjal Ettoumi and Chibane, 2015). Therefore, OHC is one of the desirable properties of proteins used in the food industry because increase in OHC can improve the flavor and feel of food. The OHC of *A. digitata* protein was determined to be 1.14 g/g or 114% indicating its potential usefulness in the food industry. Adenekan *et al*., 2017 reported a 150% OHC for *A. digitata* protein. In previous studies, when the *A. digitata* protein was added to beef patties it increased fat retention capacity and decreased the shrinking capacity of the patties (Al-Juhaimi *et al*., 2020) which is explained by the high OHC of the *A. digitata* protein and its ability to retain oil. In general, drying or shrinkage of food is caused by the loss of moisture and oil from the food and addition of proteins with good OHC allow to retain plumpness of food by trapping oil. Therefore, the high OHC of *A. digitata* protein indicate its potential usefulness as a thickener in food products.

The oil and water capacities of a protein can also be associated with its emulsification properties defined as the ability of the protein to facilitate the mixture of oil and water forming an emulsion. Formation of an emulsion results from the interaction of the hydrophobic parts of the protein with oil while its hydrophilic parts interact with water. The ability of *A. digitata* protein to interact with water and oil indicate its ability to act as an emulsifying agent in food products such as in the production of sauces, pastes, and of beverages (Adenekan *et al*., 2017)

The *A. digitata* protein contains appreciably high phenolic compounds which could attach to the protein through non covalent interactions (Rawel *et al*., 2005). The presence of phenolic compounds is expected to have some effect on the protein's functional properties, such as hydrophobicity and nutritional quality. On the one hand phenolic compounds provide nutritional benefits because of their high antioxidant activity which has a protective function against diseases such as cancer. On the other hand phenolic compounds could affect some characteristics of the food such as bitterness, flavor and color (Seczyk *et al*., 2019). Therefore, while considering *A. digitata* protein for application in the food industry its high content of phenolic compounds needs to be taken into consideration.

The solubility of *A. digitata* protein increases with increase in pH reaching a maximum at pH 9. A similar pattern was also reported by Adenekan *et al*., (2017) for *A. digitata* where solubility increased with increasing pH when tested in the range 5 to 8. A similar increase in solubility with pH was also reported for soy protein a protein which is widely used in the food industry (Were *et al*., 1997).

5.4. Hydrolysis of *A. digitata* protein and its application

Peptone generated through enzymatic hydrolysis of *A. digitata* protein supported very good growth of Gram-negative and Gram-positive bacterial strains equal or slightly better than commercial peptone. The media prepared from *A. digitata* peptone was clear and indistinguishable from the media prepared using commercial peptone. The peptone was able to also support the growth of the test strains grown in broth medium. Good growth of the test microorganisms shows that *A. digitata* peptone provides the necessary nutrients these cells normally get from commercial peptone. Uzeh *et al*., (2006) reported good growth of *E. coli*, *S. aureus*, *P. aeruginosa* and *B. subtillis* in media

containing hydrolysates of soya beans and African locust beans which was comparable to commercial peptone. Currently commercial peptone is mostly prepared from animal proteins. However, animal proteins have increasingly become very expensive making the peptone prepared from it expensive too. Therefore, *A. digitata* protein could help to replace commercial peptone for the preparation of microbiological media.

5.5. Feasibility of using *A. digitata* oil and protein: a theoretical analysis

In this study *A. digitata* seed was shown to contain about 21% oil and 39% protein. About 91% of the oil was converted to biodiesel and the remaining 9% released as byproduct mainly composed of glycerol. This means that up to 19% of the seed weight can be converted to biodiesel. Similarly, 39% of the seed weight is accounted for by protein. Assuming 90% of the protein can be isolated as pure protein, about 35% of the original seed weight could end up into pure protein. The protein can be used directly in the food industry or hydrolyzed to generate protein hydrolysates for application in the food industry or as peptone for growth of microorganisms.

Together the biodiesel and the *A. digitata* protein could account for 54% of the original seed weight. The remaining 46% of the original seed weight could be released as byproducts such as glycerol released during the production of biodiesel, the thick seed coat, carbohydrates, and other seed components. It must also be noted that the *A. digitata* pod in addition to bearing the seeds inside, contains a white pulp having several potential applications of its own.

The *A. digitata* tree is also a perineal tree estimated to stay alive for centuries. Since it grows in arid environments it thrives well under water stress. Therefore, if the biodiesel and the protein

could find commercial use and get government support, it is expected to greatly benefit the local communities. Some of the benefits could include:

1. Communities could collect and sell pod to seed collectors
2. Small seed collectors in villages could collect seed and fruit pulp and sell it to oil producers and food producers
3. The oil producers could sell to factories specialized in biodiesel production
4. The pulp and the oil cake can be supplied to larger food factories for food production

All the above activities could create jobs for the local communities. If the local community gain benefit from the tree they are expected to care for it and could be encouraged to plant more trees. This will in turn lead to ecological rehabilitation as more trees are planted and taken care of.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

The results of the study show that the baobab tree (*A. digitata)* as an important source of oil to be used as a feedstock in biodiesel production and as a source of valuable protein for food and non-food application. The biodiesel produced from the oil meets international standards indicating its potential for fuel application.

After oil extraction, the oil meal was also used for the isolation of proteins. The protein exhibited functional properties that make it suitable for application in the food industry. After enzymatic hydrolysis the protein hydrolysate was used to grow different species of bacteria and supported growth of all the test strains equal or better than commercial peptone. All these show that *A. digitata* protein could find important application in the food industry and in the production of microbiological media.

Promotion of the use of the seed oil and protein for the production of value-added products is expected to benefit local communities by creating jobs and helping them to generate income from the collection and sale of the seed and other associated products. This could in turn encourage communities to plant more trees ultimately leading to environmental rehabilitation.

6.2. Recommendations

In this study biodiesel produced from *A. digitata* seed oil was shown to have a good potential for fuel application. Utilization of the protein isolated from the oil meal cake has also been shown as

a valuable resource for food and nonfood applications considering its functional properties. The utilization of the protein could therefore greatly help to reduce the production cost of the biodiesel. In the process of biodiesel production, different other byproducts potentially useful for different applications are also generated. Therefore, to further reduce the production cost of biodiesel it is recommended that methods for their utilization are developed.

Glycerol is one of the byproducts generated during biodiesel production from *A. digitata* seed oil. Potentially the glycerol could find several industrial applications in crude form or after purification. The crude glycerol released during biodiesel production, for example, could be used as a carbon source in the fermentation industry. After purification and refining the glycerol can also find application in the cosmetic industry and many other applications in the chemical industry. Therefore, further research on the utilization of the glycerol is recommended.

During the production of *A. digitata* seeds large quantity of pulp and fruit shell are generated. The pulp is edible and could find application in the food industry. On the other hand, the hard fruit shell could be used for the production of many valuable products, one of which is activated charcoal to be used for a variety of industrial applications. Therefore, it is recommended that further research and development be done on the fruit pulp and the fruit shell.

In this study it was demonstrated that the protein isolated from the oil meal was shown to possess many functional properties for application in the food industry. These properties include water holding capacity, oil holding capacity, foaming capacity and foaming stability. However, to evaluate if the protein could indeed be valuable in the food industry, evaluation under application conditions will be necessary. Therefore, it is recommended that the protein be tested for different food applications at small and pilot scale.

The *A. digitata* is a tree with huge potential benefit for local communities. The fact that it grows under severe water stress makes it an extremely valuable plant in arid and semi-arid environments. Therefore, it is recommended that in depth research aimed at understanding the genetic diversity of the plant, methods of conservation and propagation be carried out with proper national coordination.

CHAPTER 7

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