SCREENING AND MOLECULAR CHARACTERIZATION OF MICROORGANISMS PRODUCING HYDROLYTIC ENZYMES FOR CASSAVA (*Manihot esculenta* Crantz) STARCH DEGRADATION

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DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has not wholly or in part been presented for the award of degree in Egerton University or any other institution.

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Recommendation

This thesis has been submitted with our approval as the official university supervisors.

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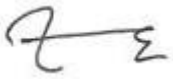
Signature…  ……. Date…16/06/2023…….

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DEDICATION

I dedicate this work to my son Alvin Wesley Muriithi Nyambura.

Matthew 7:7 & 8 Ask, and it will be given to you; seek, and you will find; knock, and it will be opened to you. For everyone who asks receives, and he who seeks finds, and him who knocks it will be opened.

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ABSTRACT

Corn starch is a traditional source of glucose in the food industry, but its use is limited due to competition with corn as a staple food. Cassava (*Manihot esculenta* Crantz), which has a high starch content, is a potential alternative starch source. However, the complex structure of cassava starch hinders its industrial application. This study aimed to isolate and identify microorganisms capable of producing amylase enzymes from diverse and unexplored environments in Kenya. The initial screening involved evaluating the ability of microorganisms to grow on minimal media supplemented with starch. Based on this screening, 70 microbial amylolytic isolates were selected. These isolates underwent a primary screening based on starch hydrolysis ratio (SHR), determined by halo formation on starch agar plates after flooding the plates with Lugol's iodine solution. This narrowed down the number of isolates to 17, which were presumed to be excellent amylase producers (SHR > 1.5) and were further used in subsequent experiments. The 17 selected isolates were cultivated in M9 starch broth media at an optimum temperature of 30°C and pH 7.0 to produce amylase enzymes. Crude amylases were extracted from the supernatant, quantified at 280 nm, and assessed for amylase activity using the 3,5-dinitrosalicylic acid (DNSA) method. Enzyme stability was tested through incubation at 30°C for 18 hours, while the effect of temperature and starch loading was evaluated by measuring activity at 45°C, 50°C, and 55°C, and with starch concentrations of 20%, 50%, and 60%, respectively. A commercial amylase was used as a control. The specific enzyme activities of the crude enzymes from the 17 isolates ranged from 0.0079 U g^{-1} to 0.0629 U g^{-1}, which were lower compared to the specific activity (1.7188 U g^{-1}) of the control enzyme. The top 5 enzymes with the highest specific enzyme activity were selected for cassava hydrolysis. These crude enzymes exhibited higher specific enzyme activity (0.0707–0.1853 U g^{-1}) than the control enzyme (0.0052 U g^{-1}) in hydrolyzing cassava starch. Among the isolates, those with the highest specific enzyme activity were identified through DNA sequencing and belonged to *Lysinibacillus* spp. (4 isolates), *Alternaria* spp. (1 isolate), and *Bacillus* spp. (2 isolates). Alkaline lakes (Lake Elementaita and Lake Bogoria) had the highest number (4) of isolates with enzymes showing the highest specific enzyme activity, followed by the gut contents of black soldier flies. The specific enzyme activity increased with temperature, and starch loading had an impact on enzyme activity. While most enzymes showed reduced activity over time at 30°C, enzymes from one isolate retained full activity at 18 hours of incubation, demonstrating thermostability. This study highlights the presence of microorganisms with amylase activity in tropical environments that can be scaled and optimized for practical applications.

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LIST OF ABBREVIATIONS AND ACRONYMS

HFCS	High Fructose Corn Syrup
DE	Dextrose Equivalent
CSR	Cassava storage roots
PDA	Potato Dextrose Agar
PPD	Post-harvest physiological deterioration
FW	Fresh weight
DM	Dry matter
KBS	Kenya Bureau of Statistics
NCBI	National Center for Biotechnology Information (NCBI)

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Starch is one of the most abundant and renewable carbohydrate resource found in plants including tuberous plants, cereals, and legumes (Sonnewald and Kossmann, 2013). It is a polymer of glucose and is the most important energy source for human nutrition and the second most abundant source of energy and carbon in plants with high demand in the food processing industry (Park *et al*., 2018; van Zyl *et al*., 2012). The starch conversion industry is the largest single consumer of starch, utilizing about 60% of total starch production. Starch, when hydrolyzed produces low molecular weight products (glucose/dextrose, maltose, maltotriose and dextrin), which could be used in industries as raw material for the production of a repertoire number of products such as glucose, glucose–fructose syrups, fructose, maltose, organic acids, and amino acids among others (Zainab *et al*., 2011). Starch is also a major feedstock in the fermentation industry where it is hydrolyzed for fermentative production of bioethanol (Favaro *et al*., 2015; Waterschoot *et al*., 2015), and has also been used in the manufacture of biodegradable films from renewable sources (Luchese *et al.*, 2017).

In the current set up, corn starch has been the main source of starch in the industries. However, as a result of unpredictable climatic variations, countries in sub-Saharan Africa are faced with diminishing corn production (Balogun *et al*., 2013). For instance, in Kenya, with a population of close to 50 million that is increasing (KBS- Kenya Bureau of Statistics, 2019), the demand for maize is 5 million metric tonnes per year. Based on the prevailing maize production rates, this means that the maize deficit is currently around 1.2 million metric tones annually. Increased reliance on imports implies that the foreign exchange reserves and resources earmarked for development will be likely diverted for the procurement of food for Kenyans (Corbould, 2013; De Zeeuw & Dubbeling, 2009; Tacoli & Agergaard 2017). Cassava is a key mandate commodity, that if used can reduce overdependence on maize (Harry *et al*., 2017). Cassava is an important staple root crop in the world and its production ranks sixth after maize, rice, wheat, potatoes and soybeans (FAOSTAT, 2013). In Africa, 140 million metric tons of cassava against 65 million metric tons of maize was produced in 2011, according to FAO statistics. Cassava ranks fourth among food staples with worldwide production of 2,769,106 tonnes per year (Uchechukwu-Agua *et al.,* 2015). Cassava is the second most important food root crop in Kenya after potatoes. Maize

production reached 3,897,000 tonnes while cassava's was 970,587 tonnes in Kenya according to FAO statistics (2019). Despite its high production in the coastal and western regions, utilization is limited to human consumption. This is a clear indicator of future directions in terms of sources of starch; cassava has become an important cash or commercial crop providing raw materials for industries (Tarawali *et al.,* 2012). According to Tonukari *et al.,* (2015), cassava is a potential alternative raw material source for the starch conversion industry since maize production is declining due to current unreliable climatic conditions (Omoyo *et al.,* 2015). Ensuring sustainable agricultural production is crucial for addressing food insecurity, alleviating poverty, and improving the lives of smallholder farmers (Donkor *et al*., 2017; Ojijo *et al*., 2016). Recognizing its importance, cassava has been identified as one of the key crops for Africa, as it plays a vital role in the livelihoods of African farmers and offers substantial potential for driving economic transformation in the continent (Feleke *et al*., 2016).

Cassava starch, either as native or modified form, is finding uses for a broad range of foods (including; high fructose syrup for confectionary, preparation of jelly, as thickening agents, in gravies, custard powders, baby food, spaghetti, macaroni, beer / beverage and baking industry) (Gbadegesin *et al.,* 2013) and non-food applications (including paper, textile, pharmaceutical, building materials and adhesives). In addition, cassava starch has extensively been utilized for the production of sweeteners and derivatives including glucose syrup, fructose syrup, sugar alcohols (e.g. sorbitol, mannitol, and organic acids such as lactic acid, fumaric, succinic and citric acid (Trakarnpaiboon *et al*., 2017). The application of cassava as renewable feedstock has now expanded to biorefinery, a facility that integrates processes and equipment to produce fuels (ethanol), chemicals and materials from biomass (Langeveld *et al*., 2010).

In sub-Saharan Africa, the major challenges that limit utilization of cassava crop include: short shelf- life, mass, source and the complex structure of cassava starch substrate (Li *et al*., 2017). Cassava roots are also bulky and heavy, and therefore expensive to transport over long distances. The roots are also perishable, and must be either consumed or processed (Aso *et al.,* 2015). Post-harvest losses in cassava value chain can be minimized by processing this perishable cassava produce into high value industrial products (Abong *et al*., 2016).

The cassava root crop is a physiological energy reserve with high carbohydrate content, ranging from 32% to 35% on a fresh weight (FW) basis, and from 81 to 88% on a dry matter

(DM) basis. Eighty percent of the carbohydrates produced is starch (Montagnac *et al*., 2009), 83% is in the form of amylopectin and 17% is amylose (Figure 1) (Rawel & Kroll, 2003).

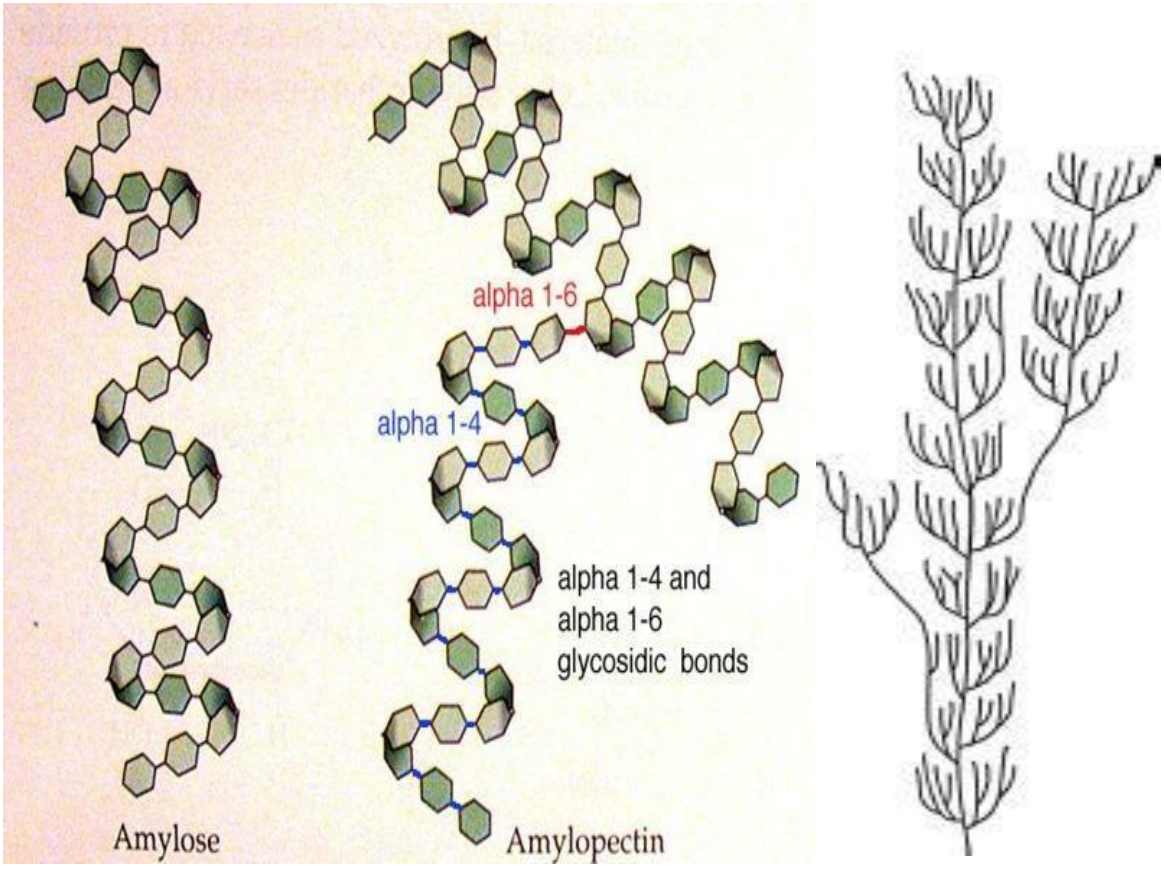

Figure 1: Chemical Structure of Amylose and Amylopectin Respectively
Source: Damodaran *et al.*, (2008) and Montagnac *et al*., (2009)

Generally, starch is a polysaccharide composed of two types of polymers – amylose and amylopectin. Amylose constitutes 20-25% of the starch molecule. It is a linear chain consisting of repetitive glucose units linked by α-1, 4-glycosidic linkage. Amylopectin constitutes 75-80% of starch and is characterized by branched chains of glucose units. The linear successive glucose units are linked by α-1, 4glycosidic linkage while branching occurs every 15 to 45 glucose units where α-1, 6 glycosidic bonds are present (Figure 1) (Damodaran *et al.*, 2008; Montagnac *et al*., 2009).

Cassava starch is a polysaccharide that has a complex structure that makes it difficult to utilize directly as a fermentable substrate for production of materials (Abdel-Rahman *et al*.,

2011). The starch conversion industry relies heavily on enzyme technology (Sarmiento *et al.*, 2015). The hydrolysate composition obtained after hydrolysis of cassava starch is highly dependent on the effect of temperature, the conditions of hydrolysis and the origin of enzyme (Sundarram & Murthy, 2014). Currently, there is an increased glucose syrup production capacity, where a 'all enzymatic process' has been realized using commercial enzymes (Table 1). Enzymes work at milder conditions, are highly specific hence significant improvements in product quality, provides higher yields, and catalyze reactions faster than chemical catalysts thus energy savings (Azmi *et al* ., 2017; Prasad, 2011).

Starch hydrolysis involves the application of a variety of enzymes including α-amylase, glucoamylase, β-amylase, isoamylase, and pullulanase (Tomasik & Horton, 2012). Alpha amylase, also known as α-(1-4)-D-glucanohydrolase or dextrogenic amylase is often used for large scale starch hydrolysis. It hydrolyses α-(1-4) glycosidic bonds in amylose and amylopectin whereas α-(1-6) linkages in amylopectin are not attacked. In the first phase of hydrolysis, starch is gelatinized then liquefied to glucose and dextrins by thermostable α-amylase at high temperatures (95–105 °C) and pH (6.0–6.5) (Xu *et al*., 2016). In gelatinization, starch is heated with excess water to increase amylopectin amorphous region and enzyme accessibility. Gelatinization increases the accessibility of the substrate and enhances the hydrolysis rate (Ruiz *et al*., 2011). After liquefaction, the resultant slurry is cooled and its pH lowered to 4.0–4.5 then glucoamylase added to break down oligosaccharides to glucose.

Glucoamylase, also known as Gamma amylase, amyloglucosidase, α-(1-4) D-glucanglucohydrolase or Exo-(1-4)-α-glucosidase cleave both α-(1-4) and α-(1-6) glycosidic bonds in amylopectin (Figure 2) to release glucose molecules (Ayoola *et al*., 2013) and is therefore able to completely hydrolyze starch to glucose. Amylases contribute to 25% of the industrial enzyme market (Arabaci and Arikan, 2018; Tambekar *et al*., 2016; Wang *et al*., 2020). Cassava starch therefore will require the synergistic action of alpha amylase and glucoamylases for its degradation (Bansal *et al.,* 2011).

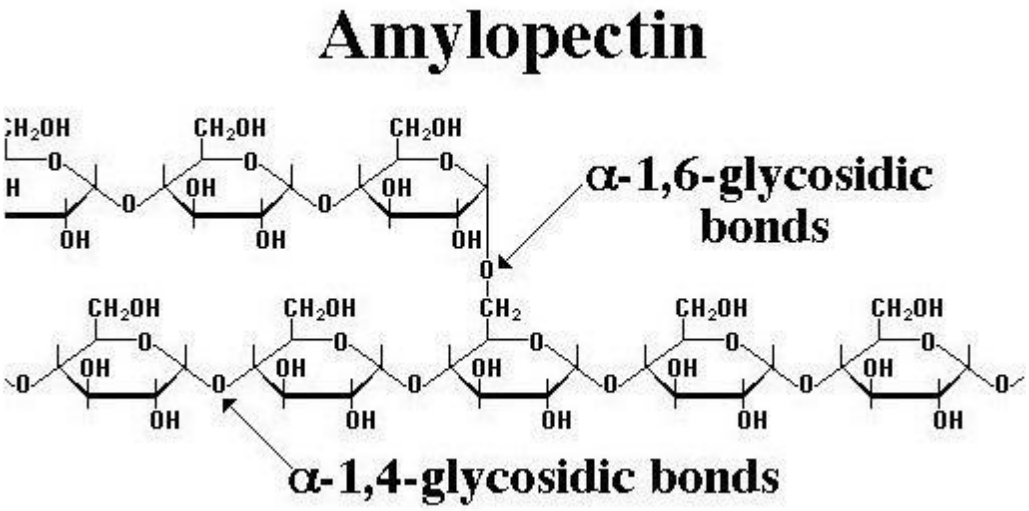

Figure 2: Glycosidic Linkages in Amylopectin

Source: Ayoola *et al*. (2013)

Amylases are derived from different sources including bacteria, fungi, actinomycetes, and plants (Table 1) (Sindhu *et al*., 2017; Tomasik & Horton, 2012). However, bacterial and fungal amylases dominate the industrial applications (Saini *et al*., 2017). This is because fungal and bacterial amylases meets industrial needs, the organisms have short growth periods, ease of bulk enzyme production, they are biochemically diverse, are safe and eco-friendly, and the organisms are genetically tractable and are versatile with environmental growth, hence yield and activity can be positively manipulated (Azmi *et al*., 2017; Wang *et al*., 2020).

Table 1: Sources of Amylolytic Enzymes

Enzyme	EC number	Source	Action
α-Amylase	3.2.1.1	*Bacillus amyloliquefaciens*	Only α-1,4 linkages are cleaved to give α-dextrins and predominantly maltose (G2),G3,G6 and G7 oligosaccharides.
		Bacillus licheniformis	Only α-1,4 linkages are cleaved to give α-dextrins and predominantly maltose,G3,G4 and G5 oligosaccharides.
		Aspergillus oryzae, Aspergillus niger	Only α-1,4 linkages are cleaved to give α-dextrins and predominantly maltose and G3 oligosaccharides.
		Bacillus subtilis (amylosacchariticus)	Only α-1,4 linkages are cleaved to give α-dextrins with maltose,G3,G4 and up to 50%(w/w) glucose.
β-Amylase	3.2.1.2	Malt barley	Only α-1,4 linkages are cleaved, from non-reducing ends, to give limit dextrins and β-maltose.
Glucoamylase	3.2.1.3	*Aspergillus niger*	α-14and α-1, 6 linkages are cleaved, from the non-reducing ends to give β-glucose.

Source: (Sindhu *et al*., 2017; Tomasik & Horton, 2012)

Diversification within the cassava industry is inevitable, as more and more cassava starch firms penetrate into the traditional corn starch markets (Hershey, 2020), and as more cassava starch firms are being launched in Kenya (Tirra *et al*., 2018). However, enzymatic hydrolysis of starch is expensive because it involves the use of imported commercial enzymes (Den Haan *et al*., 2013). Isolation and screening of microorganisms secreting amylolytic enzymes from Kenya's diverse microflora would be a cost-effective alternative for the starch conversion industry. The potential of using microorganisms as a biological source of industrially economic enzymes has stimulated interest in the exploitation of extracellular enzymatic activity in several microorganisms (Saldarriaga-Hernández *et al*., 2020).

The amylase production from different organism's exhibit broader variation since amylase production is dependent on media composition and physical parameters (de Souza & Magalhães, 2010). Moreover, the amylase enzymes in the industry are needed to work under harsh conditions such as high temperatures (for example cassava starch has high gelatinization temperature of 52-65 °C (Damodaran *et al*., 2008), low/high pH, and different salt concentrations (Gómez-Villegas *et al*., 2021). In Kenya and most of sub-Saharan Africa, enzymes for various purposes are imported (Mordor Intelligence, 2021). On the other side, Kenya is a tropical country with a diverse environment, which has a rich biodiversity of microorganisms that could be tapped for the production of various enzymes. Therefore, there is need to find diverse amylases to meet requirements of specific applications especially those that are relatively stable at elevated temperature (90–100 °C) of the starch slurry during hydrolysis. Moreover, increasing amylase titer without enhancing the overall cost of production is desirable (Miao *et al*., 2018). The present work constituted a study of screening the most efficient amylase-producing microorganisms from Kenya's diverse environment and the molecular identification of the best amylolytic isolates.

1.2 Statement of the Problem

Corn starch has become less available for industrial use as maize is a staple food for humans in Africa. Cassava has been suggested as an alternative due to its high (81-88%) starch content. However, cassava starch needs to be hydrolyzed to fermentable sugars before it can be used industrially due to its complex chemical structure and its short storage life after harvest. These two factors are important constraint that limits the full potential of cassava as a commercial food

crop in developing countries. There are many methods being used around the world to hydrolyze starch including the use of amylolytic enzymes from microorganisms. A wide range of industries including the food and beverage industry use amylases to hydrolyze starch for product manufacture. This requires a constant production of amylases and thus there is a continuous effort to produce commercial amylases. Among the amylases, microbial amylases meet industrial demands. A number of microorganisms have been identified for the production of amylases owing to the diversity of environments from where they have been isolated. In Kenya, there is a large diversity of microorganisms with some that may be having potentially more efficient amylases than the current characterized enzymes. Additionally, in industrial hydrolysis, harsh conditions of elevated temperature and low/high pH could be used for hydrolysis. For this reason, it is important to find out microorganisms with optimum activity at various harsh hydrolysis conditions. Therefore, this study aimed to screen microorganisms from Kenya's diverse microbial community, with a focus on those secreting amylases, for the hydrolysis of cassava starch. The goal was to hydrolyze cassava starch into glucose to produce various value-added chemicals and materials with industrial applications.

1.3 Objectives

1.3.1 Broad Objective

To contribute towards industrial and economic development by screening for amylolytic microorganisms with potential to hydrolyze cassava starch into fermentable sugars for industrial production of value-added products.

1.3.2 Specific Objectives

I. To isolate and characterize microorganisms from the diverse microflora in Kenya that have the potential to produce amylolytic enzymes for efficient cassava starch hydrolysis.
II. To quantify the specific activity of enzymes produced by different screened isolates for cassava starch hydrolysis.
III. To investigate the characteristics of the amylolytic enzymes produced by the screened isolates in order to optimize hydrolysis parameters.

1.4 Hypotheses

I. The diverse microflora in Kenya does not exhibit a significant difference in the production of amylolytic enzymes that hydrolyze cassava starch.
II. There is no significant difference in the specific activity of enzymes produced by different microbial isolates for the hydrolysis of cassava starch.
III. The characteristics of the amylolytic enzymes produced by the isolates have no significant effect on the hydrolysis of starch.

1.5 Justification

The utilization of cassava in the industry is often hindered by challenges such as the complex structure of cassava starch and limited knowledge of enzyme technology, which is still unexplored in Kenya. The primary objectives of this thesis were to isolate and characterize microorganisms from diverse biomes in Kenya, with the potential to produce amylolytic enzymes for efficient hydrolysis of cassava starch. The focus was on identifying microorganisms that produce superior α-amylase and amyloglucosidase enzymes, which are heat-stable and resistant to acid/alkali conditions. These enzymes enable the efficient conversion of cassava starch into fermentable sugars across a wide range of pH and temperature, while maintaining high stability.

Promoting the cassava value chain can lead to the establishment of new industries in Kenya, particularly in rural areas, for the production of various chemicals used in the food industry and fuels. This would contribute to job creation and poverty alleviation. Additionally, this study provides valuable insights into the microbial diversity of the Kenyan environment and identifies genetic resources with potential commercial value. The findings of this research are expected to generate interest within the scientific community, encouraging further studies on the unique microbial environment in Kenya. Furthermore, industries are increasingly interested in sustainable processing methods, making the screening and discovery of microorganisms producing amylase enzymes of particular importance.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Cassava (*Manihot esculenta* Crantz) is a perennial drought resistant, staple food grown in more than 90 countries in tropical and sub-tropical regions of the world. Cassava is the most important staple root crop in the world and its production ranks sixth after maize, rice, wheat, potatoes and soybeans (FAOSTAT, 2013). It is the fourth most important food crop in developing nations after rice, wheat and maize (corn) and is the world's third largest source of carbohydrates for human food (Uchechukwu-Agua *et al.,* 2015). The importance of cassava as a food and industrial crop relies on its roots since they accumulate starch (approximately 88-81% dry matter), and so, it is considered the second source of starch globally after maize (Karlström *et al*., 2016). The future of cassava in sub-Saharan Africa is linked to its diversification into industrial uses including cassava flour baked products, cassava starch conversion to glucose, animal feed and ethanol production (Li *et al*., 2017).

Cassava roots are unrivalled as the main dietary calorie for the majority of populace in sub-Saharan Africa (Gbadegesin *et al.,* 2013). In the semi-arid lands, cassava is grown largely to enhance household food security with the surplus traded in fresh form, in rural areas and major urban centers. Young cassava leaves are consumed as a vegetable (Latif *et al*., 2015) or are supplemented in livestock feeds (Vongsamphanh *et al*., 2015). There is need to explore other uses of cassava apart from its uses as food and feed. Cassava is an industrially very viable and important source of materials for processing into higher value products. Despite its great potential as a food, feed and for industrial application, its processing and marketing remains economically unexploited (Abong *et al.,* 2016a). Several factors have limited the economic development of the cassava root crop, including its high levels of cyanogenic compounds, low levels of proteins and micronutrients, and short postharvest shelf-life (2-3 days). To extend the economic value of cassava, cassava root is normally processed into cassava starch, providing an opportunity to develop value-added products for both food and industrial applications (Li *et al*., 2017).

Cassava can be grown in marginal soils, typical of low-income, small-scale farmers, with minimum input and without the need of predictable rainfall. Cassava can grow in arid and semi-

arid regions of the world and can be relied on to produce food under conditions in which other staples would fail (Lyer *et al*., 2010; Morante *et al*., 2010). It is tolerant to acid and alkaline soils, low fertility, pests and diseases, and seasonal drought. The average yield of cassava worldwide is only 12– 13 tons/Ha, but its potential yield under optimal conditions is almost seven times larger (80 tons/Ha)(FAOSTAT, 2013). According to FAO statistics (FAOSTAT, 2015), cassava world production raised to >263 million tons in 2013, a 27% increase in production during the last 10 years. From these, Asia contributed 33.5% (88.2 million tons), Africa 54.8% (144.2 million tons), and the Americas 11.6% (30.5 million tons). This trend indicates that Asia will continue growing in production and yield, while Africa, constrained mostly by viral diseases that affect the crop severely, is likely to increase the area of planting in the coming years.

Despite its agronomic advantages, cassava storage roots (CSR) are far more perishable after harvest compared to other storage root and tuber crops, such as sweet potato, true yam and potato (Compo *et al*., 2011; Waddington *et al*., 2010). Therefore, cassava is considered as a sensitive species of postharvest deterioration (An *et al*., 2012; Onyango *et al*., 2018). The rapid deterioration of CSR significantly shortens its shelf-life for fresh consumption and impacts transportation and potential for income generation. This phenomenon is known as postharvest physiological deterioration (PPD). According to studies by Salcedo *et al.* (2010), PPD in cassava is attributed to its high moisture level of 60-75% on wet basis and high respiration rate. Cassava has a very short shelf-life after harvest. Due to PPD fresh cassava has a shelf-life of only 2-3 days after harvest (Saravanan *et al*., 2016). Therefore, farmers wait to harvest the roots until ready for consumption or marketing. Rapid post-harvest physiological deterioration of cassava root has been a major constraint affecting the economic value of cassava (Kiaya, 2014).

Post-harvest losses due to PPD are estimated to be as high as 30% in sub-Saharan Africa (Djabou *et al*., 2017). A significant proportion of cassava is lost along the supply chain before it reaches the consumer markets (Naziri *et al*., 2014). Cassava is lost after harvesting and during transport, storage and processing. Reducing cassava loss and waste can contribute to environmental sustainability by lowering production costs and increasing the efficiency of the food system. At the same time, it can improve food security and nutrition (Ferraro *et al*., 2016). Diversification of cassava products is important to reduce unnecessary food loss and waste in the value chain (Abass *et al*., 2018). The Kenyan government needs to implement targeted interventions at critical stages of the value chain to reduce unnecessary cassava loss and waste (Schuster & Torero, 2016) . This can be done through continuous research to improve enzyme

technology applicable in cassava starch conversion industry (Abass *et al.,* 2018; Mtunguja *et al*., 2019). Adopting novel processing techniques using improved enzyme technology in fermentation might further increase the economic value of cassava root as well as minimize post-harvest losses (Ferraro *et al.,* 2016). Amylases have a wide application in the food industry including kiwi and apple juice clarification as well as bun making and a potential in cassava starch conversion to fermentable sugars (Rana *et al*., 2017).

Many sub-Saharan African countries including Kenya have identified food security and poverty alleviation as their priorities aligned to sustainable development goals. Given its agricultural potential, Kenya has to realize sustained substantial increase in agricultural productivity in order to overcome hunger and endemic poverty and refocus its efforts towards industrialization. Industrial application of enzymes in cassava conversion process is a great opportunity to reduce cassava loss in the value chain (Abass *et al*., 2018). Food and nutrition insecurity and poverty alleviation are some of the most important factors that the country must address in order to achieve its vision 2030. This is also going to ensure creation of jobs as well as new to the world product development.

Kenya has made major achievements in the recent past to accelerate agricultural output through research and development strategies including the improvement of marketing of cash crops such as coffee and tea (Wiggins, 2014). However, there is still need to improve marketing strategies for food crops that are well adapted to different local ecologies and well-placed to address food deficits during famines, beyond the farm level (Poku *et al*., 2018).

Cassava is an important staple food crop for subsistence mainly grown in limited acreage in the marginal and semi–arid areas that constitute over 80% of the country and is grown by resource poor small-scale farmers who are food insecure and live in poverty (Fonjong & Gyapong 2021). The agricultural potential of these marginal areas remains untapped and the consumption and marketing of cassava is still in a raw state with no value addition and is confined to local villages and nearby markets. Cassava is an annual crop grown and harvested once in a year. In recent years a substantial trade has developed in dehydrated cassava chips and pellets which are exported to Europe as a low-cost animal feed ingredient (Renuh *et al*., 2021; Wanapat *et al*., 2015). Previous studies have enlightened the scientific community and the public on the beneficial potentials of cassava as an industrial base. However, challenges of inefficient enzyme technology are a limiting factor (Sivamani *et al*., 2018).

2.2 Production and Uses of Cassava in Africa

Cassava can be used as a raw material for different purposes: it can be directly used as a food source, serve as livestock feed or be transformed for production of energy or other commodities (Tonukari *et al*., 2015). Its utilization strongly depends on any countries' socio-economical context, being that current research efforts continuously focus on increasing yields and adding value to cassava in order to tackle famine, poverty, and promote economic growth (Feleke *et al*., 2021). The importance of cassava in the agricultural economy of many tropical countries has grown remarkably in recent years and a great potential exists for cassava utilization as an industrial base in Africa (Amelework *et al*., 2021; Tonukari *et al*., 2015). The food and starch industry in Africa have taken advantage of the abundance of cassava production in the region to develop a local raw material base (Zhang *et al*., 2016).

Food insecurity is a widespread problem in several African countries where growing population pressure, combined with climate change, presents a global challenge associated with social and economic costs. About 50 percent of Africa's rural farm households and 20 percent of the urban poor are food insecure. Food insecurity arises as a result of deficiencies in three aspects: food availability, food access, and food adequacy. Food availability involves productivity and other factors in the food supply chain. Innovations in agriculture have been one of the main methods for addressing food insecurity through increased productivity of common staple crops (Bull *et al*., 2011).

Cassava is the second most important food staple in Africa after maize, and it is consumed by more than 200 million people in Africa south of the Sahara, who derive more than 50 percent of their calories from the crop. Nigeria led the global market share with about 21 percent of world cassava production in 2013 (FAOSTAT, 2013). Making cassava an industrial base by developing industries of cassava starch-derived products has been a way of obtaining a high added-value for cassava (Howeler, 2020; Kaur & Ahluwalia, 2017). Also, cassava has made a much more important contribution to national economies and provided a more stable base for industries (Anyanwu *et al*., 2015). Cassava starch and cassava "wastes" have been competing with other starches by improving the quality of its products and lowering the cost of production (Adetunji *et al*., 2015; Li *et al*., 2017). The vast availability of cassava in most African countries has been regarded as a natural plea for its exploration in order to fully maximize its potential as an industrial base for interested entrepreneurs worldwide (Kaur & Ahluwalia, 2017).

2.2.1 Uses of Cassava as a Food

The cassava plant is a valuable source of carbohydrate, protein, and vitamins (Bayata, 2019). However, these macro- and micronutrients are not well distributed in the plant. Cassava roots are rich in carbohydrates but poor in vitamins and protein, while cassava leaves are an excellent source of protein and vitamins (Latif & Müller, 2015). Since some strains of cassava produce substantial quantities of cyanide, which makes them toxic for humans and animals, processing cassava into ready-to-eat products is necessary to remove cyanogens and other antinutrients (Flibert *et al.*, 2016; Halake & Chinthapalli, 2020). However, processing reduces cassava's antinutritional content, especially when the peel is removed. Cassava crisps and chips have become popular in the urban markets in Kenya (Abong *et al.*, 2016b). A great number of production sectors in Africa that make use of maize, rice and wheat starches have carried out several experiments on cassava flour and starch, and found them to be potentially valuable substitutes (Chisenga *et al*., 2019).

2.2.2 Cassava Noodles and Spaghetti

Archaeological facts have shown that consumption of noodles as a food product dates back about 4000 years (Lu *et al*., 2014). Noodles were originally made from mung bean flour. Later, wheat flour replaced mung bean flour due to the issue of availability and cost. Nowadays, due to some health and economic reasons, gluten-free flours are now being prospected to partially or completely replace wheat flour for noodle manufacture (Purwandari *et al*., 2014).

Celiac disease is an immune disorder in which people cannot tolerate gluten because it damages the inner lining of their small intestine and prevents it from absorbing nutrients. Gluten is a protein found in wheat, rye, and barley and occasionally in some other minor products (Scherf *et al.*, 2016). A lot of foods; such as, baked food and pastas are manufactured using flour from wheat, rye, barley and oats, in which the gluten defines its functional properties. People who want to manufacture products containing gluten, have been looking for alternatives to solve this problem and to insure gluten-free products for the celiac population (Aristizábal *et al*., 2017; Pérez *et al*., 2017). Since cassava flour does not have gluten; the foods made with this flour could be one of the solutions for the development of food for gluten-intolerant consumers (Pérez *et al.*, 2017). Some research has been done in regard to substitute the gluten totally in order to produce baked goods, and pastas, quite similar in its functional properties, to those produced by

wheat flour (Ostermann-Porcel *et al*., 2017). The cookies developed were suitable for celiacs with acceptable quality and improved nutrition value.

In Africa, the alarming increase in the rate of consumption of instant noodle and spaghetti has awakened the need to source for other readily available and cheaper carbohydrate-rich substitutes (Oladunmoye *et al*., 2017) or complements for wheat (Abidin *et al.,* 2013). Instant noodle and spaghetti are carbohydrate-rich snacks made previously from wheat flour. However, the use of cassava as a preferred substitute for wheat in their production has grossly increased over the last 3 decades. The instant noodle and spaghetti industries produce millions of packs per year that are consumed by Africans and the rest of the world on daily basis (Tonukari *et al*., 2015). The cassava starch demands by these industries are in millions of tonnes, corresponding to millions of tonnes of fresh cassava roots. Although statistics of their cassava starch demand is not available now, Indonesia has a cassava starch demand of 0.045 million tonnes corresponding to about 0.20 million tonnes of fresh roots and the demand is still growing (De Souza *et al.,* 2017; Tonukari *et al*., 2015).

2.2.3 Cassava Leaves

Cassava leaves, a by-product of cassava root harvest is (depending on the varieties) rich in protein (14% - 40% dry matter), minerals, vitamins B1, B2, C and carotenes. Cassava leaves contain a number of nutritionally active factors including linamarin, oxalic acid, phytic acid, tannic acid and trypsin and chymotrypsin inhibitors. Cassava leaves as reasonable source of protein can reduce malnutrition in poor tropical and sub-tropical countries. Cassava leaves can be processed by alkaline fermentation (Hawashi *et al.,* 2019) for instance in fermented vegetable called *Ntobambodi* which is consumed in Congo and Central Africa (Kobawila *et al*., 2005). The main obstacle hampering the wide usage of cassava leaves as food is cyanogenic glucoside contents of leaves, which depending on the variety, can be six times higher than the one seen in the roots. However, heat treatment through blanching (Bradbury *et al*., 2014) as well as fermentation are used to deliberately reduce the high level of cyanogenic glucosides present in the leaves (Latif *et al*., 2019) and minimize post-harvest losses (Wafula *et al*., 2016).

2.2.4 Cassava Cakes, Bread and Biscuits

The habit of eating bread has spread throughout the world, thus making bread available in many urban centres in developing countries. The demand for gluten free bread (the most popular

yeast-leavened product) is increasing globally (Aguiar *et al*., 2021). It is one of the least expensive and yet most important staple foods in the world. Flour is an important raw material in bread making. Processing of fresh cassava roots into flour improves product palatability, reduces the cyanide content of the processed products and facilitates blending with other foods. High yielding disease resistant cassava varieties have been developed by the International Institute of Tropical Agriculture (Ohene *et al*., 2012). Cassava production trend in Africa can only be sustained with corresponding improvements in the diversification of their processing technology. Upgrading of the utilization of cassava flour in bakery and confectionery is required to exploit its industrial potentials (Uchechukwu-Agua *et al*., 2015). The application of cassava flour as a partial replacement for wheat flour in bread and cake baking, biscuits, pastries and snack foods could constitute an intervention programme in support of Sustainable Development Goals (SDGs) of the United Nations initiative in achieving food security (Adeniji, 2013).

The production cost of cake and biscuit has been considerably reduced in recent times by mixing wheat flour and cassava starch in a specified manner (Bakare *et al*., 2014). Cassava flours have now found important places in the Indian biscuit industry (Bala *et al*., 2015). Cassava is particularly used due to its good baking qualities and the fact that biscuit production is less gluten sensitive. According to Oyewole *et al*. (2010), acceptable biscuits were produced by completely substituting wheat flour with cassava flour. However, cake and biscuit industries have had good future prospects (Birch & Bonwick, 2019), and demand for cassava starch has increased as well (Mombo *et al*., 2017).

2.2.5 Cassava Starch in the Production of Confectioneries

Cassava starch can be converted to maltotriose and maltose as well as to other modified sugars and organic acids (Andriani *et al*., 2019). Starch is mostly used as an input for producing sugar syrupsin a process known as controlled enzymatic hydrolysis (Tonukari *et al*., 2015), which involves the use of either acid or α-amylase enzyme. Cutting the starch chain using acid will produce a mixture of dextrin maltose and glucose, and cassava starch is particularly suitable for this purpose (Tonukari *et al.,* 2015). Simple methods produce several maltodextrins divisible products of starch, maltose and glucose. Starch from cassava can be used to make fructose syrups and formulate gelatin capsules (Talón Argente, 2018; Yadav *et al*., 2020).

By controlling the processing greatly, cassava starch can almost be broken down to form glucose syrup. Enzymatic isomerization of glucose syrup is used for the preparation of high

fructose syrup (Amaral-Fonseca *et al*., 2021; Singh *et al*., 2017). Nigeria has a considerably great food and beverage industry that depends on glucose syrup and crystalline sugar as inputs; however, they can be transformed to high fructose syrup in the future (Clemens *et al*., 2016). Also, glucose syrup made from cassava starch can be utilized for the production of candy, soft drinks, traditional medicines and biscuits (Kaur & Ahluwalia, 2017). Research has shown that the amylose content of cassava starch is lower than that of arrowroot, but the productivity of cassava roots is much higher than that of arrowroots. Cassava starch is therefore suitable for domestic glucose and fructose syrup industries. These products (fructose and glucose syrups) can also serve as raw materials for large-scale factories (Tonukari *et al*., 2015).

Enzymatic hydrolysis is essential for the production of glucose syrups from starch because specificity of enzymes allows the sugar syrups production with well-defined physical and chemical properties and also, milder enzymatic hydrolysis results in few side reactions and less browning (John, 2017; Najiah *et al*., 2017). Cassava starch can also be used to produce gums, pastes and other types of candies; it can be used to make moulds or to dust sweets for them not to stick together. Dextrose does not allow boiled sweets to crystallize and also reduces hydroscopicity in the finished product (Hartel & Nowakowski, 2017; Tonukari *et al.,* 2015). Frozen chocolate flavored confections have been produced by treating chocolate slurries with amylases (Saini *et al*., 2017). The enzyme breaks down the chocolate starch to dextrins and thus the chocolate syrup does not become thick and has excellent stability and flow properties at room temperature and pH 5.5-7.5.

2.2.6 Treatment of Starch Processing Waste Water

Chemicals typically used in wastewater treatment include chlorine, peroxide, acids and bases, miner salts (ferric chloride, alum, etc.), and bio additives and enzymes. Many bacteria, and fungi have been reported to produce biopolymers such as polysaccharides, glycoproteins, and functional proteins with efficient bioflocculant activity (Zhao *et al*., 2012). Enzymes extracted from microbes are essential in treating the large volume of sewage and wastewater produced by metropolitan areas and industries. Recycling waste water into clean water that can be safely discharged into the environment is now possible. Termites contain microorganisms in their guts that assist in the digestion of wood, allowing the termites to extract nutrients from what would otherwise be indigestible (Tuma *et al*., 2020). Understanding of these systems helps us to manage them responsibly and as we learn more we will become ever more effective stewards.

Starch is also present in waste produced from food processing plants. Starch waste cause pollution problems (Rosseto *et al*., 2019). Biotechnological treatment of food processing waste water can produce valuable products such as microbial biomass protein and also purifies the effluent (Das & Basak, 2021). Microorganisms are being manipulated to provide a natural method for cleaning up some of the environment's worst chemical hazards (Rafeeq *et al*., 2023). The uniqueness of microorganisms and their often unpredictable nature and biosynthetic capabilities, given a specific set of environmental and cultural conditions, has made them likely candidates for solving particularly difficult problems in the life sciences and other fields as well (Kumar & Gopal, 2015). Scientists haven't only discovered microbes capable of surviving in hostile or toxic environments, but have found that microbes are also capable of immobilizing, degrading, removing or detoxifying environmental contaminants (Maqsood *et al*., 2023). Methods of manipulating these bacteria to enhance their clean-up capabilities have been discovered.

Moreover, scientists have discovered strains that can clean up toxic and radioactive materials in waste water. The emerging role of bacteria in the field of biotechnology, with countless new genes and biochemical pathways to sift for enzymes, antibiotics and other useful molecules, has generated new interest in them. These microbes often use contaminants as a food source, thereby completely eliminating toxic compounds by changing them into basic elements such as carbon dioxide and water, a process known as mineralization (Bala *et al*., 2022).

Incomplete degradation may also occur, or the partial breakdown of the original contaminant to a less complex form. Another result may be the transformation of a compound to a different chemical structure that may affect the toxicity and mobility of the original agent. Sometimes immobilization of a compound occurs where the agent is overcome by the microbe but not eliminated or altered, which is often a potential benefit but rarely a final solution (Bala *et al*., 2022).

2.2.7 Cassava-Based Animal Feeds Production

Cassava is well suited for animal feeds, as demonstrated by its utilization in many countries. In 1994, about a quarter of the global cassava production was estimated to be used as an ingredient in pork, poultry, cattle, and fish feeds (Phong *et al*., 2011). An industry that has livestock feed production as its subsidiary can utilize cassava in the production of its feed (Wang *et al*., 2011). Animal feed made of cassava has led to increased number of feeds and also

reduction of their cost in both commercial and subsistence production systems (Porter *et al*., 2017). A comparatively small amount of energy in feed extracts is caused by unavailability of cheap carbohydrate sources found in cassava. Using cassava as a substitute for maize to some extent has been an achieved goal (Kyamanywa *et al*., 2011).

Cassava peels form the bulk of residue from cassava root after post-harvest handling and processing. These peels make up to about 10% of net weight of the roots (Tonukari *et al.*, 2015). Cassava peels serves as a good source of energy in ruminant feeding systems, serving either as the main basal diet or as a supplement (Kalio *et al*., 2014). They constitute an important potential resource for animal feeds if properly processed by a bio-system. Cassava peels have been employed as an important source of carbohydrate in livestock feeds for monogastrics in various parts of the world. Since the detoxification of cyanogenic glucosides requires the presence of methionine, balanced feeds that include cassava peels must contain enough sulphur amino acids. Cassava peel meal has also been consistently incorporated into the diets of pigs as an alternative energy source. Studies by Choton *et al*. (2020), Obadina *et al*. (2006), and Ruqayyah *et al*. (2014) showed that cassava peel can be used as a substrate for microbial protein enrichment. Large quantities of cassava stems could be chopped and mixed into silage for the feeding of cattle and pigs (Ngiki *et al.,* 2014).

2.2.8 Cassava Starch in the Production of Bioethanol

Bioethanol, the most common renewable fuel today, is commonly derived from corn grain (starch) or sugarcane (sucrose) (Figure 3) (Yadav *et al*., 2018) and most recently, cassava starch. The production of bioethanol from starch and sugarcane is often criticized as it may affect the human food chain. Such bio resources are limited to be able to use for biofuel production and compete to replace or even partially supplement fossil fuels. Renewable cassava peel waste biomass from industrial processing has been used for large-scale production of alcohol-based fuels, and the process can be cost-efficient (Sánchez *et al*., 2017).

One of the limitations of this process is the complex structure of cassava starch that makes it difficult to hydrolyze to fermentable sugar (Zhang *et al*., 2016). Ethanol fermentation from starches normally requires three-stage processes namely: Liquefaction, saccharification and fermentation (Azmi *et al*., 2011). Alternative to these conventional multistage processes is by direct fermentation of raw starch using amylolytic fungus and bacterial co-culture with yeast,

that is, *Saccharomyces cerevisiae*.The amylolytic fungi and bacteria are typically found in diverse environment in Kenya. However, use of thermostable enzymes and thermophilic microorganisms for the degradation of the cassava starch offers an advantage of minimizing the risk of contamination, and it may lead to the single-step process of enzymatic hydrolysis and fermentation. Improvements in the production of ethanol has been focused on fermentation of the structurally complex carbohydrate polymers. This has been done using second generation technologies that apply metabolically engineered strains that possess a high degree of catabolic versatility (Yao *et al*., 2010). Such microorganisms have been engineered to divert carbon flux toward ethanol synthesis.

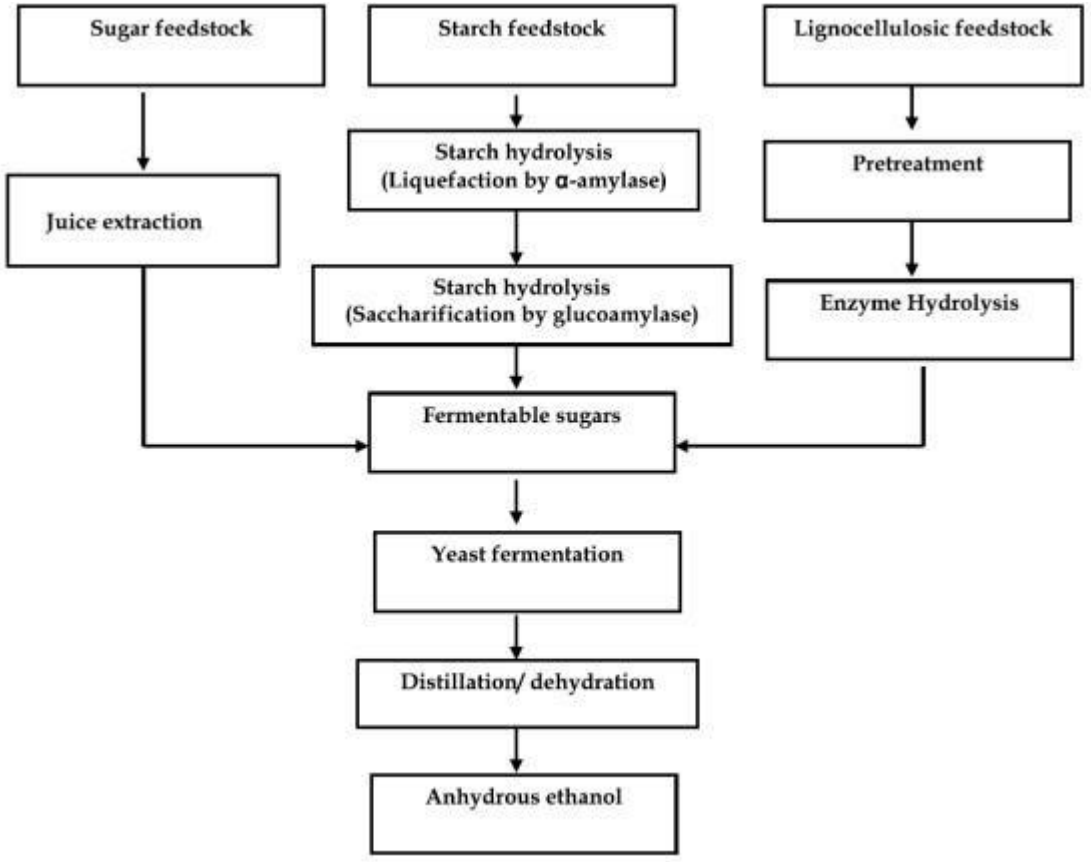

Figure 3: Schematic Diagram of Bio-Ethanol Production by Fermentation Process of Sugar, Starch and Lignocelluloses Feedstock

Source: Gbadegesin *et al.* (2013)

Bioethanol fuel is ethanol (ethyl alcohol), the same type of alcohol found in alcoholic beverages. There has been an increasing interest in using ethanol as a substitute for fossil fuels to avoid emissions from vehicles. Ethanol helps in making a smoother transition from a petroleum-based to a bio-based sustainable economics (Sivamani *et al.*, 2017). Bioethanol from plants emits carbon (IV) oxide which is absorbed by photosynthesis. Hence, pollution is considerably reduced. It can be used as fuel, mainly as a biofuel alternative to gasoline, and is widely used by flex-fuel light vehicles in Brazil, and as an oxygenate to gasoline in the United States (Adelekan, 2010; Adelekan, 2012). By blending ethanol with gasoline, the US countries oxygenate the fuel mixture so it burns more completely and reduces pollution emission. Ethanol derived from biomass is the only liquid transportation fuels that do not contribute to the greenhouse gas effect (Adelekan, 2010; Langeveld *et al*., 2010). With the development of cassava-based industries large amounts of waste solids, residues and waste water are generated during cassava processing, causing environmental problems such as contamination of water bodies and offensive odours. A highly efficient and environmentally friendly strategy of bioconversion has been developed to cope with the abundant organic-rich cassava based industrial waste. In China and Thailand, *Clostridium acetobutylicum* strain has been used for cassava starch fermentation to produce butanol, acetone and ethanol (Lin *et al*., 2019).

Cassava has been explored as a potential biofuel crop in countries like China and Thailand (Zidenga *et al*., 2012). In previous research ethanol production using inedible cassava has been done. The study was attractive because unlike edible cassava, the use of inedible cassava for ethanol production did not compete with food or feed supply. However, there was one major shortcoming in that inedible cassava has high cyanide content which is toxic to a wide range of microorganisms including fungi, bacteria and algae. In this study the main focus is on application of edible cassava using enzyme technology in industrial production of fermentable sugars used in processing of various products including ethanol.

Cassava stems have been overlooked in starch and energy production. Cassava stem contains about 30% starch (dry mass) mostly in the xylem than the phloem tissue. Up to 15% of the stem dry mass can be extracted using simple water-based techniques, potentially leading to 87% increase in global cassava starch production. Cassava stems have been used for biofuel production without the need for land use expansion. Thus, in areas with large production of cassava stems potential exists for the exportation in the form of dehydrated stems.

The cassava root has high starch content and is considered a cheap, abundant and renewable resource for production of fermentable glucose syrups and dextrins. The benefits of using cassava as a raw material for ethanol production are due to the fact that it can be planted on marginal lands where other agricultural crops such as sugarcane, rice, wheat and corn cannot be grown. In addition, it can highly tolerate drought, as it survives during dry weather when soil moisture is low with high humidity. Unlike sugarcane, it needs soil with low quality since it performs well in poor soil than any other major food plant. Several distilleries in Nigeria use cassava flour of high quality as a substrate for producing ethanol. Studies have shown that biogas can be produced from blends of cassava peels with some animal wastes. Oparaku *et al.* (2013) has also reported the production of methane from cassava peels.

During the processing of cassava into chips, flour or starch, enormous amount of wastes are generated ca. 0.47 tons for each ton of fresh tubers processed (Moshi & Nges, 2017). This waste consists of peels, wastewater and pulp that contain between 36 to 45% (w/w) of starch and from 55 to 64% (w/w) of lignocellulosic biomass. An innovative processing system is therefore essential to take into account the transformation of this waste into value added products. This will address both the environmental pollution and inefficient utilization of these resources. The starch and lignocellulosic cassava processing waste can be converted into renewable energy carriers such as biogas through anaerobic digestion (AD), bio-ethanol through fermentation and bio-hydrogen through dark fermentation (Bhurat *et al*., 2020). In the case of AD, the waste can be used directly as substrate while for fermentation; the waste must be pre-treated to release monomeric sugars, which are substrates for bio-ethanol and bio-hydrogen production. There is possibility of sequential fermentation for either bio-ethanol or bio-hydrogen and AD for biogas production thereby making use of all the fractions of the cassava waste (Sreekala *et al*., 2022).

Generation of renewable energy from cassava waste could benefit rural populations where access to electricity is very poor (Giwa *et al*., 2017). This would also reduce the dependence on firewood and charcoal that are known to provide almost 90 percent of domestic energy requirements. Such a development could help save trees, lower emissions that cause climate change and reduce the fumes from millions of tons of firewood that threaten human health, especially the health of women and children. Although deforestation and land degradation are well-known, the charcoal and firewood consumption that causes them is still on the rise.

Although, cassava flour is currently being used for alcohol production, yields are well below expectations with high levels of waste (sludge and unhydrolysed starch), blockages to heat

exchangers, and increased hydrolysis times as well as the high cost of imported enzymes for saccharification (Tonukari *et al*., 2015). These constraints can be successfully surmounted with proper mechanization and government assistance in research to discover cost friendly enzyme technology. The technological availability and awareness of Africans especially local farmers to the economic potential of utilizing cassava waste in bio-ethanol production poses a great problem (Adetunji *et al.,* 2015). The source of enzyme extraction and the conditions of operation of enzymes such as pH, temperature, reaction time, enzyme concentration, viscosity, mixing rates etc. in soluble solutions must be optimized to improve the economic and technological feasibility of the bio-process. Industrial alcohol made from cassava starch can also be used as feed-stock to produce a large amount of organic chemicals like organic acids (citric acid, lactic acid, succinic acid, and volatile fatty acids) and enzymes. Different microorganisms including *Lactobacillus plantarum* and *Lactobacillus paracasei* have been used for production of these organic acids with cassava industrial waste as the substrate in the fermentation process.

2.2.9 Cassava Beverages

Cassava can be used as a feed-stock by the brewing industry. It plays a key role in the food production and economies of several countries worldwide. Due to its starch content, alcoholic fermentation is a promising transformation process for adding value to cassava (Kaur *et al*., 2017). However, most of the existing cassava beverages are from traditional origin, with the yields and quality often poorly known or controlled due to the use of artisanal production processes (Coelho *et al*., 2020). However, use of biotechnology would be helpful in production of diverse cassava products through controlled fermentation instead of spontaneous fermentation process to ensure consistency in product quality. Enzymes are biological tools that have already been applied to cassava with the aim of bioethanol production. These tools can be shifted towards the production of alcoholic beverages, in order to design controlled processes capable of generating quality added-value products (Kaur *et al*., 2017).

When cassava surplus is available, its transformation and production of commodities can be an advisable strategy to add value to this raw material, in opposition to price reduction. One possibility is the conversion of cassava starch to ethanol, as the production of fermented alcoholic beverages is an attractive strategy for adding value to cassava surplus, suitable for developing countries, as it does not compete with food supply. Some alcoholic cassava beverages already exist, for example, cassava beer, traditionally consumed by indigenous people

in the Amazon, as well as in India, where research efforts were recently performed to valorize local beer production from cassava. Indigenous people from South America (such as in Ecuador) prepare a type of beer known as 'chicha' which is made with either corn, boiled cassava or the fruit of the palm *Bactris gasipaes* (chonta); some cassava beers include an additional chewing step before the fermentation process. A recent report showed that bacteria present in chewed cassava beers were mainly *Lactobacillus sp.* (Colehour *et al*., 2014). Researchers analyzed the microbial diversity (using culture dependent and culture independent techniques) in different types of Ecuadorian 'chicha'. *Streptococcus salivaris* and *Streptococcus mutans* are part of the human oral microbiota which were the most abundant bacteria in chewed cassava and in non – chewed cassava beers (Freire *et al*., 2016). Cassava beer, or 'chicha', is typically consumed daily by the indigenous Shuar people of the Ecuadorian Amazon (Colehour *et al.,* 2014). It is a traditional fermented beverage produced from a variety of starchy plant crops including maize, millet and cassava. 'Chicha' from cassava is typically a low alcohol beverage with 2-3% ethanol, with a milky consistency and somewhat sour flavor. It is also generically referred to as 'masato'. Preparation of 'chicha' involves peeling, washing and boiling the roots of sweet cassava until they are soft. Once soft the water is drained off and root mashed using a pestle. The brewer masticates pieces of the cassava and periodically spits into the mashed cassava. Fermentation for 1-3 days follows depending on the preference for sweet (slightly unfermented) to sour (very fermented) 'chicha'. Water is then mixed with fermented mash prior to serving. The microbial community of 'chicha' could be initiated from a variety of sources including saliva added to each new batch, tools and vessels that my contain remnants or bacterial residue from a previous ferment, the water added to thin the cassava mash, the substrate of the new material, or the household and airborne environment (Piló *et al*., 2018).

SAB Miller is a pioneer in cassava beer and in October 2011 it launched a lager, named 'Impala', produced and commercialized in Mozambique. The brewing process uses 70% cassava and 30% barley (Graffham, *et al.*, 2019). In the first 12 months of production, Impala sales were 9 million bottles (50,000 hectolitres) which equates with 2.7% of the market for beer in Mozambique. Building on the success of Impala in Mozambique, SAB-Miller launched a cassava-based beer in Ghana called "Eagle beer" in March 2013. It is believed that Eagle is a very similar product to Impala. SAB-Miller are known to be exploring the possibility of launching a cassava-based product in South Sudan. In December 2012 Guinness Ghana Limited launched a cassava-based beer in Ghana under the name of "Ruut Extra beer" with sales targeted

to more rural areas and small towns. Ruut Extra is a clear beer with a rather sweet taste due to the number of maltodextrins used in the formulation. The price of Ruut Extra is approximately 30% lower than conventional beers. Guinness are reported to be using dried cassava-grits rather than the wet-cake system favoured by SAB-Miller. In Mozambique and Ghana, a major incentive for development of cassava-based beers has been provided through reductions in excise duty for beer containing cassava (Oxford Analytica, 2015). This in turn has allowed the brewers to discount the price for cassava-based beers by 30% when compared to conventional brands. Consumers find the relatively high alcohol content and slightly sweet taste attractive.

Further cassava beverages can be mentioned, namely, cassava wines, for instance 'Parakari', popular among Wapisiana of Guyana in South America, and 'Tapai,' consumed among ethnic groups of East Malaysia. 'Parakari' production involves the use of a starch hydrolyzing amylolytic mold (*Rhizopus sp., Mucoroceae, Zygomycota*) followed by a solid –state ethanol fermentation using ambient yeast. It is the only known example of an indigenous new world fermentation that utilizes an amylolytic mold (Henkel *et al*., 2004). 'Tapai' is a traditional functional beverage which is a rich source of probiotics because it is known to contain lactic acid bacteria. It is common in Sinjai Regency. From previous studies on the microbial composition of 'Tapai' the probiotics present were found to be *Pediococcus acidilactis* and *Weissela cibaria* (Sari *et al*., 2020).

Other examples of cassava-based alcoholic beverages can be mentioned, namely, distilled cassava spirits produced in indigenous communities in Cameroon where traditional methods are employed or 'tiquira', an artisanal spirit produced in the Maranhão State in Brazil (Cereda *et al*., 2017). Another case is found in Rwanda, where a distilled alcoholic spirit named 'Kanyanga' is produced, often using cassava as raw material, also employing traditional methods. However, traditional and uncontrolled production processes of 'Kanyanga' can lead to toxic concentrations of congeners (isoamyl alcohol, isobutanol, and methanol), similarly to 'Kachasu', another traditional distilled beverage from Zambia, Zimbabwe, DR Congo, and Malawi, which led to prohibition Kanyanga distribution in Rwanda. The development of suitable starter cultures for production of such traditional beverages still remains important in order to enhance the shelf-life of the products (Lyumugabe & Songa, 2019).

The application of traditional production processes and lack of knowledge regarding the final product mostly leads to low yield, low-quality beverages, or both, therefore poorly adding value

to the raw material (Coelho *et al*., 2020). Instead, biotechnological tools, often simple and easily implementable, can be used to produce cassava beverages (Mayorga *et al.*, 2020). Several processes and tools are available for the production of ethanol from cassava starch, as seen for production of 1st or 2nd generation bioethanol, where the application of knowledge and technology allows achieving high yields. Biotechnological tools such as enzymes for the hydrolysis of starch and robust yeast for ethanol production have already been applied to cassava with the aim on bioethanol production (Cereda & dos Santo Brito, 2017; Coelho *et al*., 2020; Mayorga *et al.*, 2020).

These tools can be shifted towards the production of alcoholic cassava beverages, in order to design controlled processes capable of generating quality added-value products. Distilled spirit with 40% ethanol by value has been produced from cassava flour as raw material. The product showed desirable sensory traits, receiving good acceptance by experienced tasters, demonstrating the feasibility of the proposed process of controlled fermentation to add value to cassava surplus (Coelho *et al.*, 2020). Ethanol titers and chemical and sensory characteristics of the produced beverages are a positive indicator, highlighting the use of cassava as a promising raw material to obtain a feasible and consumer-accepted distilled spirit beverage through easily implementable biotechnological tools.

2.3 Production and Uses of Cassava in Kenya

Cassava is the second most important food root crop in Kenya. According to 2014 data by FAO, Kenya's annual cassava fresh root production was estimated at 662,405 tons, against an estimated annual demand of 301,200 tons of dried cassava and 1,204,800 metric tons of fresh roots (one ton of cassava flour is obtained from four tons of fresh cassava roots). This demand gap is what researchers have been keen to exploit to the benefit of smallholder cassava farmers in the country. According to FAO statistics of 2017, cassava production reached 1,112,000 tons under 90,394 hectares of harvested land (Figure 3). In Kenya, cassava production reached 900,000 metric tons in 2018 down from 1.2 million tons in 2017 (FAOSTAT, 2017). In comparison, maize production reached 4,000,100 tons in 2018. More than 100,000 tons of maize were imported to meet industrial demand for starch.

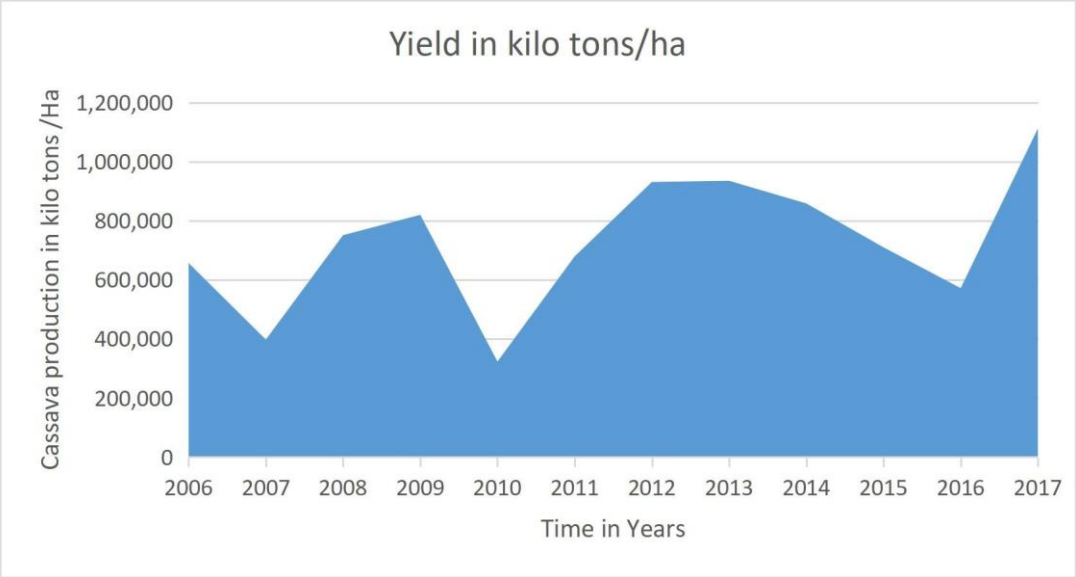

Figure 4: Cassava Production Trend Since 1961 up to 2017 in Kenya

Source: FAOSTAT, 2017

The Kenya Agricultural and Livestock Research Organization has recently released six new cassava varieties with a potential yield of 21 tons per acre double the current varieties that yield 7.5 to 10 tons per acre. The six varieties mature within six to eight months and are resistant to cassava brown streak virus and cassava mosaic virus. These two diseases have been threatening farmers' produce within the Coastal region of Mtwapa, Mpeketoni, and Msabaha. These varieties are *nzalauka, karibuni, shibe, karembo, tajirika and siri*. Following the effects of global warming and pest infestation, Kenya's main food crop maize was affected leading to a drop in production by 4.2 million bags in 2017. Farmers can now grow these drought-tolerant and disease resistant cassava varieties in a bid to boost food production and improve food security in the country (Anderson *et al*., 2016; Githunguri *et al*., 2014).

Despite its high production in the Kenyan coastal and western regions, cassava utilization is limited to human consumption. Cassava roots are grown under subsistence farming and consumed locally directly as cooked (boiled or roasted) starch food, custard and other forms (Uchechukwu-Agua *et al*., 2015). Some of it is peeled and ground and milled into cassava flour and used as a: thickener using the paste properties of starch (as in soups, baby foods, sauces and gravies);filler contributing to the solid content of soups, pills and tablets and other pharmaceutical products and fee cream; binder, to consolidate the mass and prevent it from drying out during cooking (as in sausages and processed meats); stabilizer, owing to the high

water-holding capacity of starch (as in fee cream). Excess cassava produce is utilized as animal feed.

Cassava roots are locally available natural source of starch and commercially unexploited in the production of glucose in Kenya. In Canada and Europe, industrial utilization of cassava starch has been practiced using chemical hydrolysis and recently using commercial enzymes (Woiciechowski *et al*., 2002). Enzymes have been used preferable over chemical catalysts in many industrial processes due to the increasing knowledge of the technical and economic advantages together with the need for environmentally safe technology. Currently, the global production of amylases has reached 65% and is continuously increasing (Simair *et al*., 2017). Amylases can be obtained from several sources such as plant, animal and microbes such as bacteria and fungi (Abd-Elhalem, 2015; Murakami *et al.,* 2008).

Nowadays the potential of using microorganisms as a biological source of industrially economic enzymes has stimulated interest in the exploitation of extracellular enzymatic activity in several microorganisms (Samaranayake *et al.,* 2018). Microbial amylases have a wide range of industrial application due to their broad biochemical diversity,feasibility of mass culture, high enzymatic stability under extreme conditions and ease of genetic manipulation (Sreena and Sebastian, 2018; Vijayalakshmi *et al*., 2012). Due to the importance of amylases, isolation of new microbial producers capable of producing amylases provides potential new sources of enzymes (Aullybux and Puchooa, 2013). Microbial sources of amylase are preferred because of its plasticity and vast availability. Genetically improved amylolytic enzymes have been used for industrial enzymatic hydrolysis of starch (Samaranayake and De Silv, 2017). However, in developing countries like Kenya, the cost of production would be too high if processors would rely on imported commercial enzymes. Application of innovative processes in cassava starch hydrolysis will save this situation in Kenya.

2.4 Limitations of Industrial Use of Cassava Starch in Fermentation in Kenya

Cassava starch is composed of unbranched amylose (17-25%) and branched amylopectin (75-83%), both of which can be hydrolyzed acidically or enzymatically (either with pure enzymes or amylase-producing microorganisms) to release their constituent glucose and maltooligosaccharides (Zhu, 2015). Both products are easily transported across the cell membrane and metabolized by microorganisms such as *Saccharomyces cerevisiae* to produce ethanol.

Starch gelatinization in water is a process of breakdown of the intermolecular association between amylose and amylopectin molecules at solid state (granules) with heating (Riuz *et al*., 2011). Starch grains are heated with excess of water to increase amylopectin amorphous region and enzyme accessibility. Alternatively, starch grain is heated in presence of 0.016 mol /L sodium acetate buffer (pH 4.0). This starch solution is then heated at 66 ºC under centrifugation at 390 rpm for 30 minutes. According to Doporto *et al*. (2012) the onset of gelatinization of cassava starch was about 52 °C whereas cassava flour from the same root material was 67-71 °C. This indicates that presence of other materials like protein,fat and fibre in cassava flour influence gelatinization behavior.

Liquefaction is carried out by thermal stable alpha amylases that hydrolyze the chemical bond α-(1-4) of starch, producing dextrin, maltose, maltotriose and maltopentoses. This step takes place at 80-110 ºC. This enzyme processing of starch allows a rapid reduction in the viscosity of the solution. The degree of liquefaction is assessed by determining the Brookfield viscosity. Saccharification takes place at 60-70 ºC after glucoamylase treatment (Riuz *et al.,* 2011). The degree of saccharification is determined by the rate and extent of reducing groups /glucose formation. Glucoamylase simultaneously hydrolysis the bonds α-(1-4) and α-(1-6) in starch resulting in a higher glucose yield.

In previous studies, alpha amylase enzyme was added to carry out liquefaction process. This enzyme had optimum activity at pH 5.0 and 80 °C. The reaction was monitored until the viscosity of the gelatinized starch reduced due to partial starch hydrolysis to dextrins. Glucoamylase completed the starch hydrolysis process (saccharification) to glucose which was the ultimate product. The optimum activity of glucoamylase was pH 4.5 and 70 °C. Disappearance of cloudiness was used to determine the end point of the reaction of cassava starch hydrolysis. However, this method had shortcomings because it was difficult to detect slight differences in the level of hydrolysis (Johnson *et al*., 2010).

Amylolytic enzymes are obtained from different sources. Though amylases originate from different sources (plants, animals and microorganisms), the microbial amylases are the most produced and used in industry, due to their productivity and thermostability (Rana *et al*., 2013). Thermostable bacterial enzymes which are suited for liquefaction and saccharification processing steps performed at high temperature are identified in this research study.

2.5 Overcoming the Limitations of Industrial Application of Cassava Starch in Kenya

Cassava is a source of food security, not only because it can be grown on less productive land, but because it is a source of income for producers and generally a low-cost source of food (Giller *et al.,* 2021). The development of industrial application for cassava can contribute substantially to poverty alleviation, especially for resource-constrained households, and can increase household food security (Wahab *et al*., 2022). One way of overcoming limitations in the application of cassava is using a demand-driven approach to promote and develop cassava-based industries (Agbachom *et al*., 2019). This is possible with the assistance of a coalition of groups and individuals interested in developing the cassava industry.

Increasing demand for glucose as a sweetener is growing drastically (Grembecka, 2015). Glucose from cassava starch hydrolysis has been used as a raw material for other food industries namely brewing, soft drinks, confectionaries and baking industry (Kaur & Ahluwalia 2017). These industries rely purely on glucose and not any other starch polymers. In Kenya, the starch conversion industry depends on commercial amylase enzymes which are imported (Muriithi *et al*., 2022). This has a high cost implication on the processors.Promotion of innovative research with the aim of looking for cost effective enzyme sources is a venture worth attempting (Osmakova *et al*., 2020).

The potential source of thermal stable amylolytic enzymes e.g. α, β and ϒ amylase can be the diverse microorganisms from Kenya's environment (Cira-Chávez *et al*., 2018). Hot springs, forest soil,decomposing waste and the alimentary canal of black soldier fly larvae as well as termites are habitats for the desired microorganisms secreting amylolytic enzymes (Deepika and Satyanarayana, 2013; Hreggvidson *et al.,* 2012). Halophiles are also excellent sources of enzymes that are not only salt stable but also can withstand and carry out reactions efficiently under extreme conditions (Kumar *et al.,* 2012). Extreme terrestrial, naturally formed alkaline and saline (halo alkaline) environments such as soda lakes and hot springs are now recognized as hot spots of microbial diversity (Santini *et al*., 2015). The results of a study by Liu *et al*. (2014) demonstrated that the hypersaline ecosystems hosted surprisingly diverse eukaryotic microbial community. Analyses of pyrosequencing data revealed that the dominant genera were *Dunaliella*, *Alternaria* and *Chlamydomonas*, which dominated the microbial assemblages in the waterlogged sediments, the salt crusts and the saline loess from the lake banks, respectively (Liu *et al*., 2014). Studies by Bhatt *et al*. (2020) showed that halophilic amylases my reduce contamination risk and contribute to low cost amylase production.

2.6 The Need to Expand Research on Isolation and Identification of Microorganisms for Industrial Hydrolysis of Cassava Starch in Kenya

Research needs to be emphasized in order to identify microorganisms producing superior thermostable amylolytic enzymes with high activity. This is because the commercial enzymes in the industry are not best suited to carry out cassava starch hydrolysis owing to its high gelatinization (52-65 °C), liquefaction (80-110 °C) and saccharification (60-70 °C) temperatures (Ruiz *et al*., 2011). Thermostable alpha-amylase is active and stable at high temperatures, such as during gelatinization and liquefaction of starch (Far *et al.,* 2020). Thermostable enzymes are thus a potential candidate for industrial application in starch pre-treatment (Dutta & Suresh Kumar 2023). Few studies so far have been done in Kenya on enzyme technology that is applicable in the starch industry.

A major limitation in research is over dependence on imported commercial enzymes (Aso *et al*., 2013) as well as lack of advanced technology to aid in molecular characterization for rapid identification of microorganisms. Industries end up being closed due to lack of improved enzyme technology (Taheri-Kafrani *et al*., 2021) which has a significant implication on the production cost. Consequently, a lot of downstream processing and purification of products also contributes to the cost. The cost of enzymes is one major factor determining the economics of a biocatalytic reaction and it can be reduced by continuous isolation of hyper producers and finding the optimum conditions for their production (Sireena and Sebastian, 2018).

Enzymes are ideal catalysts for the starch conversion industry (Miao *et al.,* 2018). This is because of several reasons namely: enzymes are specific, have ability to work under mild conditions, enzymes make it possible to increase the reaction rate, they operate without contamination by microorganisms and purity of end product is guaranteed (Ramesh *et al*., 2020). Over the last years, extremophiles with its different categories (Figure 5), thermophiles (high temperature), acidophiles (low pH), alkaliphiles (high pH), halophiles (high salinity), and psychrophiles (low temperature), have fascinated researchers in many fields, due to their ability to withstand and function under extreme conditions (Kumar *et al.,* 2022).

Worldwide, geothermal areas which are favorable habitats for thermophilic organisms are limited to a restricted number of sites (Mohammad *et al.,* 2017). In Kenya there are several hot springs which are habitat for microorganisms producing enzymes applicable in the industry (Kour *et al.,* 2019). In these conditions' microorganisms have to cope with extremes temperature, low humidity, and low availability of nutritional compounds. Thermal environments on the

earth's surface also experience evaporation, and thus many environments have elevated salinity and therefore halophilic inhabitants (Rodriguez-Valera, 2020). These conditions reduce biodiversity but some bacteria develop survival strategies in order to adapt to such stress (Rajendran, 2015). In Kenya, studies to isolate and identify organisms for industrial use is limited. The aim of the present study was to therefore to establish a continuous research line for screening, isolation, and characterization of new extremophilic microorganisms that can possess high biotechnological potential.

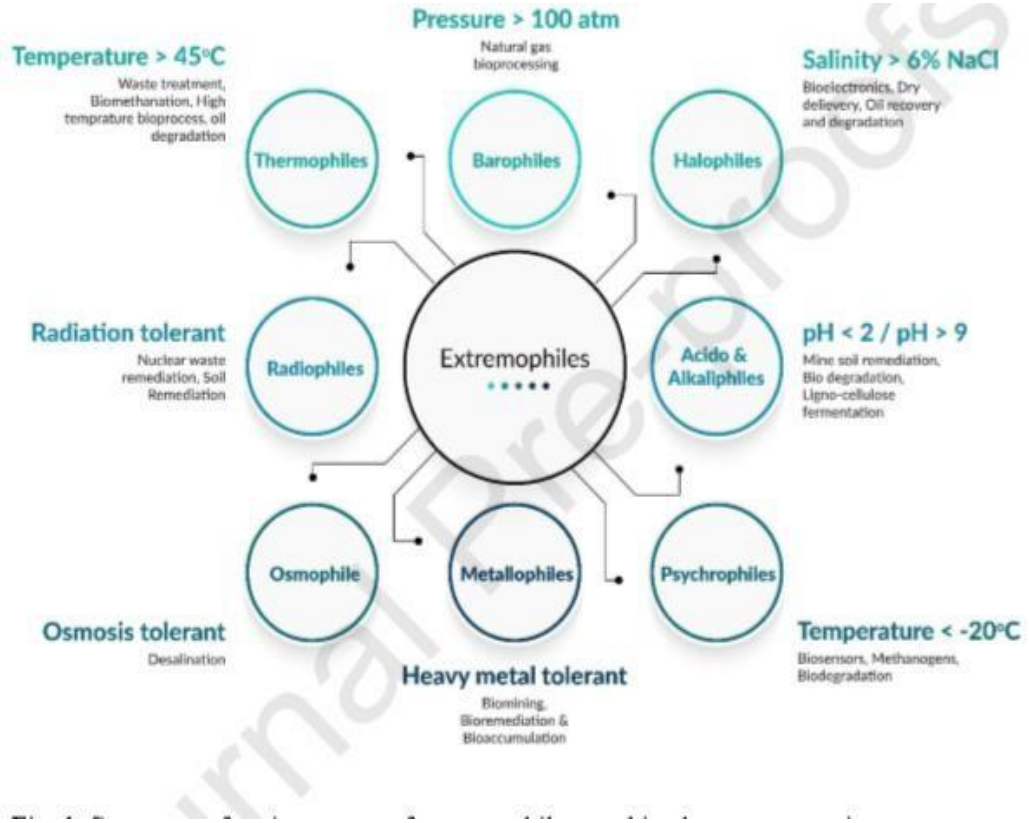

Figure 5: Summary of Various Types of Extremophiles Used in Enzymatic Process

Source: Suresh *et al*. (2020).

The adaptation of thermophilic microorganisms to harsh habitats makes thermophiles and their thermostable proteins suitable for various industrial and biotechnological applications (De Castro *et al*., 2016; Raddadi *et al.,* 2015). The growing demands for amylases for biotechnological applications show an urgency to produce the enzyme at large scale with reduced cost and time. Alpha –amylase is the most important industrial enzyme which

contributes up to 65% of the industrial enzyme market (Simair *et al*., 2017). Although amylases are produced by plants, animals and microorganisms, microbial sources have better potential applications due to their stability and economic viability.

Survival of thermophilic microorganisms at high temperatures is because of various adaptations in physiological systems and genetics as stress response to stabilize homeostasis (Wang *et al*., 2015). Few examples are production of DNA- binding proteins, activation of heat shock proteins, activation of reactive oxygen species and efficient repair damage (Ranawat and Rawat, 2017). Other survival mechanisms at thermophilic environment involve amino acid substitutions, hydrophobic cores (Bezsudnova *et al*., 2012), interactions among subunits (Pang and Allemann, 2007), and inactivation of spores by high hydrostatic pressure (Sarker *et al*., 2015) and by adjusting membrane fatty acid compositions (Yoon *et al*., 2015) and also by maintaining membrane fluidity using thermosensors e.g. DesK (Cybulski *et al*., 2015). Another worth mentioning fact of thermophilic proteins is substantial rise in the proportion of alpha helices and beta strands, with a decline in irregular region; with moderately thermophilic proteins, alpha helical increase is dominant whereas in extremely thermophilic ones, beta strand rise is more extensive (Chakravorty *et al.*, 2017).

Amino acid composition is another distinguishing factor among extreme thermophilic proteins and moderately thermotolerant proteins (Barik, 2020). In moderately thermophilic proteins, lysine content is less than arginine content, but in extremely thermophilic proteins, lysine residues are more in number because of their requirement of stronger electrostatic interactions and lysine is a charged residue (Taylor & Vaisman, 2010). Amino acid residues which are not high temperature tolerant namely methionine and asparagine, are less in extreme thermophilic proteins. However, the important phenomena, i.e. lysine succinylation and lysine propoinylation which are important for protein function, are not different in extremely thermophilic and moderately thermophilic proteins. These are common protein functions which are not dependent on temperature tolerance (Okanishi *et al*., 2017).

High temperature is also related to strong association between ions. The despairs for the ion pair decrease with an increase in temperatures. In thermostable proteins, internal water molecules (bridging water molecules) are present which ensures that ions are not fully desolated at high temperature. Hence it was established that salt bridges are very important in the design of thermostable proteins (Bikkina *et al*., 2017). Up regulation of proteins which are responsible for protection against heat stress i.e. heat shock proteins (HSPs), is common in all thermophiles.

Chaperon proteins which are up regulated in thermophilic strains *Thermoanaerobacter tengcongensis* and *Thermotoga maritime* in response to heat stress include: Gro EL, Gro ES, Dna K and Grp E (Chen *et al*., 2012; Wang *et al*., 2012). In conclusion, a range of factors contribute to thermostability in proteins and it varies from protein to protein.A single factor cannot be accounted for thermal stability and despite extensive research in this area, a clear understanding to formulate a theory for protein thermostability is still elusive (Kumar *et al.*, 2019).

CHAPTER THREE
MATERIALS AND METHODS

Research was conducted in the Guildford Dairy Institute Food Microbiology and Biochemistry laboratories of Egerton University, and Kenya Agricultural and Livestock Research Organization Njoro Biotechnology laboratory.

3.1 Sampling and Sampling Sites

Amylase producing microbes are ubiquitous but for this research study, substrate rich environments were preferable (Table 2). Samples were collected between May and August 2020 from seven locations in Kenya targeting isolation of organisms with diverse amylases. The sampling locations were Gilgil potato processing company (water from waste treatment lagoons-targeting high-activity amylase) on (0°29'32.19"S; 36°19'2.28"E), Lake Bogoria (saline and alkaline water from the hotsprings-targeting alkaline and thermostable amylases) on (0°13'33" N; 36°05'41" E), Lake Elementaita (alkaline water-targeting alkaline amylases) on 36° 14' 23.92" E; 0° 26' 33.47" S, Menengai crater (volcanic soil-targeting high-activity amylases) on 35° 28', 35°36'E, and 0°13', 1°10'S, and Nakuru municipal waste dump site ('Gioto') (rotten organic waste-targeting high-activity and thermostable amylases) on 0° 15' and 0° 31'S, and 36° 00' 36°12' E, and International Centre of Insect Physiology and Ecology (ICIPE), Nairobi (Black soldier fly gut as well as termite gut-targeting high-activity amylases) (Luang-In *et al*., 2019). Three sub-sampling sites were identified and samples were randomly drawn aseptically using falcon tubes and then pooled to get six representative samples. The water, soil/waste or mud samples were put in iceboxes to maintain temperature at 4°C then transported to the Microbiology laboratory at Egerton University for analysis.

3.2 Sampling plan

Out of the six sampling sites, eighteen composite samples were randomly drawn aseptically using falcon tubes (50 mL), pooled, and homogenized to obtain six uniform representative samples. The isolation of amylolytic enzyme producing microorganisms was done with an aim of getting unique enzymes with desirable properties as shown in table 2 above. Due to the high gelatinization temperature (52-65 ºC) of cassava starch (Damodaran *et al.,* 2008), heat stable enzymes were a desirable characteristic as well as high activity which would translate to high

yielding enzymes. The sampling procedure was adapted to each habitat. The temperature and pH of the sampling sites were also be taken and recorded. Samples from the hot springs were reserved in keep its hot thermos flasks to maintain the sampling temperatures. Soil samples from Menengai crater were taken from 3-5cm depth after removing 5cm from the ground surface These samples were collected in sterilized bottles.Samples from other sites were kept at 4 ºC in an ice box. Isolation using primary media formulation was done within 24 hours after sample collection.

3.3 Isolation of Microorganisms with Ability to Hydrolyze Starch

At the laboratory, 25 g/ml of the six homogenized samples drawn from the environment were mixed with 225 ml of buffered peptone water, vortexed, and then followed by serial dilution up 10^{-6} to using buffered peptone water. One hundred microliters from each tube containing the sample (dilutions) from each sampling site was transferred aseptically to M9 starch agar medium plates containing: $NaHPO_4$ (6.7g/l), KH_2PO_4 (3g/l), NaCl (0.05g/l), NH_4Cl (1g/l), $MgSO_4$ (0.24g/l), $CaCl_2$ (0.01g/l), carbon source (4g/l), Agar (15g/l) and the samples were evenly spread (surface plating) (Aygan *et al.*, 2008). The plates were incubated for 48 hours at 37 °C.

After 48 hours, colonies (indicative of starch-hydrolyzing organisms) were sub-cultured repeatedly for purification by streaking on Nutrient agar for 24 hours at 37 °C for bacteria. The bacterial strains were also scrutinized by observing the colony morphology. This included the physical appearance of the bacterial colonies on nutrient agar medium. This medium was selected because it is a non selective and non differentiating medium which allows the growth of maximum types of bacterial strains and due to the absence of any selective components in the medium, the appearance of the bacterial colonies are not affected (Patel *et al.*, 2019).

Purified isolates were harvested after 18 hours of incubation at 30 °C on Nutrient agar and preserved on 6% sucrose solution at -20 °C for subsequent characterization. For the isolation of fungi, Rose Bengal differential media was used. Pink coloured colonies were picked using sterilized toothpick and cultured on Potato Dextrose Agar (PDA) media after which they were selected on M9 starch agar medium.

3.3.1 Primary Screening of Amylase Producing Microorganisms

The 70 amylolytic isolates preserved under sucrose were drawn using a sterilized wire loop and streaked on nutrient agar then incubated for 24 hours at 37 °C or PDA then incubated for 5

days at 24 °C for bacteria and fungi, respectively. The colonies from nutrient agar/PDA were picked using a sterilized wire loop and streaked on the M9 starch agar medium plates (10 g l^{-1} corn starch) then incubated at 37 °C. After 48 hours of incubation, the plates were flooded with 1% Lugol's iodine reagent, which is a starch indicator for 20 minutes. Excess Lugol's iodine reagent was drained off by distilled water and the plates observed for the formation of a clear halo zone around the colonies against a blue-black background. A clear halo zone around the colonies indicated amylase production and was used for the determination of starch hydrolysis ratio (SHR) as indicated in Equation 1 (Abd-Elhahlem *et al*., 2015; Pranay *et al*., 2019). SHR was used for the ranking of the hydrolysis potential of the amylases produced by the isolates.

$$SHR = \frac{\text{Clear halo zone diameter (mm)}}{\text{Colony growth diameter (mm)}} \quad \text{..... Equation 1}$$

3.3.2 Secondary Screening for Amylase Producing Microorganisms

Specific enzyme activity formed the basis for tertiary screening. The 17 best ranked amylolytic microorganisms from the primary screening were used for enzyme extraction process. This process involved the streaking of the pure 17 isolates on nutrient agar then incubating for 18 hours at 30°C. Colonies from nutrient agar were picked using sterilized wire loops and inoculated onto 2 ml of M9 starch broth media [$NaHPO_4$ (6.7g/L), KH_2PO_4 (3g/L), NaCl (0.05g/L), NH_4Cl (1g/L), $MgSO_4$ (0.24g/L), $CaCl_2$ (0.01g/L), and corn starch (4g/L)] then incubated at 30°C for 24 hours in a shaker incubator at a rotation speed of 180 rpm. This was used as an inoculum for 10 mL then ultimately 100 mL of M9 starch broths media [corn starch (4g/L)]. Amylase enzymes production was scaled up to 100 mL in 1L Erlenmeyer glass flasks which were incubated for 72 hours at 30°C in a shaker incubator at a rotation speed of 180 rpm. After 72 hours, centrifugation (1000 rpm/ 15 min) was done to separate the crude enzymes in the starch broth from the organisms. The quantity of the crude enzymes was estimated by measuring absorbance at 280 nm wavelength using a spectrophotometer. Soluble starch solution (0.3% w/v) was prepared based on a study by Mesbah and Wiegel (2014) that demonstrated inhibition of amylase activity by soluble starch concentrations greater than 0.3%.

3.3.3 Determination of Specific Activity of the Enzymes From Different Screened Isolates

Amylase activity assay was performed using 3, 5-Dinitrosalicylic acid (DNSA) method with some modifications. In previous studies the DNS method was used to determine the amylase activity of each bacterial isolate. Isolate showing highest activity was chosen. In this study, enzyme activity was assayed by reducing sugar formed by the enzymatic hydrolysis of soluble starch. Starch was used as a substrate at a concentration of 1% in 0.05M phosphate buffer at pH 6.9. Crude enzyme sample was mixed with substrate solution and incubated at 37 °C for 10 minutes. The reaction was controlled by adding 1ml of 3,5 Dinitro salicylic acid solution. After that the test tube was kept in boiling water bath for 10 minutes and cooled. The absorbance was read at 540 nm against blank (Vaidya *et al.,* 2015). For this research some modification done were as follows: the assay mixture contained 500 µL of corn starch (0.3% w/v), 500 µL of 0.1 M phosphate buffer, and 100 µl of appropriately diluted crude enzyme solution with pH adjusted to 7.0. The assay mixture was incubated at 40 °C for 10 minutes then the reaction was stopped by the addition of 1 ml of 3, 5-dinitrosalicylic acid reagent. Two milliliters (2 mL) of distilled water

was also added. The mixture was boiled for 5 minutes and the solution was rapidly cooled in ice water. Absorbance was taken at 540 nm in UV-VIS spectrophotometer. Glucose was used as a standard to prepare the calibration curve. Specific enzyme activity was taken as the amount of enzyme required to catalyze the release of reducing sugars equivalent to 1 μmol of D-glucose per minute under assay conditions. Commercial amylase (α-fungal amylase from Bio-medica laboratories limited, Nairobi, Kenya) was used as a standard for comparison purposes.

3.4 Selection Based on Efficiency in Cassava Starch Hydrolysis

The five best crude enzymes were inoculated on 11 identified cassava varieties as well as modified cassava starch for further comparison. These 11 randomly selected cassava varieties were as follows: NYAR JICA, MM961 1817, MIEYERA, MASISA, MM961 0013, MM961 4884,010 MBA, MM961 7680, MADAM, MM961 2480 and KME-1. Cassava was collected from specific identified farmers. It was sun-dried, peeled and milled into cassava flour. Cassava starch solution (3%) was prepared and sterilized at 121 °C/15 minutes. The cassava starch was put in a water bath at 52-65 °C for six minutes to allow for gelatinization. The crude enzymes were inoculated further on the earlier prepared gelatinized starch. The rate of starch hydrolysis was monitored by testing for reducing sugars using 3, 5-dinitrosalicylic acid (DNSA) reagent (Miller, 1959). This method is based on the color which forms when sugar reacts with 3, 5-dinitrosalicylic acid (DNSA) to form 3-amino-5-nitrosalicylic acid (red brown complex).

The sucrose does not react with DNSA; therefore, it must be broken down into simple sugars like glucose first. The red-brown color was detected at 540 nm. The deeper the colour intensity the higher the concentration of reducing sugars. Blank sample containing starch treated with no microorganism was used for comparison. Commercial amylolytic enzymes were purchased and used as a control with which the efficiency of hydrolysis were compared. The enzymes with high activities / stabilities were selected and further characterized.

3.4.1 Phenotypic and Genetic Characterization of Amylase-Producing Microorganisms

Phenotypic and genetic characterization of amylase-producing microorganisms is vital for understanding their diversity, enzymatic capabilities, and industrial potential. Phenotypic characterization involves studying observable traits like morphology, physiology, and growth rates, providing valuable information for selecting suitable microorganisms and optimizing their cultivation conditions. Genetic characterization especially identification contributes to our

understanding of the evolutionary relationships and genetic diversity of amylase-producing microorganisms, shedding light on their adaptations and safety.

3.4.2 Phenotypic Identification

Phenotypic identification of the selected isolates was carried out according to their morphological (Gram and endospore staining) and biochemical tests (catalase test and starch utilization).

3.4.3 Molecular Characterization of the Selected Microbial Strains

The identity of of the seven microbes with the most efficient starch-hydrolyzing enzymes were studied using Polymerase Chain Reaction (PCR) of rDNA (Ribosomal Deoxyribonucleic acid) genes. PCR-amplification of 16S or 23S rDNA were used to visualize microbial diversity (Ravindar & Elangovan, 2013). DNA was extracted from isolated microorganisms using DNA extraction kit (Promega kit) then quantified using Nanodrop Spectrophotometer. The extracted DNA was used as a template in the PCR assays. Extraction of the DNA was followed by PCR amplification of the ITS (Internal Transcribed Spacer) regions of the 16S rDNA and 23S rDNA genes for bacteria and fungi, respectively using universal primers. For bacteria universal primers 27F and 1492R were used, while for fungi universal primers ITS1 and ITS4 were used for amplification.

The Amplicons were electrophoresed with 1% agarose gel, purified using QIA quick PCR purification kit (Qiagen), and then sequenced using Genetic analyzer (Applied Biosystems 3130 XL, Switzerland) by Inqaba Biotechnical industries, South Africa. BLAST (Basic Local Alignment Search Tool) was used to determine the most similar sequences in the public sequence databases (GenBank database) (www.ncbi.nlm.nih.gov/genbank/).

3.4.4 Phylogenetic Analysis

The 16S rDNA and 23S rDNA gene partial sequences were submitted to the ribosomal database project to obtain the taxonomic assignment and the relative abundance of the different microbial groups. The consensus sequences were aligned and used to generate a phylogenetic tree based on the neighbor-joining and bootstrap methods on the National Center for Biotechnology Information (NCBI) database. The consensus sequence was deposited in the GenBank database and they were assigned unique accession numbers.

3.5 Characteristics of the Amylolytic Enzymes From the Isolates

The characteristics of amylolytic enzymes from isolates can be evaluated by studying their temperature and starch loading rate effects on specific enzyme activity. This provides insights into enzyme efficiency and optimal conditions for starch breakdown, aiding industrial applications and process optimization. Evaluating stability at 30°C helps select enzymes suitable for prolonged activity at this temperature.

3.5.1 Determination of the Effect of Temperature and Starch Loading Rate on the Specific Enzyme Activity

The activity of the amylolytic enzyme obtained from BSF (C4) was evaluated at different temperatures (45 °C, 50 °C, and 55 °C) and percentages of starch loading (20%, 50%, and 60%). The enzyme was incubated at a specific temperature and starch loading for 30 minutes after which reducing sugars were quantitated by DNSA reagent (as described in section 3.4) and the values obtained used to determine specific enzyme activity.

3.5.2 Evaluating the Stability of Enzymes of Isolates at 30°C

The enzymes produced by the isolates were evaluated to determine their stability by incubating them at 30 °C as the commonly used temperature to evaluate enzyme stability and pH 7.0 (neutral pH). Thereafter, sampling was done at intervals of 6 h then enzymatic activity determined as described in section 3.4.

3.6 Statistical Data Analysis

Statistical analysis was carried out in triplicate and the data subjected to ANOVA (Analysis of variance) using PROC GLM (Generalized Linear Model Procedure) in SAS software (2006) version 9.4. ANOVA was used to evaluate the differences between enzymatic activities of the isolates. Mean separation at 95% confidence level ($P<0.05$) using Tukey's Honestly Significant Difference (HSD) was done. This was because this method was more stringent compared to other methods and had limited chances of type one error occurring. The relationship between protein yield (purified enzyme) and glucose accumulation was assessed using linear regression.Genstat 16th edition analytical tool was used for the graphical representation.

CHAPTER FOUR
RESULTS

4.1 Initial Screening Procedure

In order to identify microbial strains with amylase production capabilities, an initial screening procedure was conducted using a cost-effective minimal growth medium, namely M9 medium. The M9 medium was supplemented with starch to serve as a selective agent and sole carbon source, allowing only those microbes capable of producing amylases to grow. Various bacteria and fungi isolated from diverse environments were tested for their ability to produce amylases by inoculating them onto M9 agar plates supplemented with pure corn starch. Negative control plates without starch, but inoculated with the microbial samples, were also included to assess the specificity of growth. As a result, only the M9 agar plates supplemented with starch supported the growth of microbial strains capable of producing amylases. No growth was observed on the negative control plates without starch. A total of 70 amylase-producing microbial strains were isolated from the samples. These strains appeared as distinct colonies on the M9 agar plates, indicating their ability to utilize the added starch as a carbon source for growth.

4.2 Primary Screening for Amylase Producing Microorganisms

In this case, the starch hydrolysis ratio (SHR) of the 70-amylase producing microbial isolates was used to narrow down the number of isolates with high amylolytic activity. All the isolates exhibited extracellular amylase activity as depicted by the presence of clearance zones or 'halos' around their colonies after staining with Lugol's iodine reagent (Figure 6).

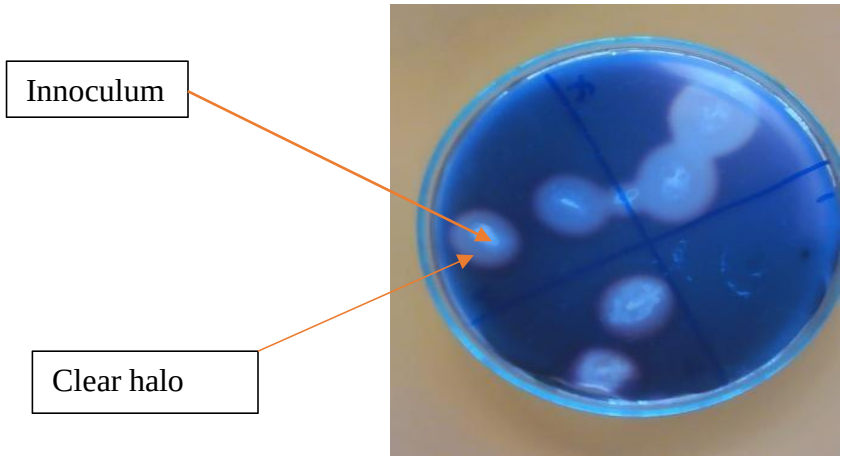

Figure 6: Starch Agar Plate Showing Halo Around Colonies after Staining with Lugol's Iodine Reagent

The size of the halo zone is known generally to be proportional to amylase production (Alam & Ruchika, 2011; Gómez-Villegas *et al*., 2021). The halo zone formation and SHR was then used as a semi-quantitative method for classifying the isolates as excellent producers of amylases (SHR>1.5), very good producers (SHR >1 but <1.5), good producers (SHR>0.5 but <1), and poor producers when no clear zones were obsrved. Based on this criterion, 17 microbial isolates falling into the categories of excellent producers of amylases were considered for secondary screening and further experiments (Table 3).

Table 2: Starch hydrolysis ratio of the selected amylolytic microbial strains (Excellent amylaseproducers**)**

Sample Code	Colony/Inoculum No.	Mean Halo zone (mm)	Mean Colony diameter (mm)	Starch hydrolysis ratio (SHR)
C.2	2	7.3 ± 0.30^{e}	2.4 ± 0.07^{fg}	3.1
C.4	4	8.5 ± 0.26^{d}	2.5 ± 0.06^{f}	3.4
C.5	5	3.8 ± 0.10^{l}	1.0 ± 0.00^{j}	3.8
C.6	6	3.8 ± 0.10^{l}	1.0 ± 0.00^{j}	3.8
C.14	9	2.5 ± 0.10^{m}	1.0 ± 0.00^{j}	2.5
C.16	16	6.8 ± 0.28^{fg}	2.3 ± 0.05^{g}	3.0
C.21	21	6.5 ± 0.10^{g}	1.8 ± 0.10^{h}	3.7
C.27	27	4.8 ± 0.10^{k}	1.3 ± 0.09^{i}	3.8
C.29	29	6.3 ± 0.05^{h}	2.5 ± 0.17^{fg}	2.5
C.42	42	7.3 ± 0.15^{e}	1.8 ± 0.09^{h}	4.1
C.64	54	14.0 ± 0.22^{a}	6.8 ± 0.00^{a}	2.1
C.61	60	11.3 ± 0.14^{b}	3.5 ± 0.00^{d}	3.2
ps.r	68	6.0 ± 0.08^{i}	1.0 ± 0.05^{j}	6.0
ts.r	66	6.0 ± 0.14^{i}	3.3 ± 0.05^{e}	1.8
bs.r	67	5.5 ± 0.19^{j}	1.3 ± 0.05^{i}	4.4
el2.r	69	7.0 ± 0.00^{f}	4.0 ± 0.11^{c}	1.8
bg1.r	70	9.0 ± 0.12^{c}	5.5 ± 0.17^{b}	1.6

Means (of three replicate measurements) in a column with similar superscript are not significantly different at $p \leqq 0.05$.

4.3 Secondary Screening for Amylase Producing Isolates and Specific Enzyme Activity of Enzymes from the Isolates

In secondary screening of the strains, the crude enzymes produced by the 17 excellent isolates of primary screening were used to determine specific enzyme activity. The specific enzyme activities ranged between 0.0079 U g^{-1} for colony number 69 and 0.0629 U g^{-1} for colony number 70 and were lower compared with the control enzyme whose activity was 1.7188 U g^{-1} (Table 4). Ranking based on specific enzyme activity identified the top seven isolates (colonies number 4, 5, 9, 27, 42, 66, and 70) that were taken for cassava starch hydrolysis, and phenotypic and genotypic characterization.

Table 3: Specific enzyme activity of crude enzymes from isolates classified as excellent amylase producers (against corn starch) and specific enzyme activity of top enzymes against cassava starch hydrolysis.

Colony Number	Specific Activity (U g^{-1}): Corn starch	Specific Activity (U g^{-1}): Cassava
2	0.0253 ± 0.004^{cd}	N. A
4	0.0619 ± 0.031^{a}	0.0707 ± 0.014^{cd}
5	0.0563 ± 0.013^{a}	0.0947 ± 0.11^{c}
6	0.0183 ± 0.000^{de}	N. A
9	0.0362 ± 0.010^{c}	0.1853 ± 0.018^{a}
16	0.0239 ± 0.017^{c}	N. A
21	0.0217 ± 0.016^{de}	N. A
27	0.0305 ± 0.016^{c}	N. A
29	0.0244 ± 0.008^{cd}	N. A
42	0.0499 ± 0.008^{b}	0.0728 ± 0.006^{d}
54	0.0128 ± 0.008^{de}	N. A
60	0.0282 ± 0.007^{cd}	N. A
66	0.0327 ± 0.002^{cd}	N. A
67	0.0224 ± 0.002^{de}	N. A
68	0.0182 ± 0.004^{de}	N. A
69	0.0079 ± 0.005^{de}	N. A
70	0.0629 ± 0.001^{b}	0.1347 ± 0.028^{b}
IND. A	1.7188 ± 0.000^{f}	0.0052 ± 0.01^{e}

IND. A: industrial amylase used as a control. NA: Not assayed

Means (of three replicate measurements) with the same superscript letter in a column are not significantly different from each other at $p \leqq 0.05$.

4.4 Selection Based on Efficiency in Cassava Starch Hydrolysis

From the 17 excellent amylolytic microbes from the secondary screening, the top 5 (Colonies number 4, 5, 9, 42, and 70) were selected and used for the hydrolysis of cassava flour to demonstrate their potential application in starch hydrolysis (Table 4). All the crude enzymes exhibited higher specific enzyme activity compared with the control (industrial amylase). The enzyme with the highest activity was that obtained from colony number 9 (0.1853 U g^{-1}) followed by colony number 70 (0.1347 U g^{-1}), 5 (0.0947 U g^{-1}) and 42 (0.0728 U g^{-1}), while colony number 4 had the least specific enzyme activity (0.0707 U g^{-1}). Unlike the control (industrial pure enzyme), the specific activity of the isolates during cassava hydrolysis was higher than that for corn starch hydrolysis.

4.5 Morphological Characteristics of the Isolates

The top 7 amylolytic microorganisms were characterized on the basis of color, configuration, margin, elevation, and mucus on their colonies (Table 5). The isolates exhibited highly heterogeneous culture characteristics, which were a reflection of the diverse microbial flora of the sample sites. Most of the isolates producing high-activity amylases were from Lake Elementaita (2) and Lake Bogoria (2) while the potato processing factory waste, termites' gut, and BSF each had a representative. Most of the isolates had clear colonies (4) that were mostly spreading or round in shape and ranging from small to very large in size. Most colony margins of the isolates were smooth (3), irregular (2) while others were regular (1) or lobbed (1). Most isolates also had shiny and mucoid colonies that were elevated (5) while one isolate had an elevated and dry colony and another isolate's colony was shiny and elevated.

On biochemical characterization, all the isolates were catalase and Gram positive, which appeared as rods (long/short, thin/thick) with most (4) having an endospore. One isolate (colony number 70), was assigned as a yeast while the rest of the isolates were presumptively assigned as members of the *Bacillus* genus, based on their properties (Ravindar and Elangovan, 2013; Retnaningrum and Purwestri, 2016).

Table 4: Phenotypic Characteristics of the Most Potent Amylolytic Microbial Isolates

Colony number	Sampling Site	Colour	Shape	Size	Margin	Gram staining	Catalase test	Others	Potential Identity
4	BSF gut	Clear	Spreading	Very large	Irregular	G+, long rods with endospore	+	Mucoid, shiny, elevated	*Bacillus* sp.
5	L. Elementaita	Clear	Spreading	Small	Regular	G+, long thin rods	+	Mucoid, shiny, elevated	*Bacillus* sp.
9	L. Elementaita	Clear	Spreading	Very large	Irregular	G+, long thin rods with endospore	+	Mucoid, shiny, elevated	*Bacillus* sp.
27	Potato factory waste site	Cream	Spreading	Very large	Lobbed	G+, long thick rods	+	Mucoid, shiny, elevated	*Bacillus* sp.
42	L. Bogoria	Clear	Spreading	Large	Smooth	G+, short rods with endospore	+	Shiny, elevated	*Bacillus* sp.
66	Termite gut	White	Round	Medium	Smooth	G+, short rods with endospore	+	Mucoid, shiny, elevated	*Bacillus* sp.
70	L. Bogoria	White	Round	Medium	Smooth	Yeast cells	+	Elevated, dry	Yeast

BSF; Black soldier fly

4.6 Molecular Identification of the Most Efficient Strains

After the phenotypic characterization of the seven isolates producing amylases with amylolytic activity, the identification of the isolates was not certain. Therefore, genotypic characterization was carried out to identify the strains up to the species level. The 16 S rDNA sequences of the six strains and 5.8 S rRNA-Internal Transcribed Sequence (ITS) gene of the isolate (colony number 70) (Appendix 1) were compared to sequences available in the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) in GenBank and the Ribosomal Database Project (RDP) database. The isolate's sequences exhibited a high degree of similarity to sequences in the database, enabling identification of the isolates. Most (4) of the isolates were identified to belong to the genus *Lysinibacillus* while two isolates belonged to the *Bacillus* genus and one isolate was identified as *Alternaria* spp. (Table 7 and Figure 6). The 16S rDNA and 5.8S rDNA sequences were deposited into the GenBank database with accession numbers presented in Table 4. One strain (colony number 27) was not assigned accession number due to poor quality DNA sequence though it had high similarity (>83%) with *Bacillus cereus* strains.

Table 5-Isolate Identity and Accession Numbers Deposited in the Genbank

Colony Number	Habitat	Isolate identification	GenBank accession No.
4	BSF gut	*Lysinibacillus* sp. strain EGER20	**MW720604**
5	L. Elementaita	*Bacillus* sp. strain EGER21	**MW720605**
9	L. Elementaita	*Lysinibacillus* sp. strain EGER22	**MW720606**
27	Potato factory waste	*Bacillus cereus*	**NA**
42	L. Bogoria	*Lysinibacillus* sp. strain EGER26	**MW725245**
66	Termite gut	*Lysinibacillus* sp. strain EGER27	**MW725246**
70	L. Bogoria	*Alternaria* sp. strain EGER23	**MW721021**

NA; Not assigned

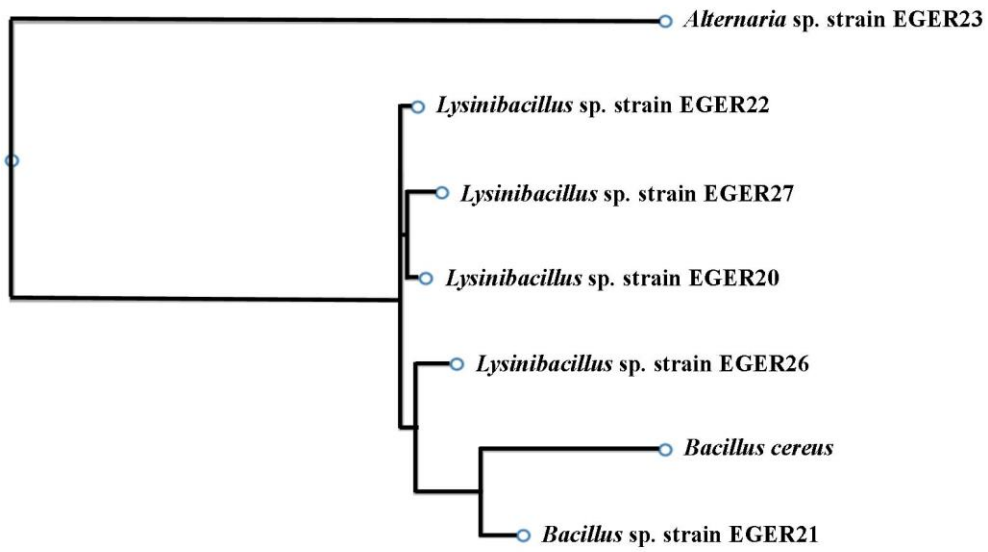

Figure 7-Neighbor-Joining Tree Based on 16S rRNA/ 5.8S Sequences of the Isolates Obtained from BLAST Search Showing the Position of Isolates.

4.7 Characteristics of the Amylolytic Enzymes from the Isolates

In this study, the effect of temperature and starch loading rate on the specific enzyme activity of the isolated amylolytic enzymes, as well as their stability at a constant temperature of 30 °C, which is a critical factor for their practical application in various industries was investigated.

4.7.1 Effect of Temperature and Starch Loading Rate on the Specific Enzyme Activity

The enzymatic activity of the amylolytic enzymes derived from black soldier fly (BSF) was evaluated under various conditions, including different temperatures and percentages of starch loading at pH 7 and the findings are summarized in Table 7. When the starch loading was set at 20%, the specific enzyme activity displayed a significant increase as the temperature was raised. In contrast, when the starch loading was set at 50%, the specific enzyme activity decreased as the temperature increased. The enzyme appeared to be less efficient at higher temperatures under this starch loading condition. Interestingly, at 60% corn starch loading, there was no discernible pattern regarding the effect of starch loading on the specific enzyme activity at different temperatures. This suggests that the starch loading may have a less significant impact on the

enzymatic activity under these conditions. However, regardless of the temperature and starch loading combination, higher starch loading consistently resulted in higher specific enzyme activity. This indicates that increasing the amount of starch available for enzymatic reaction enhances the enzyme's activity. However, for detailed characterization, the enzymes from the other sources could be evaluated.

Table 6: Effect of Temperature and Starch Loading Rate on the Specific Enzyme Activity of a Representative Amylase Enzyme Obtained from an Isolate from the Black Soldier Fly Larvae.

ISOLATE	Temperature (℃)	% corn starch	Specific enzyme activity (U g^{-1})
BS	55	20	0.239
BS	55	50	0.059
BS	55	60	0.280
BS	50	20	0.044
BS	50	50	0.103
BS	50	60	0.217
BS	45	20	0.0082
BS	45	50	0.212
BS	45	60	0.242

4.7.2 Stability of Enzymes of Isolates at 30 °C

To assess the stability of the enzymes produced by the isolates, an incubation experiment was conducted at 30 °C and pH 7.0. The enzymes were sampled at 6-hour intervals, and the results are presented in Table 8. After 6 hours of incubation, the enzymes from 12 isolates, including the control (industrial enzyme), maintained their original specific enzyme activity. This indicates that these enzymes remained stable over the initial 6-hour period. Upon reaching the 12-hour mark, only 5 isolates, along with the control, retained their full enzymatic activity. The enzymes from these isolates demonstrated higher stability compared to the others. By the 18-hour mark, only two samples exhibited full enzymatic activity. The control enzyme continued to display full activity, as did the enzyme from a specific isolate, identified as *Lysinibacillus* spp. strain

EGER26 and labeled as colony number 42 (C42). Notably, this isolate was obtained from Lake Bogoria.

Table 7:-Stability of Enzymes from the Isolates at 30 °C

INNO.	Specific enzyme activity at 0 h	Specific enzyme activity at 6 h	Specific enzyme activity at 12 h	Specific enzyme activity at 18 h
C.27	0.1508[a]	0.1213[a]	0.0448[c]	0.0719[b]
C.5	0.1549[a]	0.1466[a]	0.0973[b]	0.0006[c]
C.29	0.1182[a]	0.1007[a]	0.959[a]	0.0201[b]
C.64	0.2122[a]	0.0751[b]	0.0544[b]	0.0224[c]
TS rose	0.1443[a]	0.1451[a]	0.064[b]	0.0068[b]
C.2	0.0719[a]	0.0228[b]	0.0018[d]	0.0088[c]
C.61	0.0772[b]	0.0767[b]	0.1244[a]	0.0282[c]
BGMB rose	0.1297[a]	0.1608[a]	0.0049[b]	0.0064[b]
PS rose	0.2218[a]	0.2307[a]	0.0439[b]	0.0082[c]
EL2MB	0.1400[a]	0.1066[b]	0.0072[c]	0.0195[c]
C.16	0.0774[a]	0.0498[b]	0.0180[c]	0.0070[d]
IND.AMY	0.0014[a]	0.0007[a]	0.0014[a]	0.0047[a]
C.4	0.122[a]	0.1419[a]	0.0373[b]	0.0033[c]
FC. rose	0.2087[a]	0.1646[a]	0.2096[a]	0.0088[b]
C.14	0.2114[a]	0.2091[a]	0.2132[a]	0.0234[b]
C.42	0.0973[a]	0.0650[b]	0.0173[c]	0.1277[a]
BS rose	0.1402[a]	0.0913[b]	0.0334[c]	0.0454[c]
C.21	0.1297[b]	0.1431[b]	0.1917[a]	0.0365[c]

Values having similar superscript letters in a row are not significantly different at p=0.05

CHAPTER FIVE
DISCUSSION

5.1 Sampling

Amylases play a crucial role in various industrial applications, particularly in the food industry, where they are utilized for starch hydrolysis (Pascon *et al*., 2011; Ullah *et al*., 2021). However, the current use of less active and unstable amylases in food production contributes to high manufacturing costs (Gómez-Villegas *et al*., 2021). Therefore, there is a need to screen for diverse amylases that are better adapted to the harsh conditions encountered during food processing. The objective of this study was to conduct bioprospecting for amylase enzymes from different environments in Kenya.

The initial screening procedure involved cultivating amylolytic microbes on M9 minimal Agar medium supplemented with starch as the sole carbon source. This method enabled the isolation of 70 microbes presumed to be producing amylase enzymes. The selection of M9 minimal medium was based on the principle that these microbes would require a carbon source for growth. Consequently, the growth of isolates on M9 media supplemented with starch indicated their ability to hydrolyze starch (Mamo and Gessesse, 1999; Msarah *et al*., 2020; Pascon *et al*., 2011). The successful isolation of amylase-producing microbial strains confirmed that the M9 medium, along with the pH and temperature conditions employed, provided a suitable environment for screening amylase-producing microorganisms.

5.2 Primary Screening for Amylase-Producing Microorganisms

The isolates were then subjected to a primary screening, which was based on halo zone formation around their colonies and starch hydrolysis ratio (SHR). All the isolates exhibited extracellular amylase activity as depicted by the presence of clearance zones or ‘halos’ around their colonies after staining with Lugol’s iodine reagent, that was used to compute the SHR. In this analysis, the activity of the enzymes produced by the organisms is revealed by the size of the halo, measured in millimeters (mm), where the bigger the halo, the higher the activity produced (Thebti *et al*., 2016). The starch hydrolysis ratio ranged from 1.6 to 6.0 for 17 isolates, which indicated that they fell into the category of excellent amylase producers. The SHR ratios were

diverse (1.6–6.0) compared with 3.4 to 4.0 reported by VaseeKaran *et al*. (2010), 2.7–8.3 reported by Pranay *et al*. (2019), and 1.5 reported by Alkando and Ibrahim (2011) for *B. licheniformis*. The results from this study indicated that the isolates produced amylases with varying amylolytic potentials.

5.3 Secondary Screening for Amylase-Producing Microorganisms

The secondary screening process aimed to evaluate the enzyme production and activity of the 17 amylase-producing isolates identified during the primary screening. After cultivating the isolates, the supernatant was used to assess enzyme activity using soluble corn starch as the substrate. The specific enzyme activity of the extracellular enzymes produced by the isolates ranged from 0.0079 to 0.0629 U g-1. These values were lower compared to both the commercial amylase enzyme control (1.7188 U g-1) and values reported in previous studies (Abd-Elhalem *et al*., 2015; Msarah *et al*., 2020; Pranay *et al*., 2019). The higher enzyme activity observed in the control sample compared to the isolate samples can be attributed to the fact that the control is a purified enzyme, whereas the samples obtained were crude enzymes. The level of enzyme purity directly affects its specific activity, which is the ratio of enzyme activity to the concentration of the protein used in the enzymatic activity assay (Msarah *et al*., 2020). Consequently, the values obtained in this study were lower compared to specific activity values reported for *Bacillus* strains by Pranay *et al*. (2019) (13.68 – 18.20 U g-1) and Luang-In *et al*. (2019) (0.21-0.91 U mg-1). However, the values were within the same range as those reported for *Bacillus amyloliquefaciens* by Abd-Elhalem *et al*. (2015).

The lower and varied specific activity values observed in our study may be attributed to suboptimal conditions for enzyme production and assay, including pH and temperature, which were not optimized in this study. Amylase-producing enzymes exhibit optimal growth and enzyme production under specific pH and temperature conditions (Mamo and Gessesse, 1999; Msarah *et al*., 2020; Xu *et al*., 2019). Therefore, it is plausible that by optimizing these conditions, amylases with higher activity can be produced.

5.4 Selection Based on Cassava Starch Hydrolysis

When enzymes from some of the isolates were applied in the hydrolysis of raw cassava, they exhibited higher specific enzyme activity than that of the purified enzyme (control) and higher

than towards purified corn starch. This is because amylases exhibit different substrate specificity towards different starches, whose amlose to amylopectin ratios and starch structure are different (Xu *et al*., 2016). Enzymes that hydrolyze raw starch could thus be economically attractive since they broaden the range of starch sources for direct saccharification with reduced cost (Gangadharan *et al*., 2009).

5.5 Specific Enzyme Activity of the Isolates Enzymes

The enzyme activities reported for the isolates in this research are significant when compared to the activities reported in other studies. However, since growth and enzyme production optimization and enzyme purification were not conducted, the isolates could be of great interest especially since most (4) isolates were obtained from an alkaliphilic environment (Lake Elementaita and Lake Bogoria). Production and harvesting of the enzyme in great quantities is therefore desirable for enzyme characterization and evaluation of biotechnological application potential.

5.6 Morphological Characteristics of the Isolates

The characterization of the top 7 amylolytic microorganisms based on colony characteristics and biochemical properties provided insights into the diversity and nature of the isolated strains. The heterogeneous culture characteristics observed among the isolates reflected the diverse microbial flora present in the sample sites. This suggests that the sources from which the isolates were obtained harbor a wide range of microbial species capable of producing high-activity amylases.

Understanding the characteristics and properties of the isolated strains was crucial for subsequent steps in enzyme production and application. It provided a foundation for selecting the most promising isolates for further studies, such as optimization of growth conditions, scale-up of enzyme production, and exploration of their industrial applications. By harnessing the diversity and capabilities of these amylolytic microorganisms, it may be possible to develop improved and tailored amylase enzymes for various industrial sectors, including the food industry and biotechnology.

5.7 Genotypic Characterization of the Isolates

To gain a more precise identification of the isolated strains, the obtained 16S rRNA and 5.8S rRNA gene sequences were subjected to GenBank BLAST search analyses. The results revealed significant similarities between the gene sequences of the isolates and known organisms in the database. One strain showed >98% similarity to *Alternaria* sp., another strain displayed >98% similarity to *Bacillus* sp., and four strains exhibited >98% similarity to *Lysinibacillus* sp. Additionally, one strain displayed >83% similarity to *Bacillus cereus* strain. These findings provided valuable insights into the taxonomic affiliations of the isolated strains.

To further analyze the gene sequences, they were imported into MEGA software for alignment and subsequent construction of a phylogenetic tree. The resulting tree displayed shorter evolutionary distances among members of the same genera, indicating their close relatedness. It is worth noting that members of *Alternaria* species, such as *Alternaria alternata* and *A. tenuissima*, have been previously reported for their ability to produce amylases (El aty and Mostafa, 2015; Shafique *et al*., 2010). Similarly, members of the *Bacillus* genera, including *B. subtilis*, *B. stearothermophilus*, *B. amyloliquefaciens*, *B. licheniformis*, *B. coagulans*, *B. polymyxa*, *B. mesentericus*, *B. vulgaris*, *B. megaterium*, *B. cereus*, *B. halodurans*, and other *Bacillus* spp., have been extensively studied and utilized for amylase production (Abd-Elhalem *et al*., 2015; Luang-In *et al*., 2019; Pranay *et al*., 2019).

Notably, the isolates belonging to the *Lysinibacillus* spp. were initially identified based on colony morphology and biochemical characterization, which suggested their affiliation with the *Bacillus* genera. However, recent taxonomic revisions have resulted in the reclassification of *Lysinibacillus* spp. as distinct from *Bacillus*. The *Lysinibacillus* isolates in this study were specifically obtained from Lakes Elementaita and Bogoria, which are natural alkaline environments with a pH exceeding 9.0 (Mwirichia *et al*., 2010). These soda lakes provide unique ecological niches that favor the survival and adaptation of *Lysinibacillus* spp.

The identification of these strains is crucial for understanding their genetic and enzymatic potential, as well as their ecological relevance. By identifying the specific genera to which the isolates belong, researchers can draw upon existing knowledge and literature to gain insights into their physiological characteristics, enzyme production capabilities, and potential applications. The identification of *Alternaria* and *Bacillus* species, known for their amylase production, highlights the promise of the isolated strains in the biotechnological industry. Moreover, the

presence of *Lysinibacillus* spp. in the alkaline soda lakes of Kenya adds to the growing body of knowledge regarding the unique microbial diversity and adaptation strategies in extreme environments. Further investigations into the metabolic capabilities and functional traits of these isolates may provide valuable insights into their survival mechanisms and potential biotechnological applications.

5.8 Characteristics of the Amylolytic Enzymes from the Isolates

This section discusses the results of the study, which focused on investigating the influence of temperature and starch loading rate on the specific enzyme activity of the isolated amylolytic enzymes. Additionally, the stability of these enzymes at a constant temperature of 30 °C, which is crucial for their practical application in various industries, is also examined and discussed.

5.8.1 Effect of Temperature and Starch Loading Rate on the Specific Enzyme Activity

The optimization of hydrolysis temperature is a critical factor in maximizing the activity of amylase enzymes. In this study, the specific enzyme activity at 20% starch loading was found to increase with temperature, with the highest activity observed at 55 °C. This finding is consistent with previous research indicating that the majority of amylase enzymes exhibit enhanced activity at higher temperatures (Egas *et al*., 1998). The temperature-dependent increase in enzyme activity can be attributed to the enhanced enzymatic kinetics and substrate accessibility at elevated temperatures.

Interestingly, at higher starch loading levels, the activity of the amylase enzymes was either reduced or the effect of temperature on activity was not apparent. This observation aligns with findings reported in the literature (Božić *et al*., 2017) and can be attributed to substrate or product inhibition. At high starch concentrations, the accumulation of substrate or its hydrolysis products may hinder the enzymatic reaction, resulting in reduced activity. To overcome this inhibition, higher enzyme concentrations may be required to achieve efficient hydrolysis of the starch substrate.

The starch processing industry typically employs mashes with starch loadings ranging from 25% to 33%. It is worth noting that most amylase enzymes exhibit optimal efficiency at starch loadings below 25% (Tawil *et al*., 2012). Consequently, there is ongoing research to identify amylases that can efficiently hydrolyze higher starch loadings. Such enzymes would offer

significant advantages to the starch processing industry by allowing for increased starch utilization and improved process efficiency.

5.8.2 Stability of Enzymes of Isolates at 30 °C

The amylase enzyme's activity showed a decline over time when incubated at 30 °C. This decrease in activity could be attributed to the denaturation or decomposition of the amylase enzymes, possibly due to interactions with other components present in the medium, as reported by Özdemir *et al*. (2011). While several enzymes from the isolates retained activity at 6 hours, only the enzymes from isolate C42 maintained full residual activity at 18 hours, similar to the control. This suggests that the control enzyme may have been carefully selected for its stability, making it suitable for industrial applications. Notably, the isolate *Lysinibacillus* spp. strain EGER26, which exhibited enzymes with full residual activity at 18 hours, was obtained from Lake Bogoria, a soda lake. Soda lakes are characterized by dense populations of aerobic organotrophic and alkaliphilic bacteria, supported by primary productivity, and have received limited attention until recently. The presence of *Lysinibacillus* spp. in such alkaliphilic environments indicates that the enzymes they produce could be of significant importance, particularly due to their potential for high thermostability, pH stability, and salinity tolerance (Luang-In *et al*., 2019; Tambekar *et al*., 2016).

This study is a starting point for commercial amylase production. However, to make the production of amylases cost-effective, additional research is essential to characterize the enzymes produced by the isolates. This characterization may include optimum growth and enzyme production by the isolates, thermostability, pH stability, and the effect of metal ions on the enzymes.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

(i) Based on the findings, it can be concluded that Kenya's diverse microflora harbors a rich collection of amylolytic microorganisms. These isolates have been successfully characterized at the molecular level and have been identified as unique compared to those currently utilized in the industry. This suggests that further exploration of Kenya's microflora may lead to the discovery of novel and valuable enzymes for efficient cassava starch hydrolysis.

(ii) The screened microbial isolates demonstrated superior performance in cassava starch hydrolysis compared to pure industrial amylases, even in their crude form. The specific enzyme activity and starch hydrolysis ratio indicated the high efficiency of these enzymes. Notably, isolates from Lake Elementaita (*Alternaria* spp. and *Lysinibacillus* spp.) and Lake Bogoria (*Lysinibacillus* spp.) showed particularly promising results. Therefore, it is recommended to conduct further studies, such as genetic engineering, to enhance the efficiency of these isolates and potentially optimize their application in industrial settings. Additionally, the isolates from black soldier fly larvae showed high specific enzyme activity and demonstrated resilience to extreme temperatures, making them potential candidates for further investigation and optimization.

(iii) The optimal conditions for amylolytic enzyme production were determined to be a temperature of 30 °C and a pH of 7.0, achieved through submerged fermentation in a shaker incubator for 72 hours. The crude enzymes extracted from black soldier fly larvae exhibited exceptional thermophilicity, with the highest specific activity observed at temperatures of 55- 60 °C and pH 7.0. Moreover, these enzymes demonstrated the ability to efficiently hydrolyze high concentrations of corn starch, indicating their suitability for industrial applications where high substrate concentrations and extreme temperatures are involved. The enzymes also exhibited rapid glucose production, fulfilling an essential requirement in industrial processes.

6.2 Recommendations

(i) To ensure the suitability of the enzymes for industrial application, it is highly recommended to pursue the purification of the crude enzyme extracts. Purification will enhance their stability, specificity, and overall performance.

(ii) Further research on black soldier fly larvae as a potential source of amylases for industrial applications, particularly in cassava starch hydrolysis, should be conducted. This research could focus on optimizing enzyme production, exploring genetic modifications, and investigating downstream processing techniques. Such efforts will contribute to the development of efficient and cost-effective enzymatic processes for the starch processing industry.

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APPENDICES

Appendix 1: DNA Consensus Sequences

i. Colony No 4 from the BSF gut: Accession No: **MW720604**

1 TCATGGTGAAACACTGAAAGACGGTTTCGGCTGTCGCTATAGGATGGGCC CGCGGCGCAT
61 TAGCTAGTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACC TGAGAGGGTG
121 ATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAAT
181 CTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGAGTGAAGAA GGGTTTCGGT
241 TCGTAAAACTCTGTTGTAAG GGAAGAACAA GTACAGTAGT AACTGGCTGT ACCTTGACGG
301 TACCTTATTAGAAAGCCACG GCTAACTACG TGCCAGCAGC CGCGGTAATA CGTAGGTGGC
361 AAGCGTTGTCCGGAATTATT GGGCGTAAAG CGCGCGCAGG GGTTTCTTAA GTCTGATGTG
421 AAAGCCCACGGCTCAACCGT GGAGGGTCATTGGAAACTGG GAGACTTGAG TGCAGAAGAG
481 GATAGTGGAATTCCAAGTGT AGCGGTGAAA TGCGTAGAGA TTTGGAGGAA CACCAGTGGC
541 GAAGGCGACTATCTGGTCTG TAACTGACAC TGAGGCGCGA AAGCGTGGGG AGCAAACAGG
601 ATTAGATACCCTGGTAGTCC ACGCCGTAAA CGATGAGTGC TAAGTGTTAG GGGGTTTCCG
661 CCCCTTAGTGCTGCAGCTAA CGCATTAAGC ACTCCGCCTG GGGAGTACGG TCGCAAGACT
721 GAAACTCAAAGGAATTGACG GGGGCCCGCA CAAGCGGTGG AGCATGTGGT TTAATTCGAA
781 GCAACGCGAAGAACCTTACC AGGTCTTGAC ATCCCGTTGA CCACTGTAGA GATATAGTTT
841 CCCCTTCGGGGCAACGGTGA CAGGTGGTGC ATGGTTGTCG TCAGCTCGTG TCGTGAGATG
901 TGGGTTAGTTCCGCACGAGC GCCAACCCGT GGATCTTAGG TSGCATCATT TTAGTGGCAC
961 CTCTAAGTGACTGCGATGAC AACCGAGAGG GTGGGTATGA CGTCATTAT

ii. Colony No 5 from Lake Elementaita: Accession No: **MW720605**

1 CCTGCCCATA AGACTGGGAT AACTCCGGGA AACCGGGGCT AATACCGGAT AACATTTTGA
61 ACGCATGGTT CGAAATTGAA AGGCGGCTTC GGCTGTCACT TATGGATGGA CCCGCGTCGC
121 ATTAGCTAGT TGGTGAGGTA ACGGCTCACC AAGGCAACGA TGCGTAGCCG ACCTGAGAGG
181 GTGATCGGCC ACACTGGGAC TGAGACACGG CCCAGACTCC TACGGGAGGC AGCAGTAGGG
241 AATCTTCCGC AATGGACGAA AGTCTGACGG AGCAACGCCG CGTGAGTGAT GAAGGCTTTC
301 GGGTCGTAAA ACTCTGTTGT TAGGGAAGAA CAAGTGCTAG TTGAATAAGC TGGCACCTTG
361 ACGGTACCTA ACCAGAAAGC CACGGCTAAC TACGTGCCAG CAGCCGCGGT AATACGTAGG
421 TGGCAAGCGT TATCCGGAAT TATTGGGCGT AAAGCGCGCG CAGGTGGTTT CTTAAGTCTG
481 ATGTGAAAGC CCACGGCTCA ACCGTGGAGG GTCATTGGAA ACTGGGAGAC TTGAGTGCAG
541 AAGAGGAAAG TGGAATTCCA TGTGTAGCGG TGAAATGCGT AGAGATATGG AGGAACACCA
601 GTGGCGAAGG CGACTTTCTG GTCTGTAACT GACACTGAGG CGCGAAAGCG TGGGGAGCAA
661 ACAGGATTAG ATACCCTGGT AGTCCACGCC GTAAACGATG AGTGCTAAGT GTTAGAGGGT
721 TTCCGCCCTT TAGTGCTGAA GTTAACGCAT TAAGCACTCC GCCTGGGGAG TACGGCCGCC
781 AAGGCTGAAA CTCAAAGGAA TTGACGGGGG GGCCCGCACA AGCGGTGGAG CATGTGGGTT
841 TAATTCAAGG CAACGCGAAG AACCTTACCA GGTCTTGACA TCCTCTGAAA ACCCTAGAGA
901 TAGGGTTCTC CTTCGGGAGC AGAGTGACAA GTGGTGCATG GGTGTCGTCA GCTCCGTGTC
961 GAGAAGATTG TTGGGGTTAA GATCCCGTAC GAACAGCGTA CCCTTTGATC CTAAGTTGGC
1021 CGTCCTTCAG TTGGG

iii. Colony No 9 from Lake Elementaita: Accession No: **MW720606**

1 TGAAAGACGG TTTCGGCTGT CGCTATAGGA TGGGCCCGCG GCGCATTAGC TAGTTGGTGA
61 GGTAACGGCT CACCAAGGCG ACGATGCGTA GCCGACCTGA GAGGGTGATC GGCCACACTG
121 GGACTGAGAC ACGGCCCAGA CTCCTACGGG AGGCAGCAGT AGGGAATCTT CCACAATGGG
181 CGAAAGCCTG ATGGAGCAAC GCCGCGTGAG TGAAGAAGGA TTTCGGTTCG TAAAACTCTG
241 TTGTAAGGGA AGAACAAGTA CAGTAGTAAC TGGCTGTACC TTGACGGTAC CTTATTAGAA
301 AGCCACGGCT AACTACGTGC CAGCAGCCGC GGTAATACGT AGGTGGCAAG CGTTGTCCGG
361 AATTATTGGG CGTAAAGCGC GCGCAGGTGG TTTCTTAAGT CTGATGTGAA AGCCCACGGC

421 TCAACCGTGG AGGGTCATTG GAAACTGGGA GACTTGAGTG CAGAAGAGGA TAGTGGAATT
481 CCAAGTGTAG CGGTGAAATG CGTAGAGATT TGGAGGAACA CCAGTGGCGA AGGCGACTAT
541 CTGGTCTGTA ACTGACACTG AGGCGCGAAA GCGTGGGGAG CAAACAGGAT TAGATACCCT
601 GGTAGTCCAC GCCGTAAACG ATGAGTGCTA AGTGTTAGGG GGTTTCCGCC CCTTAGTGCT
661 GCAGCTAACG CATTAAGCAC TCCGCCTGGG GAGTACGGTC GCAAGACTGA AACTCAAAGG
721 AATTGACGGG GGCCCGCACA AGCGGTGGAG CATGTGGTTT AATTCGAAGC AACGCGAAGA
781 ACCTTACCAG GTCTTGACAT CCCGTTGACC ACTGTAGAGA TATGGTTCCC CTTCGGGGGC
841 AACGGTGACA GTGTGCATGA TGTCGTCAGC TCGTGTCGTG AGATGTTGGT AGTCCGCACG
901 AGCGCAACCC GTGGATCTTA GTGGCCATCA TTAGTGGCAC TCTAAGTGAC TGCGTGCACC
961 ACCCGAGAGG TGGGGATGAC CGTCAATCGA T

Iv. C27_27-F (Colony 27 from Potato factory waste) Forward sequence

GAACCGCACTASAGTGTGAGTCGAGCGATGGATTAAGAKCTTGCTCTTATGAAGYTAGCGGCGGAC
GGGTGAKGAACACGTGGGTAACCTGYCCATAAGACTGGGATAACTCCGGGAAAGCGGGGCTAATAC
CGGATAACMTTTTGAACCGCATGGWTCKAAATTGAAAGGSGGMTTCGAYTGTCMCTTATGGATGGA
CCCGCGTCGCATTAKCTAGGTGGTGAGGTAACGGCTCACCAAGGSRACSATGCAKARCCRACCTGA
GAGGGTGATCGGMCACACTGGGACTGAKACWCSGSCCAGACTCCTACGGGAGGCAKSARYAGGGA
ATCTTCCSCAATGGACGAAATKCTGACGGAGCAACGCCGSGYGAKTGATGAARGCTTTCGGGTCGTA
AAACTCTGKTGTTASGGAAKAACAASTGCTAATTGAATAAGCTGGCACCTTGACGGKACCTAACCARA
AAGGCACCGSTAACTACGKGCCAACAGACGSGGYAATACKTARGYGGAAAKMGTGATCCGGAATTA
TTGGKMGKAAAGTGCGCRCAKGKGGTTTCTTATGCCTGATGTGAAATCCCACGGTTCATCCGWGAA
TGGTCATTGRAAACTGGGAAACTTGAGTGCAAAATASGAAAGTGGAATTCCATGTGTARCGGAGAAA
TGCGTARASATATGGAGGAACACCASTGGCTGAAGGCRACTTTCTGGTCTGTAACTGACACTGAGGG
CGAGAAAGCGTGRKKGAGCACACCTGGATTAKAYACCCTGGAAACSCCACKCCRCTAAACCGATGA
GTGCTAWKKGKTMKAKGGTGTCCGCCCTTTAGYGMTRAAGWTAMCGCMTKRAGCACTCCGCCTGG
GRGASTACRGCCSMAAKGMTKARACTCAAGGCRTTGACGGSGGGCCCGCACRAGSGKTGGAKCAT
GTGGGTCTAATACGAACSYWACSCTGAASATCCTTACCWGGGTMTTGACMTCCWCTRGAAAYCCTA
GAKWWAGGATYTCTCCTTCAGGAGCATGMTTGACATGGTSGGTGCATKYGGTKGTCGTKCAGMAT

>C27_1492-R(Colony 27 from Potato factory waste) Reverse sequence

CATWAGGGRGCYKKSTCCYTAAGGTTACCCYACCGACTTCKGGTAAACAAACTCTCKGGGGGTGAC
GGGCGGGGTGTACAAGGCCCGGGAACGTATTCACCGCGGMATGCTGATCCGCGATTACTASCGATT
CCAGCTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAAAACGGTTTTATGAGATTAGCTCCA
CCTCGCGGKCTTGCASCTCTTTGWACCGYCCATTGWASMACGKGTGTASCCCAGGTCATAAGGGGC
ATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTARAGTGCCCAAC
TTAATGATGGSAACTAARATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACG
AGCTGACRACAACCATGCACCACCTGTCACTCTGCTCCCGAAGGAGAAGCCCTATCTCTAGGGTTTT
CARAGGATGTCAAGACCTGGKAAGGGTCTTCSCGTTGCTTCSAATTAAACCACATGCTCCACCGCTT
GGGCGGGCCCCCGYCAATTCCTTTGAGTTTCAGCCTTGSGGMCGKACTCCCCAGGGGGAGTGMTT
AATGCGTTAACTTCAGCACTAAAGGGCGGAAACSCTCYAACACTTAGCACTCATCGTTTACGGCGTG
GACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGYGTCAGTWWCAGRCCA
GAARGTYGCCTTCGCCACTGGTGTTCCTCCATATCTCTACGCATTTCACCGCTACACMTGGGAATTC
CACTYWTCCTCTTCCTRYACTCWAGTCTCMCCAGATATCCAATGACMCTCCMMRGTTGARGCYGRG
GGMTTTCACATCMGACTTAAKAAACCMCCTGCGCGCGCTTTACGCCCARTAAWWTCCGGATAACGC
TSGCCACCTACKGWATTACCGCGGCTGCTGGCACRTAGTAARCCGGGGCTTCTGSRTAGGTACCGT
CMAGGKAKCYRCMTGAWTCAACTAGMASTWGCTTCCTCCCYTAMMCTACAAGAGTTTTACGACCAG
AAGCCTTCATCACTCMACGCGGCATGCCTGCGATTCAGACTCGTCAATGGSGMAGATTCCTTACTGC
TGCCCTCCCGATAG

V. Colony 42 from L.Bogoria. Accession No. **MW725245**

1 GCATTAGCTA GTTGGTGAGG TAACGGCTCA CCAAGGCGAC GATGCGTAAC CGACCTGAGA
61 GGGTGATCGG CCACACTGGG ACTGAGACAC GGCCCAGACT CCTACGGGAG GCAGCAGTAG
121 GGAATCTTCC ACAATGGGCG AAAGCCTGAT GGAGCAACGC CGCGTGAGTG AAGAAGGTTT
181 TCGGATCGTA AAACTCTGTT GTAAGGGAAG AACAAGTACA GATAACTGGC TGTACCTTGA
241 CGGTACCTTA TTAAAAAGCC ACGGCTAACT ACGTGCCACA GCCGCGGTAA TACGTAGGTG
301 GCAAGCGTTG TCCGGAATTA TTGGGCGTAA AGCGCGCGCA GGCGGTCCTT TAAGTCTGAT
361 GTGAAAGCCC ACGGCTCAAC CGTGGAGGGT CATTGGAAAC TGGGGGACTT GAGTGCAGAA
421 GAGGAAAGTG GAATTCCAAG TGTAGCGGTG AAATGCGTAG AGATTTGGAG GAACACCAGT
481 GGCGAAGGCG ACTTTCTGGT CTGTAACTGA CGCTGAGGCG CGAAAGCGTG GGGAGCAAAC
541 AGGATTAGAT ACCCTGGTAG TCCACGCCGT AAACGATGAG TGCTAAGTGT TAGGGGGTTT
601 CCGCCCCTTA GTGCTGCAGC TAACGCATTA AGCACTCCGC CTGGGAGTAC GGTCGCAAGA
661 CTGAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGCATGTG GTTTAATTCG
721 AAGCAACGCG AAGAACCTTA CCAGGTCTTG ACATCCCGTT GACCACTGTA TAAATATAGT
781 TTCCCCTTTC GGGGCAACGG TGACAGGTGG TGCATGGTGT CGTCAGCTCG TGTCGTGAGA
841 TGTTGGTTAG TCCCGCAACG ATTCGCAACC CTTGATCTAG TG

Vi. Colony 66 from termite gut. Accession no. **MW725246**

1 CCGACCTGAG AGGGTGATCG GCCACACTGG GACTGAGACA CGGCCCAGAC TCCTACGGGA
61 GGCAGCAGTA GGGAATCTTC CACAATGGGC GAAAGCCTGA TGGAGCAACG CCGCGTGAGT
121 GAAGAAGGAT TTCGGTTCGT AAAACTCTGT TGTAAGGGAA GAACAAGTAC AGTAGTAACT
181 GGCTGTACCT TGACGGTACC TTATTAGAAA GCCACGGCTA ACTACGTGCC AGCAGCCGCG
241 GTAATACGTA GGTGGCAAGC GTTGTCCGGA ATTATTGGGC GTAAAGCGCG CGCAGGTGGT
301 TTCTTAAGTC TGATGTGAAA GCCCACGGCT CAACCGTGGA GGGTCATTGG AAACTGGGAG
361 ACTTGAGTGC AGAAGAGGAT AGTGGAATTC CAAGTGTAGC GGTGAAATGC GTAGAGATTT
421 GGAGGAACAC CAGTGGCGAA GGCGACTATC TGGTCTGTAA CTGACACTGA GGCGCGAAAG
481 CGTGGGGAGC AAACAGGATT AGATACCCTG GTAGTCCACG CCGTAAACGA TGAGTGCTAA
541 GTGTTAGGGG GTTTCCGCCC CTTAGTGCTG CAGCTAACGC ATTAAGCACT CCGCCTGGGG
601 AGTACGGTCG CAAGACTGAA ACTCAAAGGA ATTGACGGGG GCCCGCACAA GCGGTGGAGC
661 ATGTGGTTTA ATTCGAAGCA ACGCGAAGAA CCTTACCAGG TCATGACATC CCGTTGACCA
721 CTGTAGAGAT ATAGTTTCCC CTTCGGGGCA ACGTGACAGG TGGTGCATGT TGTCGTCAGC
781 TCGTGTCGTG AGATGTTGAT AGTACCCGCA TCGAGCGCAA TCCATGATCT AGTGCCATCA
841 TTTAGTGGTC ACGTCTAAGT GACTGGCGAT GACGACGGAG ACGGTGGGAA TGACGATCAA
901 CATCC

Vii. colony 70 from L. Bogoria. Accession No: **MW721021**

1 TACAAGGGGG GGGGCGGGCT GGACCTCTCG GGGTTACAGC CTTGCTGAAT TATTCACCCT
61 TGTCTTTTGC GTACTTCTTG TTTCCTTGGT GGGTTCGCCC ACCACTAGGA CAAACATAAA
121 CCTTTTGTAA TTGCAATCAG CGTCAGTAAC AAATTAATAA TTACAACTTT CAACAACGGA
181 TCTCTTGGTT CTGGCATCGA TGAAGAACGC AGCGAAATGC GATAAGTAGT GTGAATTGCA
241 GAATTCAGTG AATCATCGAA TCTTTGAACG CACATTGCGC CCTTTGGTAT TCCAAAGGGC
301 ATGCCTGTTC GAGCGTCATT TGTACCCTCA AGCTTTGCTT GGTGTTGGGC GTCTTGTCTC
361 TAGCTTTGCT GGAGACTCGC CTTAAAGTAA TTGGCAGCCG GCCTACTGGT TTCGGAGCGC
421 AGCACAAGTC GCACTCTCTA TCAGCAAAGG TCTAGCATCC ATTAAGCCTT TTTTCAACTT
481 TTGACCTCGG ATCAGGTAGG GATACCCGCT GAACTTAAGC ATATCAATAA GCGGAGGAA

Appendix 2: Field work photographs

Sample collection at L.Bogoria and L.Elementaita

Specific enzyme activity determination

Optimization enzyme activity in food microbiology lab of Guild ford Dairy Institute

Appendix 3:Research Publication (Muriithi et al., 2021)

RESEARCH ARTICLE

Applied Research

Amylolytic microorganisms from diverse tropical environments: Isolation, identification, and amylase production

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Center of Excellence in Sustainable Agriculture and Agribusiness Management (CESAAM) of Egerton University

Abstract

This study aimed to identify microbes producing amylases from diverse unexplored environments in Kenya. Screening based on microorganisms' ability to grow on minimal media (M9) supplemented with starch resulted in the selection of 70 amylolytic isolates. The isolates were then subjected to primary screening based on starch hydrolysis ratio (SHR) obtained by halo formation on starch agar plates after flooding plates with Lugol's iodine solution. SHR narrowed down the isolates' number to 17 (SHR > 1.5), which were used for subsequent experiments. The 17 isolates were grown on M9 starch broth for the production of amylases. Crude amylases and commercial enzyme (control) were assayed for amylase activity using the 3,5-dinitrosalicylic acid method. Specific enzyme activities of crude enzymes ranged between 0.0079 and 0.0629 U g^{-1}, which were lower than 1.7188 U g^{-1} of the control. Five enzymes with the highest specific enzyme activity were used for cassava hydrolysis. All crude enzymes exhibited higher specific enzyme activity (0.0707–0.1853 U g^{-1}) than the control (0.0052 U g^{-1}) in hydrolyzing cassava. Alkaline lakes had the highest number (4) of isolates whose enzymes had the highest specific enzyme activity, while isolates from potato processing plant waste, termite gut, and black soldier fly gut had a representative each. Isolates with enzymes having the highest specific enzyme activity were identified by DNA sequencing and belonged to *Lysinibacillus* sp. (four isolates), *Alternaria* sp. (one isolate), and *Bacillus* sp. (two isolates). The study demonstrated the presence of microorganisms in tropical environments with amylase activity that can be scaled and optimized for practical applications.

KEYWORDS
alkaline lake, amylase-producing microorganisms, amylases, specific enzyme activity, starch hydrolysis

INTRODUCTION

Starch is one of the most abundant and renewable carbohydrate resources found in plants, including tuberous plants, cereals, and legumes [1]. It is a polymer of glucose and is the most important energy source for human nutrition and the second most abundant source of energy and carbon in plants with high demand in the food processing industry [2,3]. The starch conversion industry is the largest single consumer of starch, utilizing about 60% of total starch production. Starch can be used to produce a repertoire of products in the food processing industry, such as glucose, glucose–fructose syrups, fructose, maltose, organic acids, and amino acids among others. Starch is

Appendix 4:Data Analysis for 13 cassava varieties hydrolysis by 5 excellent amylolytic microorganisms Anova tables

```
ata Josephine;
input Var $ Innoculum Reps Glucose;
Cards;
A      70     1      0.1377
B      70     1      0.1121
C      70     1      0.1186
D      70     1      0.1319
E      70     1      0.1118
F      70     1      0.1322
G      70     1      0.1172
H      70     1      0.1189
I      70     1      0.1172
J      70     1      0.1302
K      70     1      0.1244
L      70     1      0.1325
M      70     1      0.266
A      71     1      0.0026
B      71     1      0.0021
C      71     1      0.0025
D      71     1      0.0027
E      71     1      0.0033
F      71     1      0.0027
G      71     1      0.0021
H      71     1      0.0034
I      71     1      0.0026
J      71     1      0.0031
K      71     1      0.0024
L      71     1      0.0023
M      71     1      0.005
A      4      1      0.0687
B      4      1      0.0785
C      4      1      0.0532
D      4      1      0.0615
E      4      1      0.0598
F      4      1      0.0719
G      4      1      0.0599
H      4      1      0.0909
I      4      1      0.0809
J      4      1      0.0755
K      4      1      0.0567
L      4      1      0.0561
M      4      1      0.1032
A      5      1      0.0854
B      5      1      0.0999
C      5      1      0.0968
D      5      1      0.0909
E      5      1      0.0933
F      5      1      0.0729
G      5      1      0.0936
H      5      1      0.1036
I      5      1      0.0867
J      5      1      0.0888
K      5      1      0.1007
```

L	5	1	0.0933
M	5	1	0.1232
A	9	1	0.174
B	9	1	0.1766
C	9	1	0.1777
D	9	1	0.1766
E	9	1	0.1776
F	9	1	0.1734
G	9	1	0.187
H	9	1	0.1818
I	9	1	0.1703
J	9	1	0.1771
K	9	1	0.1901
L	9	1	0.2089
M	9	1	0.236
A	42	1	0.073
B	42	1	0.0678
C	42	1	0.0716
D	42	1	0.0767
E	42	1	0.0663
F	42	1	0.0766
G	42	1	0.071
H	42	1	0.0714
I	42	1	0.0728
J	42	1	0.0702
K	42	1	0.0688
L	42	1	0.0678
M	42	1	0.0905
A	70	2	0.1383
B	70	2	0.01114
C	70	2	0.1189
D	70	2	0.1312
E	70	2	0.1138
F	70	2	0.1319
G	70	2	0.1189
H	70	2	0.1179
I	70	2	0.1175
J	70	2	0.1298
K	70	2	0.1261
L	70	2	0.1305
M	70	2	0.2647
A	71	2	0.0026
B	71	2	0.002
C	71	2	0.0025
D	71	2	0.0027
E	71	2	0.0033
F	71	2	0.0027
G	71	2	0.0021
H	71	2	0.0033
I	71	2	0.0026
J	71	2	0.0031
K	71	2	0.0025
L	71	2	0.0024
M	71	2	0.005
A	4	2	0.0685
B	4	2	0.0779
C	4	2	0.0539

D	4	2	0.0613
E	4	2	0.0613
F	4	2	0.0708
G	4	2	0.0617
H	4	2	0.0905
I	4	2	0.0807
J	4	2	0.0766
K	4	2	0.0569
L	4	2	0.0569
M	4	2	0.1027
A	5	2	0.0848
B	5	2	0.0997
C	5	2	0.0965
D	5	2	0.0907
E	5	2	0.0938
F	5	2	0.0743
G	5	2	0.0944
H	5	2	0.1039
I	5	2	0.0883
J	5	2	0.0888
K	5	2	0.1002
L	5	2	0.0938
M	5	2	0.1234
A	9	2	0.1745
B	9	2	0.1766
C	9	2	0.1781
D	9	2	0.1755
E	9	2	0.176
F	9	2	0.1729
G	9	2	0.1896
H	9	2	0.1823
I	9	2	0.1719
J	9	2	0.1781
K	9	2	0.1907
L	9	2	0.2074
M	9	2	0.2377
A	42	2	0.072
B	42	2	0.068
C	42	2	0.072
D	42	2	0.0761
E	42	2	0.0678
F	42	2	0.0767
G	42	2	0.071
H	42	2	0.071
I	42	2	0.0744
J	42	2	0.0694
K	42	2	0.0692
L	42	2	0.0678
M	42	2	0.0915
A	70	3	0.138
B	70	3	0.1107
C	70	3	0.1203
D	70	3	0.1325
E	70	3	0.1145
F	70	3	0.1312
G	70	3	0.1175
H	70	3	0.1196

I	70	3	0.1169
J	70	3	0.1295
K	70	3	0.125
L	70	3	0.1315
M	70	3	0.2633
A	71	3	0.0026
B	71	3	0.002
C	71	3	0.0025
D	71	3	0.0027
E	71	3	0.0033
F	71	3	0.0027
G	71	3	0.0021
H	71	3	0.0034
I	71	3	0.0026
J	71	3	0.0031
K	71	3	0.0024
L	71	3	0.0023
M	71	3	0.005
A	4	3	0.0691
B	4	3	0.0781
C	4	3	0.0535
D	4	3	0.0619
E	4	3	0.0599
F	4	3	0.0713
G	4	3	0.0619
H	4	3	0.0905
I	4	3	0.0805
J	4	3	0.0757
K	4	3	0.0574
L	4	3	0.0565
M	4	3	0.1029
A	5	3	0.0846
B	5	3	0.1002
C	5	3	0.0973
D	5	3	0.0902
E	5	3	0.0944
F	5	3	0.0753
G	5	3	0.0938
H	5	3	0.1028
I	5	3	0.088
J	5	3	0.0899
K	5	3	0.0997
L	5	3	0.0928
M	5	3	0.1218
A	9	3	0.1749
B	9	3	0.1755
C	9	3	0.1776
D	9	3	0.1759
E	9	3	0.1781
F	9	3	0.1739
G	9	3	0.1886
H	9	3	0.1813
I	9	3	0.1729
J	9	3	0.1759
K	9	3	0.1917
L	9	3	0.2068
M	9	3	0.2366

```
A       42      3       0.0724
B       42      3       0.0686
C       42      3       0.0714
D       42      3       0.0757
E       42      3       0.068
F       42      3       0.0773
G       42      3       0.0714
H       42      3       0.0708
I       42      3       0.073
J       42      3       0.0699
K       42      3       0.068
L       42      3       0.0692
M       42      3       0.0919
;
Proc print;
Proc glm;
class Var Innoculum     Reps;
model Glucose= Var      Innoculum Reps Var*Innoculum;
run;
```

Appendix 5: Research Permit from NACOSTI

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Appendix 6: Glucose callibration curve