INVESTIGATION OF MAERUA SHRUB *(Maerua subcordata)* AND YEASTS FROM FINGER MILLET (*Eleusine coracana)* FOR PRODUCTION OF CHEAP AND EFFICIENT BIOETHANOL IN KENYA

BY

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DECLARATION

Declaration by the Student

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DEDICATION

I dedicate my work to my parents, my spouse Dorcas Cheruiyot, my children Mercy Jebchumba, Brenda Jebet, Naomi Jepkoech, Alex Kiploman and Brian Kipkech who have always supported me throughout the study.

ABSTRACT

Bioethanol is an attractive energy source in comparison to fossil fuels because it is renewable and environmentally friendly. The first generation bioethanol is unsustainable due to high cost of food crops and causes food insecurity. In addition, there is need to search for a yeast with high ethanol producing. Yeast with high adaptation to adverse conditions increase ethanol productivity causing economic viability. This study investigated the use of maerua shrub and fermentation using finger millet malt yeasts (Y1 and Y2) as a cheap and efficient way of producing bioethanol. Morphological and microscopic characteristics of purified cultures were determined. The physiological characterization of the yeast were done by looking at the effect of temperature, ethanol concentration and glucose content on the growth of the yeasts using optical densities determined by UV-visible spectrophotometer at 600 nm. The concentration of sugar was determined by titrimetric technique using standardized Fehling's solution. Samples were fermented for 48 hrs at 35 ^{o} C, distilled, oxidised and analysed using UV-Visible spectrometer at 595nm. Statgraphics centurion was used in data analyses. The isolated yeasts Y1 showed white colony, Y2 creamy colour and both indicate multilateral budding, characteristic of *Saccharomyces* strain. The mean optical densities on growth of yeast at various temperature were Y1 (0.4201), Y2 (0.5097) and *Saccharomyces cerevisiae*, Y3 (0.9287), p = .467, ethanol concentration on yeasts were Y1 (0.8434), Y2 (0.4185) and Y3 (0.9672), p = .117, had no significant difference, while glucose concentration for Y1 (0.8329) which was significantly lower compared to both Y3 (1.1726) and Y2 (1.3907), p = .0045. The reducing sugar content in (mole/L); maerua shrub (0.3906), sorghum (0.4426) both were comparable, but significantly lower than cassava (0.7760), maize (0.7054) and sugarcane molasses (0.8980), p = .0001. The ethanol concentration and productivity (g/L/h) from various plants were; cassava (64.052±0.098, 1.334 g/L/h), maize (66.670±0.227, 1.389 g/L/h), sorghum (62.382±2.148b, 1.300 g/L/h) and maerua shrub (61.988±0.160, 1.291g/L/h) which were remarkably higher compared to sugarcane molasses (49.978 g/L, 1.041g/L/h) when fermented by Y2. Y2 gave higher ethanol production than Y1 which produced lower productivity in relation to (Y3). The yeast Y2 isolated from finger millet malt can be used in fermentation and Maerua Shrub to be used as sugar source for bioethanol.

Table of Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

ETBE	Ethyl tertiary butyl ether
GHG	Greenhouse gases
ICRISAT	International Crop Research Institute for Semi-Arid Tropics
LSD	Least significant difference
MEA	Malt extract agar
nm	nanometre
PDA	Potato dextrose agar
SDA	Sabouraud dextrose agar
SHF	Separate hydrolysis and fermentation
Sp	species
SSF	Simultaneous saccharification and fermentation
TBP	Tri-butyl phosphate
YEPD	Yeast extract peptone dextrose

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CHAPTER ONE

INTRODUCTION

1.1. Background of the Study

1.1.1. Biofuels and its categories

Biofuels are chemicals that contain energy generated and derived from biomass which is a sustainable and renewable source of energy. Biofuel includes bioalcohols mainly bioethanol, biodiesel and biogas. Biowastes and lignocellulosic feedstocks can be processed to biofuel (Abbas & Al-zuhairi, 2020).

The common biofuel sources are the plant biomass especially crops which are first generation biofuels. Microbial feedstock can produce second, third and fourth generation biofuel when various methods are used (de Souza Candeo, Sydney, Hashimoto, Soccol & Sydney, 2020)

Biofuels can also be produced from microalgae due to their considerably high levels of carbohydrate, high rate of replication, high photosynthetic efficiency and its ability to remediate waste water (Musa *et al*., 2019). Algae do not require arable land, fertilizers, or fresh water, and consequently, they would not compromise food supply or cause a major environmental problem.

1.1.2. Bioethanol

Bioethanol is ethyl alcohol produced through biomass conversion via biochemical processes (Tran *et al*., 2019). The biochemical processes involved include hydrolysis and fermentation of biomass to yield bioethanol. The bioethanol-gasoline blend is used as a source of energy, also used to synthesize ethyl tertiary butyl ether (ETBE), both are

blended with gasoline to reduce pollutant levels such as CO, NO_X in vehicles (Lim *et al*., 2019).

1.1.3. Importance of bioethanol

Biofuels are good alternative sources of energy because they are non-toxic, biodegradable and they release much lesser greenhouse gases than conventional energy sources. Bioethanol is used as transportation fuel in its pure form or when mixed with gasoline which enhances octane number in gasoline. The oxygenated fuel-mix reduces air pollution (Wibowo *et al*., 2020).

Bioethanol application in compression-ignition engine is enhanced by blending biodiesel-diesohol which shows good performance especially when dual fuel operation system is used a maximum of 80 % ethanol mass ratio can be attained when loads are increased without engine failure (Han *et al*., 2020).

Alcohol based hand sanitizer are prepared using ethanol, isopropyl alcohol and n-propanol with solutions containing alcohol above 60 % v/v being most effective in inactivating SARS- CoV- 2 also commonly known as Covid - 19 (Golin *et al*., 2020).

Bioethanol is used as cooking fuel with special bioethanol stoves that are currently in the market. This fuel reduce air pollution significantly from within the house as compared to fuels such as kerosene as a result use of bioethanol reduces cardiovascular health problems (Olopade *et al*., 2017).

1.1.4. Efficiency of bioethanol

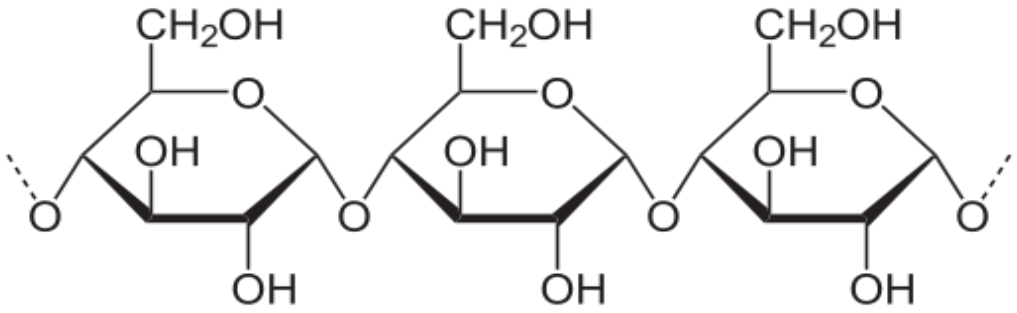
Octane level manipulation is done by increasing octane that causes reduction in the risk of engine knock. The higher the octane level the more compressible the fuel (Rodriguez-Fernandez *et al*., 2020). Knock resistance of engines is improved by high octane number and heat of evaporation of ethanol (Mourad & Mahmoud, 2019). Blends of up to E20 in vehicles can be used without any change of the engine set up (Tibaquira *et al*., 2018).

1.1.5. Bioethanol and environment

Greenhouse gas and aerosol emissions are associated with climate change (Lelieveld *et al*., 2019). The use of biodiesel and bioethanol significantly decrease emitted greenhouse gases from engines because they are oxygen containing fuels. Particulate number and particulate matter emission decreases remarkably when oxygen rich fuel blends with gasoline (Liu *et al*., 2019).

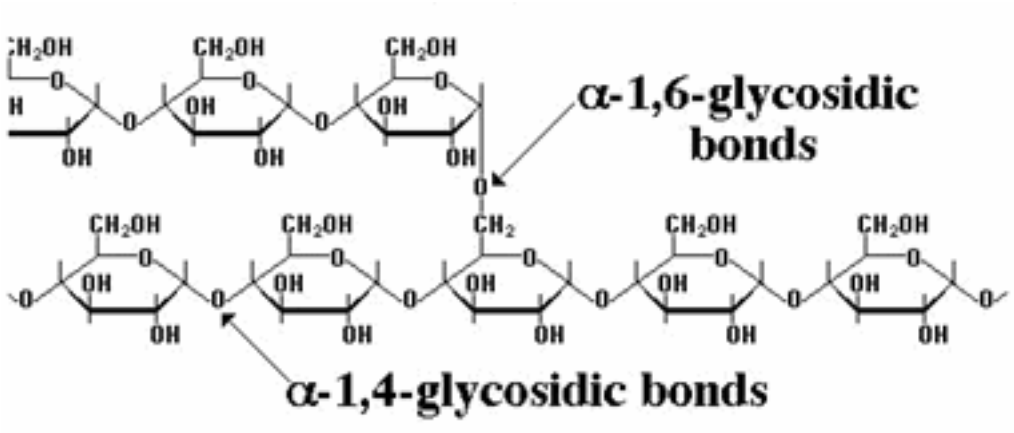
1.2. Structure of starch

Starch is a biomaterial with a variety of uses because of its properties. Starch naturally constitute about 20-30 % amylose and 70-80 % amylopectin. Amylose (Figure 1.1) is composed α-1, 4-glycosidic bonds which form straight chain while amylopectin (Figure 1.2) is branched polymer made of α-1, 4-glycosodic with branched chain bonded by α-1, 6-glycosidic bonds (Gangoiti *et al*., 2020). At 20 – 30 glucose molecules of amylopectin has α-D-1, 6-glycosidic linkages (Plaza-Vinuesa *et al*., 2019).



Source Ghanbarzadeh &Hadi (2013)

Figure 1.1: Structure of amylose molecule



Source Ghanbarzadeh & Hadi (2013)

Figure 1.2: Structure of Amylopectin

1.3. Starch hydrolysis

1.3.1. Enzyme hydrolysis of starch

Endo-acting enzymes for example α-amylase hydrolyses α-1, 4-glucosidic bond inside the starch chain yielding linear and branched oligosaccharides (Lim, Oslan and Oslan,

2020). Alpha-amylase enzymes are synthesized by organisms. Exoamylases such as glucoamylases and α-glucosidases cleaves glycosidic linkages from non-reducing end producing glucose, while β-amylases cleaves second α-1, 4 bonds only producing maltose (Oluwadamilare, Oluwatofunmi, Dzorbenya & Adekunle, 2019).

The hydrolysis of α-1, 6-glucosidic linkages is catalysed by debranching enzymes but they do not hydrolyse α-1, 4-glycosidic linkages (Cifuente, Comino, Trastoy, D'Angelo, & Guerin, 2019). Industries that process starch require a temperature tolerant α-amylase enzyme because the procedures are performed at temperature above 50 ^{o} C (Lim *et al*., 2020).

The thermal and enzymatic pretreatment methods for starch are the most efficient method to enhance the yield of hydrolysate (Nguyen, Chu-ky, Luong, & Nguyen, 2020). The α-amylase enzyme having greater capacity to hydrolyse raw starches at low temperature increases the use of starch (Fang *et al*., 2019). Chemical reactivity of starch takes place in polyhydroxyl group of the glucose monomer.

1.3.2. Acid hydrolysis

Starch concentration plays a significant role in glucose yields than other conditions such as acid concentration and temperature in optimized hydrolysis of cassava starch (Azmi, Yusuf, Jimat & Puad, 2016). Sugar release from starchy substrates involves two reaction processes in which cells are acid processed then followed by enzyme treatment (Daroch, Geng & Wang, 2013).

Acid hydrolysis yields high levels of glucose, however the challenge is the eventual removal of acid from products. Acidic degradation is a common method used to cleave glycosidic linkage than alkali hydrolysis. Sulphuric acid is more attractive mineral acid because of high ethanol yields from its hydrolysate than acetic acid (Phwan *et al*., 2019).

1.4. Lignocellulosic biomass

1.4.1. Pretreatment of Lignocellulose

Lignocellulosic feedstocks contain cellulose, hemicellulose and lignin. Pretreatment methods are used in improving conversion of biomass (Zoghiami & Paes, 2019). Pretreatment methods can be done at low temperatures to recover cellulose, hemicellulose, and lignin (Lorenci *et al*., 2020). Autohydrolysis pretreatment uses water as the solvent its merits include increase in overall sugars yields, decrease in furfural formation and high lignin purity (Conrad & Smirnova, 2020).

The resistance to pretreatment by softwood is remarkably higher than most agricultural or herbaceous crops and residues therefore there is no best method of processing lignocelluloses due to varied biomass. Consequently, for any proposed method should be founded on thorough investigation on techno-economic assessment (Galbe & Wallberg, 2019).

Biological hydrolysis of lignocelluloses is mostly done using cellulase and hemicellulase enzymes. This method is commonly used because it is environmentally friendly, no chemical or energy involved (Ferdeș, Dincă, Moiceanu, Zăbavă, Ștefania & Paraschiv,

2020) and cannot be inhibited by furfural and its derivatives (Tsegaye, Balomajumder & Roy, 2019).

1.4.2. Saccharification of cellulose

Saccharification involves conversion of cellulose to glucose by using enzymes or acids. Enzyme saccharification exposes internal chains of cellulose and hemicelluloses and these changes in structure causes significant effect in the cellulose conversion of water pretreated sugarcane bagasse (Ladeira, Bordignon-Junior, Laufer, Specht, Ferrier, & Kim, 2020). Hemicellulose is composed of polymers which are made up of pentose sugar such as xylose, and hexose sugar such as mannose (Florez-Pardo *et al*., 2019).

The main challenge in valorisation of lignicellulose is resistance to enzyme hydrolysis (Zoghlami & Paes, 2019). The major steps in lignocellulose degradation and subsequent fermentation are by using acid hydrolysis, immobilized enzyme or biocatalysts which increases degradation efficiency and stability (Singhvi & Kim, 2020).

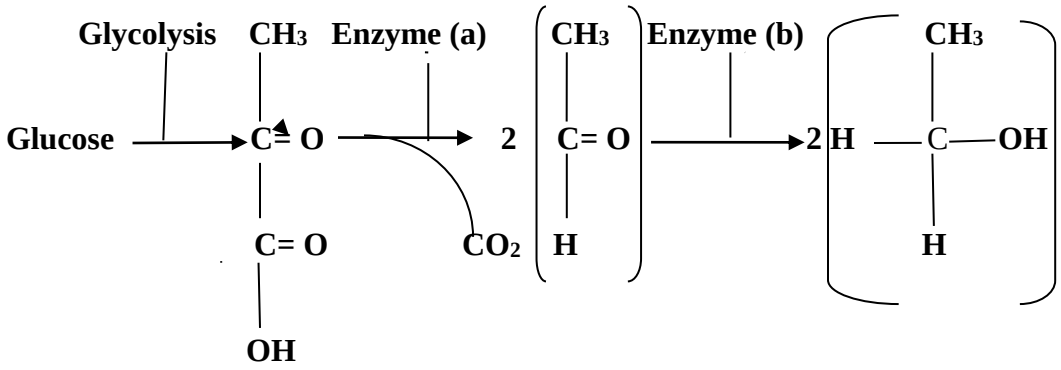
1.4.3. Fermentation

Fermentation is a biochemical reaction that breaks down carbohydrates to various products such as alcohol that involve the use of microorganisms (Martínez-Espinosa, 2020) which have various commercial and domestic uses. Glycolysis is a process in fermentation that breaks down glucose molecule to give two pyruvate molecules (Chaudhry & Varacallo, 2020).

Fermentation is an important process used in improving nutritional value. For example it is used to lower anti-nutrients in cereal food (Budhwar, Sethi & Chakraborty, 2020). Some bacteria such as *Escherichia coli* can ferment glucose and co-ferment xylose and glucose to ethanol (Fernández-Sandoval, Galíndez-Mayer, Bolívar, Gosset, Ramírez & Martinez, 2019). Lactic acid bacteria are involved in improving dairy products through fermentation (Mathur, Beresford & Cotter, 2020). Industrial wastes such as potato wastes can be used as biomass for yeasts, enzymes and organic acids, therefore allowing reduction in environmental pollution (Kot *et al*., 2020). The products of anaerobic respiration include alcohols, organic acids and also hydrogen gas (Darwin, Cord-Ruwisch & Charles, 2018). The fermentation products that are useful include ethanol (Zhang & Lis, 2020) and hydrogen as potential fuels (Sarangi & Nanda, 2020) lactic acid is used in bioplastic production (Morao & de Bie, 2019).

The Acetone, Butanol and Ethanol production by *Clostridia* could be done using optimized conditions. Acetone, butanol, and ethanol can be produced using lignocellulose (Molina-Guerrero, Valdez-Vazquez, Sanchez, Vazquez-Castillo & Vanzquez-Nunz, 2020). Butanol is a promising biofuel alternative compared to ethanol and methanol an idea supported by (Birgen, Durre, Preisig, & Wentzel, 2019).

Figure 1.3, show a summary of the processes involved in ethanol fermentation in which two molecules of ethanol is formed from one glucose molecule.



Pyruvic acid Acetaldehyde Ethanol

Key: Enzyme (a) Pyruvate decarboxylase; Enzyme (b) Alcohol dehydrogenase.

Source; http://www.themadscienceblog.com/2013/05/biology-and-beer.html

Figure 1.3: Ethanol fermentation from glucose

1.4.4. Production methods and challenges

Bioethanol is mainly produced from food crops and it is the transition to the advanced generations of bioethanol for example non-food crops which are required to solve the challenge of sustainability (Susmozas *et al*., 2020). The lignocellulosic ethanol is more expensive than fossil fuel due to complex and expensive conversion processes (Rosales-Calderon & Arantes, 2019). Second-generation biofuels are derived mainly from lignocellulosic feedstock which can generate great amount of energy in a sustainable and environmentally friendly way (Miskat *et al*., 2020).

1.4.5. Economic viability of some feedstock

Bioethanol production from food-based biomass shows the negative impact on the food market and the agricultural sector. The food safety should be considered when using food products for biofuel production because food shortage can result when such consideration is not done (Brinkman *et al*., 2020). Lignocellulose based feedstock are promising bioethanol raw material because they are of low cost and readily available, however their hydrolysis is expensive, therefore there is a need for a biorefinery strategy and high value chemical from lignocellulose (Rosales-Calderon & Arantes, 2019).

Reduction in ethanol cost requires critical improvement of source of heat energy and reduction in enzyme cost (Cheng *et al*., 2019). The integration of distillation with other methods lowers distillation cost (Zentou, Abidin, Yunus, Awang, Biak & Korelskiy, 2019). Fermentations by *S. cerevisiae* in sea weed hydrolysate produce low ethanol levels which makes the process uneconomical (Kostas, White & Cook, 2020). Crop residues are part of second generation biofuel which can generate sustainable and eco-friendly bioethanol because they are available in large quantities (Miskat *et al*., 2020).

Marine algae are of high potential for bioethanol production because of their high growth rate and do not require land for their cultivation therefore sparing land for crops. However their application is limited by expensive pretreatment cost and nitrogen supplements used during fermentation (Sulfahri, Dirayah, Alexandra, Asmi & Tassakka, 2020).

1.5. Statement of the Problem

Despite being a more attractive, renewable and environmentally friendly source of energy than fossil fuels, high cost has hindered bioethanol production. First generation bioethanol is expensive because it is produced from food crops, which are costly and they account for more than half of its cost. In addition, the use of food crops in bioethanol production would cause food insecurity a situation that increases their cost further, hence there is need to search for new source of starch for production of bioethanol.

The cost of bioethanol is exacerbated by lack of efficient yeasts that can produce ethanol of higher concentration compared to current industrial yeast (*Saccharomyces cerevisiae*). Yeast that produces ethanol of higher concentration would reduce the cost of production. Yeasts that are not efficient produce low ethanol concentration that would be economically unsustainable. High quantity of fermentation enzymes leads to high ethanol concentration which is cheap to extract from broth. There is need to investigate yeast(s) that can produce high concentration of bioethanol.

1.6. Justification

There is increase demand for alcohol based sanitizers currently because of Covid 19 disease. There is necessity to seek alternative feedstock to produce cheap bioethanol because its current high cost is an obstacle to its production. Currently in Kenya bioethanol is produced from sugarcane molasses which is first generation feedstock that increases the cost of bioethanol due to the high cost of food crops. This displaces sugar production causing food insecurity. The use of lignocellulosic feedstock is challenging

due to high cost of pretreatment despite the fact that they are readily availability. Therefore it is necessary to research on other non-food crops for bioethanol synthesis.

The production of bioethanol uses industrial yeast strain *S. cerevisiae*. Kasavi *et al*., (2012) concluded that using yeasts that yields high ethanol concentration reduces the cost of production. Therefore it is also necessary to investigate the fermentation abilities of potential wild yeasts in comparison to commercial yeast *Saccharomyces cerevisiae*.

1.7. Objectives of the Study

1.7.1 General Objective

The overall objective is to investigate a novel starchy plant and some yeasts for application in the production of cheap bioethanol.

1.7.2. Specific objectives

The specific objectives of the study are to:

i) Culture and characterize yeasts from finger millet (*Eleusine coracana)* malt.

ii) Evaluate temperature, ethanol and glucose tolerance of isolated yeasts verses commercial yeast (*Saccharomyces cerevisiae*).

iii) Determine reducing sugar levels in maerua shrub (*Maerua subcordata*) in comparison to cassava (*Manihot esculenta*), sorghum *(Sorghum bicolor*), maize (*Zea mays)* flour and sugarcane molasses.

iv) Identify most efficient ethanol producer amongst maerua shrub, cassava, sorghum, maize and sugarcane molasses when fermented using yeasts.

v) Compare the ethanol productivity from isolated yeasts verses *Saccharomyces cerevisiae* when they ferment maerua shrub and other sugar sources.

1.8. Research Questions

i) Does finger millet malt contain yeasts?

ii) Are the yeasts in finger millet malt more tolerant to temperature, ethanol and glucose than commonly used commercial yeast?

iii) What is the reducing sugar concentration in Maerua shrub relative to other sugar sources?

iv) Which plant is the most efficient ethanol producer when various yeasts species are used to ferment?

v) Does the yeasts species in finger millet malt fermentation produce higher ethanol productivity compared to commonly used yeast types?

CHAPTER TWO

LITERATURE REVIEW

2.1. Biofuels

Biofuels are energy sources that originate from biomass, they include solids, liquids and gases. Bio-alcohols, biogas, bio-oils and biohydrogen are examples of biofuels. All four generations of biofuels ranging from first to fourth generation are conventional sources of biofuel (de Souza, 2020).

First generation employ methods that use food crops to produce various forms of biofuels, however it is associated with many disadvantages such as food insecurity because it competes with food supply unlike other generations (Kurowska, Marks-Bielska, Bielski, Kryszk & Jasinskas, 2020).

The second generation biofuels involves utilizing non-food crops such as lignocelluloses, industrial wastes, forestry residues and agricultural feedstock, one of the challenges of using this generation of biofuels is abiotic stress which necessitates the use of advanced technology so as to meet commercial standards (Ozsoz, Ibrahim & Coston, 2019).

Third generation biomass is from algae and are suitable for biofuel production because of high carbohydrates and lipid levels. The algae include microalgae and macroalgae in which they are all potential biofuel feedstock. Microalgal feedstock produces third generation biofuels (Sadatshojaei, Wood & Mowla, 2020).

Cyanobacteria can produce third and fourth generation biofuels, fourth generation biofuels involve production from genetically engineered feedstock (Farrokh, Sheikhpour, Kasaeian, Asadi & Bavandi, 2019). Microalgae remove CO_2 through the process of photosynthesis which involves sequesterization of CO_2 therefore reducing global warming (Molazadeh, Ahmadzadeh, Pourianfar, Lyon & Rampelotto, 2019).

2.2. Bioethanol extraction approaches and distillation

Azeotropic distillation is important in maximizing yields and minimizing waste for environmental and economic sustainability (Valentini & Vaccaro, 2020). Azeotropic distillation is an effective separation technique because of high separation capacity and simple to apply, however the challenge is the high cost of energy used in the process (Zentou *et al*., 2019).

Pervaporation is a process which is economically competitive for industrial applications because it is cost effective compared to molecular sieves (Amornraksa, Subsaipin, Simasatitkul & Assabumrungrat, 2020).

Fluidic oscillator is a process that injects hot air microbubbles causing evaporation, the microbubbles flow upward into the mixture. The fluidic oscillator consumes lower energy, in comparison to other microbubble methods because of reduced size of bubbles with insignificant increase in energy (Desai, Hines, Riaz, & Zimmerman, 2018).

Bioethanol production becomes economically viable when production costs are reduced, therefore there is a necessity of using a cheaper method of separation (Susmozas *et al*., 2020). The challenge of fermentation is the accumulation of bioethanol in the

fermentation reactor that affects cell membrane of yeasts cell (Eardley & Timson, 2020) and hence inhibits the activity of microorganism. A way of overcoming this problem is integrating coupled systems to recover bioethanol from broth and control inhibition (Zentou *et al*., 2019).

2.3. Current trends on bioethanol production

2.3.1. Advances in bioethanol feedstock

The macroalgae are also candidates for bioethanol and biobutanol production because they contain high carbohydrate content and little or no lignin (Dave, Selvaraj, Varadavenkatesan & Vinayagam, 2019). The challenge associated to sea weed cultivation is ensuring that the carrying capacities are within limits and distribution within a species relates to same altitudes to maintain conservation (Campbell *et al*., 2019).

The factors to consider when choosing microalgae strains for bioethanol production are the carbohydrate concentration and cost effectiveness of pretreatment method. Merit of using microalgae is high production rate, however the challenge is the high cost of cultivation (Silva & Bertucco, 2019).

2.3.2. Economic prospects in Kenya and beyond

Some countries have produced bioethanol from lignocellulosic biomass, however in Kenya little investigation has been done and applied (Otieno & Ogutu, 2020). Studies on sustainable ethanol production and use in Kenya were undertaken since 2005 (Dalberg,

2018). It was found that for Kenya to produce bioethanol from molasses the problem of GHG emissions and the high energy requirement than its energy content should be solved for its production to be viable (Mbothu, Mutwiwa, Eshton & Abu-bakar, 2018).

The baggasse from sugarcane and sweet sorghum developed by Kenya Sugar Board and ICRISAT, are ideal crops to be cultivated to produce bagasse for bioethanol feedstock (Machandi, Gathitu & Kihoro, 2013).

Bioethanol manufactured and marketed for cooking is taxed as alcoholic beverages so reduction on taxation would improve sustainability and use of bioethanol (Dalberg, 2018)

The test on the use of bioethanol succeeded considerably in most countries like Madagascar, Ethiopia and Nigeria (Lambe, Jüriso, Wanjir & Senyagw, 2015). The success in Brazil and USA was because of the government's dedication to provide support for the sugar industries and/or for fuel blending programmes, also establishment of institutional frameworks and formulation of favourable policies.

2.3.3. Challenges of bioethanol production

The physiological stresses that affect yeasts during fermentation are high temperature (35-45 ^{o} C) and ethanol concentration over 15 % (Ivit, Longo & Kemp, 2020). *S. Cerevisiae* does not grow in media containing concentration above 14 % ethanol which leads to inhibition (Kechkar *et al*., 2019). Stress tolerant yeasts improve ethanol production and lowering the overall cost. Thermotolerant yeast isolates lowers the cooling cost and reduces contamination during fermentation (Ndubuisi *et al*., 2020).

The other challenge is the capability of the yeasts to utilize pentose during fermentation, because yeasts such as *S. cerevisiae* are unable to ferment pentose sugar to bioethanol (Martins *et al*., 2018). Genetic engineering and co-culturing of yeasts are ways of solving problems of yeast fermentation. Genetic engineering uses DNA technology in up-regulating genes that are tolerant to various inhibitory conditions (Wang *et al*., 2020).

Ethanol production using cellulosic material is stagnating because of its technical difficulties and high cost of production, despite it being a promising biofuel producer (Padella, O'Connell & Prussi, 2019).

2.4. Review of some experimental methods

2.4.1. Culturing and isolation of yeast

Fungi have a wide range of habitats and numerous survival mechanisms (Hyde *et al*., 2019). YPD medium and enrichment media uses chloramphenicol for inhibiting bacterial growth. The media commonly used for culturing include potato dextrose agar, malt extract agar, and sabouraud dextrose agar with antibiotics to inhibit bacterial growth (Black, 2020). The colonies with distinct features are sub-cultured on media supplemented with antibiotic to obtain pure isolates (Shi, Qiu, Wang, Zhang, Wang & Sun, 2020).

2.4.2. Yeast characterization

The standard morphological and physiological tests are used in yeast characterization. The tests include morphology, surface characteristics, presence of pseudomycellium,

ascospore formation, vegetative reproduction and growth in 10 % NaCl +50 % yeast extract. Biochemical fermentation tests include the sugar maltose, glucose, sucrose and lactose (Monapathi, Bezuidenhout & James, 2020).

Purification of yeast colonies involves continuous sub-culturing on potato dextrose agar + chloramphenicol slants incubated for 48hours and stored at a temperature of 8 ^{o} C (Black, 2020). Morphological characterization of the cells involve preparation of a thin smear of isolate on a clean slide mounted in a drop of cotton blue in lactophenol and a cover slip placed on the slide and observed under a microscope (Patra, Das, Das & Thatoi, 2020). Physiochemical characterization involves investigating growth in Yeast dextrose with minimal medium on glucose concentration and temperature (Prado *et al*., 2020). CHROM agar Candida is a medium based on species-specific enzyme activity, used to isolate and identify Candida species (Surain & Aggarwal, 2019). Classification of yeasts involves the use of morphological, physiological and biochemical characteristics of yeasts.

2.5. Yeasts

2.5.1. *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is superior to other microorganisms in some physiological characteristics regarding ethanol production in industries. *S. cerevisiae* is a commonly used yeast for ethanol fermentation and has high ethanol tolerance compared to other yeasts (Ruchala, Kurylenko, Dmytruk & Sibirny, 2020). *S. cerevisiae* is safe for

consumption and is widely used in biofuel production because it is amenable to systems and synthetic biology tools (Oh & Jin, 2020).

Co-culture involves growing two yeasts in a reactor which help improve ethanol production, productivity and yields while pure cultures show lesser ethanol yields (Naseeruddin, Suseelendra & Venkateswar, 2019).

Wild yeast *S. cerevisiae* KL17 produced ethanol concentration of 96.9g/L because it can simultaneously ferment glucose and galactose (Kim, Ryu, Huh, Hong, Kang, & Chang, 2014) an indication that natural yeasts can produce high ethanol concentration from sugars. Some wild yeasts of the strain *S.cerevisiae* are novel strains because of their high fermentation kinetics and ability to ferment in harsh conditions. This findings show gaps for research on ethanol production by wild yeasts because of presence of various yeasts in different habitats.

It was found that microbiota connections and intraspecific distribution is related to similar altitudes (Moreira, Erica, Vieira, Silveira, Silveira & Martins, 2020). Genomic studies of wild yeasts have been in the increase because of understanding of their ecology and phylogeography (Libkind *et al*., 2020).

Glucose is most abundant sugar contained in lignocelluloses followed by xylose in biomass (Zhao, Xian, Liu & Zhao, 2020). *Saccharomyces cerevisiae* poorly utilizes D-xylose and L-arabinose (Nijland & Driessen, 2020). *S. cerevisiae* is a preferred biocatalyst due to its fermentation capacity, adaptability to adverse osmotic pressure and

pH (Parapouli, Vasileiadis, Afendra & Hatziloukas, 2020). Yeast fermentation ability is greatly determined by the environmental factors, as well as genetics and ecological niche.

2.5.2. Non-saccharomyces yeast

Non-*Saccharomyces* yeast improves the freshness and quality of wine, the selection of yeast strains is a very important factor in obtaining freshness in wine (Morata, Escott, Loira, Del Fresno, González& Suárez-Lepe, 2019). Some non-*Saccharomyces* yeast cannot cope with fermentation conditions in wine as compared to *S.cerevisiae*. *S. cerevisiae* strain takes shorter time to consume sugar especially in orange wine when compared to non-*Saccharomyces* yeasts. Non-*Saccharomyces* yeast fermentations have characteristics such as higher concentration of residual sugar and lower ethanol levels (Hu, Wang, Ji, Liu, Chen & Zhang, 2018).

2.6. Review of feedstock plants

2.6.1. Maerua shrub (*Maerua subcordata*)

Plate 2.1: Picture of Maerua shrub (*Maerua subcordata*) (Source: Author, 2021)

Maerua subcordata is a wild shrub which belongs to the family *Capparaceae* (Hiben *et al*., 2020) found in arid and semiarid land of East Africa, especially in burned regions of grassland (Strauch & Eby, 2012). The plate 2.1 above shows a picture of maerua shrub.

The shrub has root tubers which the locals use for treatment of turbid water (Megersa, Beyene, Ambelu & Woldeab, 2014). *Maerua subcordata* root tubers contain macromolecules of starch (300 mg/mL), proteins and mineral salts. Starch is composed of a highly branched macromolecule called amylopectin which is 70-80 % and amylose 20-30 % (Mavura, Chemelil, Saenyi & Mavura, 2008). *Maerua subcordata* is shrub under research to investigate its potential to be a second generation bioethanol source. The plant

has presence of significant amount of amylopectin and amylose. It is a non-food source because of the presence of mild toxic substances.

2.6.2. Sorghum (*Sorghum bicolor*)

Sorghum is a staple food of the poorest communities of the world, therefore monitoring the factors that affect its production is important for food security (Mundia, Secchi, Akamani & Wang, 2019). Sweet sorghum is more advantageous than other varieties because it produces grains for human consumption and juice for ethanol. The bagasse of sweet sorghum is used as silage for livestock feed (Dong, Li, Xu, Wang, Chen & Li, 2020) this role competes with bioethanol production from bagasse. The commercial viability of sweet sorghum for bioethanol production is determined by high sugar containing varieties, transportation costs and storage (Kanakaraju, Uma, Vani, Kumari, Srindar & Umakanth, 2020).

Sorghum is a promising biofuel source because of higher biomass production and wide adaptation. The sweet sorghum juice does not have inhibitory substances that hinder yeast growth, however mineral elements such as nitrogen and phosphorus are necessary for optimum growth of yeasts (Volodko, Ivanova, Kulichkova, Lukashevych, Blume & Tsygankov, 2020).

Sebayang *et al*., (2017) obtained the reducing sugar to be 175.94 g/L, a value almost similar to experimental value (174.29 g/L) and the ethanol content obtained from sorghum starch using *Saccharomyces cerevisiae* at suitable conditions was 82.11 g/L, which is nearer the average value from experiments (81.52 g/L).

2.6.3. Cassava (*Manihot esculenta*)

Cassava is a crop that is grown widely, a carbohydrate source, providing daily food to millions of people in Africa (Szyniszewska, 2020). Cassava is a drought resistant crop that grows in regions with low nutrient availability. The high ethanol concentration from cassava was (110.88 g/L) makes it a suitable biomass for bioethanol source (Adeleye, Sharafadeen, Mobolaji, Olusegun & Abideen, 2020).

Agricultural wastes from crops such as cassava peels are available and cheap therefore they can be used as biofuel feedstock. Sustainable bioethanol production from cassava depends on obtaining energy from by-products such as stems and leaves in comparison to yields from single product (Pabon-Pereira, Slingerland, Hogervorst, van Lier & Rabbinge, 2019).

2.6.4. Maize (*Zea mays*)

The use of maize as biofuel feedstock causes a rise in prices of maize and its products such animal feeds (Lee, Featherstone, Nayga & Han, 2019).

Ethanol concentration from maize ranges between (122.6 - 126.9 g/L), the variation is caused by differences in amylose content which cannot be hydrolysed efficiently by the enzyme (Pradyawong, Juneja, Sadiq, Noomhorm & Singh, 2018).

Maize can be used in the production of ethanol for fuel and other valuable products. The starch fermentation produces substances that are used in the feed industry. Cellulosic

ethanol is synthesized from lignocellulose, a substance that is resistant to enzyme degradation (Pandey, Shrestha, Khanal, Adhikari, & Kunwar, 2019).

2.6.5. Sugarcane (*Saccharum officinarum*)

Sugarcane produces various products such as sugar, molasses and residues for bioethanol production. Sugarcane molasses is a cheaper feedstock because of its high sucrose levels. The bioethanol concentration obtained from molasses at optimum conditions was reported as 11% (v/v) which is acceptable level of ethanol in industries (Darvishi & Abolhasan, 2019). Sugarcane bagasse is renewable and can produce bioethanol, because of its high amount of cellulose. Sugarcane bagasse is reported to have produced ethanol of an average of 9.07% which show improvement in its production and positive impact on the economy (Portero-Barahona, Mayorga, Martín-Gil, Martín-Ramos& Barriga, 2020).

Sugarcane molasses produce first generation biofuel which are used as food and animal feeds (Tan, Pongsathon, Chee, Lai, Abu-Bakar & Paramasivam, 2019). Rasmey, Heba, Omar, Abdul and Akram, (2018) obtained ethanol content of 9.55% from fermentation of sugarcane molasses. The low ethanol concentration from sugarcane molasses could be caused by the low minerals content and high sugar levels.

2.7. Extraction and Analysis

2.7.1. Reducing sugar analysis

Reducing sugar is sugar with aldehyde or ketone group. Sugar concentration is assayed using copper reduction method (Gandhi, Bankar, Vishwakarma, Satpute & Upkare, 2017). 3, 5- Dinitrosalicylic Acid method is another method that can be used to detect the carbonyl (C=O) functional group. Concentration of fermentable sugars is determined using a High Performance Liquid Chromatography device due to its separation system and rapid analysis of monosaccharides, disaccharides and oligosaccharides in food (Crha & Pazourek, 2020).

2.7.2. Ethanol extraction methods

Distillation is a widely used technique in separation of bioethanol, however, the challenge is its high cost (Zentou *et al*., 2019). Combining distillation with other methods reduces the cost of distillation and improves the separation efficiency of the alternative techniques (Gao, Wang, Li, Xie, He, & Wang, 2019). The specific gravity of a distilled solution of ethanol is determined by weighing using load cell and the volume is measured by ultrasonic sensor, then specific gravity is calculated from the mass and volume (Susanti, Rohman, Rusmin, & Pristianto, 2019). The other method that is fast, simple and precise is gas chromatography (Mihretu, Gebru, Mekonnen, Asgedom & Desta, 2020).

The alternative method of extracting bioethanol is solvent extraction by use of a solvent such as (Tri-n-butyl phosphate) and determination of concentration in aqueous solution by dichromate oxidation (Erguden, 2019).

2.7.3. UV-Vis spectrophotometric analysis of ethanol levels

UV/visible spectrophotometric method are suitable to small amounts of samples (Battu, Gandu & Nenavath, 2020). Solvent extraction - dichromate oxidation and detection using spectrophotometer is an appropriate method to quantitatively measure ethanol in fermented samples. The reaction takes place as shown in the equation below.

$$3CH_3CH_2OH + 2Cr_2O_7^{2-} + 16\ H^+ \longrightarrow 3CH_3COOH + 4Cr^{3+} + 11\ H_2O \qquad (1)$$

Spectrophotometric methods based on oxidation of ethanol with dichromate are advantageous because of its low cost (Sriariyanun, Mutrakulcharoen, Tepaamorndech, Cheenkachorn, & Rattanaporn, 2019).

CHAPTER THREE

MATERIALS AND METHODOLOGY

3.1. Requirements

3.1.1. Chemicals and Reagents

The following chemicals and reagents were used in this study; Absolute ethanol (AR), Sulphuric acid (1.84 g/cm^3) (AR) (Griffchem Fine chemicals), Fungal alpha amylase (Loba chemie), Potato dextrose agar (Titan Biotech LTD), Potassium dichromate AR (Griffin &George). The other chemicals and reagents were Sodium hydroxide pellets, Zinc sulphate, Barium hydroxide, Yeast (*Sacchromyces cerevisiae*), D-Glucose monohydrate, Hydrochloric acid (1.18g/cm^3), Methylene blue, Yeast extract, Peptone (AR), Copper sulphate (AR) and Potassium sodium tartrate (AR)

3.1.2. Apparatus

The apparatus include: 50 mL burette, 1 mL and 25 mL pipettes, 10 mL and 100 mL measuring cylinder, 250 mL Erlenmeyer flasks, Thermometer (-10 to 110 ^{o} C), Fractionating column, Liebig's condenser, Boiling tubes, 60 mL plastic containers and Teat pipette.

3.1.3. Equipments

UV-Visible spectrophotometer (Spectroscan 30, UK), Rotary shaker, pH meter (Hanna), Autoclave, Incubators (Carbolite), Centrifuge (Hettich), Oven (Mettler), Weighing

balance (Ohaus). Mantle (Winkler), Hot plate (Labtech), Cuvettes (1cm path length), Shaker incubator (Aerotrom) and Compound light microscope (CETI).

3.2. Sampling area for Maerua shrub

The plants were collected from Kaplelwo village, Emining division, Mogotio subcounty in Baringo County. The area lies at about 0^{o} 28' N and 35^{o} 58'E. The altitude is 1067 metres above sea level, with annual rainfall of 512 mm occurring in two seasons (Ezenwa, Omondi, Nwagbara, Gbadebo, & Bada, 2018). The sampling area has various indigenous plants such as acacia plants, maerua shrub, *Aloe vera* and many others plants. The residents use wild yeasts from roots and fruits of some plants for beer and wine brewing. The study area was chosen because of wide spread use of wild yeasts which elicit scientific research and also availability samples.

3.3. Sample collection and identification

Samples were collected randomly from farmers who had the required crops and were willing to give out their crops for investigation and identified by a botanist from University of Eldoret. Crops from a specific variety were collected from farmers. Maerua shrub root tubers were chosen because it contains high concentration of starch than the other parts of the plant. Roots of maerua shrub were dug out from indigenous forest after getting consent from the area chief. About 4 Kg of each fresh cassava and maerua shrub tubers were weighed and washed in clean fresh water. A sample of about 500 g finger millet grains was purchased from a farmer. The yeast species have been in the study area for longer period of time and are wild residents of finger millet (Aljohani,

Samarasinghe, Ashu & Xu, 2018) therefore expected to be well distributed in the study area because of similarity in ecological conditions. About 2 L sugarcane molasses in plastic container was purchased at an agrovet in Eldoret town. Exactly 100 g standard yeast was supplied by Agrochemicals Company of Kenya at Muhoroni. About 1 kg of maize, sorghum, cassava and maerua shrub, 500 g of finger millet, 2 L sugarcane molasses, 100 g of commercial yeast were required for laboratory procedures.

3.4. Sample Preparation

Root tubers of Maerua shrub and cassava, also grains of maize, finger millet and sorghum were collected from various areas within the study area, the plant parts were chosen because they contain high concentration of starch. Tubers were cleaned using tap water, peeled, chopped to cubes of about 5 cm x 5 cm, then together with sorghum and maize they were dried in the sun for about 7 hours a day for two days. They were taken to the laboratory where they were oven-dried at 80 ^{o}C for 3 days (Janket *et al*., 2020). Maerua shrub, maize, sorghum and cassava samples were ground using electric grinder with 1 mm mesh sieve.

Exactly 500 g of finger millet grains were mashed in 1 liter of distilled water in a plastic bucket for 24 hrs. The water was drained off and kept in perforated bag to germinate at 30 ^{o}C for 72 hours in an incubator. Sprouting millet was spread on a tray and dried on the sun for 6 hours. Dry finger millet malt were ground to powder in an electric grinder with 1 mm sieve, it is stored in air tight dry clean plastic containers.

3.5. Yeast isolation from millet malt

3.5.1. Yeast isolation

Exactly 1 g of commercial yeast (*S. cerevisiae*) was placed in 50 mL sterile 2 % dextrose solution to activate the yeast, then the ground finger millet malt was placed in sterile 50 mL distilled water, both were in two different 100 mL beakers which were sealed, labelled as (FM) for finger millet malt and (SC) for commercial yeast then incubated at 30 ^{o}C for 24 hours (Yuma, 2020).

Exactly 9.75 g of PDA was weighed, dissolved in distilled water to make 250 mL of media, was autoclaved, then 3 mg of streptomycin was added to inhibit bacterial growth (Waters &Tadi, 2020). After 24 hours, 0.1mL of (FM) and (SC) were serially diluted by 10^{8} folds then placed on PDA for 48 hours, incubated at 35 ^{o} C. The plates were also labelled (FM) then (SC) in triplicate.

3.5.2. Purification

Colonies of two isolated yeasts from (FM) having white and creamy colour were streaked on three PDA plates using a sterilized loop. The plates were sealed to avoid contamination, labelled in triplicates as QY1 with white yeast colony, QY2 with creamy yeast colony of isolated yeast and QY3 that was (SC), incubated for 48 hours in an incubator maintained at temperature of 35 ^{o} C (Mateus, Sousa, Coimbra, Rogerson, & Simões, 2020). Colonies of purified cultures were observed, aseptically cultured on PDA slants then incubation at 30 ^{o} C for exactly two days and then kept in a refrigerator

maintained at 4 ^{o} C (Yuma, 2020). All procedures were performed under sterile conditions.

3.6. Yeast characterization

3.6.1. Morphological characterization

The isolated yeast QY1, QY2 and commercial QY3 on PDA plate were identified using morphological characteristics such as colony colour, nature, appearance, elevation and margin.

3.6.2. Microscopic characterization

These were done by mixing yeast colony with two drops sterilized de-ionized water on a slide then dried (Karki *et al*., 2017). Lactophenol cotton blue stain was used to stain the specimens, dried, viewed using a microscope (Santana *et al*., 2018).

3.7. Physiological characterization

A loop full of yeasts was inoculated into 50 mL broth media in 100 mL conical flasks labelled Y1, Y2 and Y3, shaken at 150 rpm and 30 ^{o} C, for 48 hours which is optimum conditions for yeast growth. Yeast strains can grow well in substrate with pH between 3.0- 6.0 and temperature range of 30 – 35 ^{o} C The YEPD broth contained, yeast extract, 3 g; peptone, 10 g; dextrose, 10 g ; water 1L; pH of the broth was 6.0.

3.7.1. Determination of temperature tolerance

Exactly 2 ml of YEPD broth containing isolated yeasts strains and reference strain *Saccharomyces cerevisiae* were inoculated to 20 mL YEPD broth in Erlenmeyer flasks labelled TY1, TY2 and TY3. It was then incubated at 25, 30, 35, 40 and 45 ^{o}C statically for 48 hours. Cellular growth was measured using optical density from a UV- Visible Spectrophotometer (Spectro scan 30, UK) maintained at 600 nm (Gong, Yang, Yang & Gu, 2020), path length of the sample in cuvette was 1cm. YEPD broth was used as blank.

Mathematical expression of Beer Lambert law is

$$\boldsymbol{O.D} = \frac{\boldsymbol{A}}{\boldsymbol{L}} \qquad \textbf{(2)}$$

Key: *O.D.* = optical density, *A*=absorbance, *L*=path length of sample.

Therefore values obtained from the instrument were optical densities in absorbance units per centimetre, Au/cm.

3.7.2. Determination of ethanol tolerance

The effect of ethanol concentration on growth of yeast strains were tested by inoculating exactly 2 ml of broth culture of each strain in Erlenmeyer flasks labelled RY1, RY2, and RY3 with 20 mL YEPD broth containing 6, 9, 12, 15, 18 % alcohol (v/v) in triplicates (Alabere, Ogbonna & Williams, 2020). The bioethanol concentration obtained at optimum conditions is 11% (v/v) which is acceptable level of ethanol in industries (Darvishi & Abolhasan, 2019), therefore it is necessary to investigate ethanol tolerance within and near this concentration. Flasks and their contents were in an incubator whose

temperature was maintained at 30 ^{o} C and shaken for 48 hours, these are optimum conditions for incubation. Optical density of 2 mL of the initial inoculums and the samples were determined after 48 hours using UV-visible spectrophotometer at 600 nm. YEPD broth was used as blank.

3.7.3. Determination of glucose tolerance

The isolated yeasts and *Saccharomyces cerevisiae* were tested for their growth in 20 mL YEPD broth (in triplicates) containing 10, 20, 30, 40, 50 % glucose concentration. The optimum glucose tolerance for most yeasts range 20-30 % therefore it was important to investigate glucose tolerance around this range. Yeasts to be used in industrial production of bioethanol should show marked tolerance to glucose. The flasks were labelled SY1, SY2 and SY3. Exactly 2 mL of samples containing yeast cultures were inoculated in triplicate and shaken at 150 rpm at 30 ^{o} C for 48 hrs. Optical density of 2 mL of the initial inoculums and the samples were determined after 48 hours using UV-visible spectrophotometer at 600 nm and YEPD broth was used as blank.

Optical density of each experiment were obtain by getting the difference between optical density after 48 hours of experimental samples and initial optical density then mean values were considered. Formula (3) below was used in getting the values.

$$\boldsymbol{O.D = Final\ O.D - Initial\ O.D} \qquad \textbf{(3)}$$

3.8. Reducing sugar concentration

3.8.1. Standardizing Fehling's solution

Fehling's solution was standardized by transferring accurately measured 5.0 mL of each Fehling's solutions, were titrated with glucose solution. Three standard solutions were prepared by dissolving 2.5 g of glucose to make 250 mL solution in volumetric flask. Titration was done at about 80 ^{o} C until the colour of methylene blue disappears this is modified method by (Owuama & Owuama, 2021). Titration was repeated three times to find out whether the results are reproducible then results were tabulated.

3.8.2. Determination of reducing sugar concentration

Reducing sugar levels in the plant samples was necessary to compare the contents of Maerua shrub to that of Cassava, Sorghum, Maize and Sugarcane molasses because it is a main factor in ethanol production. Triplicate 250 mL Erlenmeyer flasks were labelled A - E then 25 g of the samples were placed as follows; A Maerua shrub, B Cassava, C Sorghum, D Maize, E Molasses. Exactly 120 cm^3 of water was added, autoclaved for 15 minutes then cooled. To hydrolyse the samples they were treated with 1 g of α- amylase and pH was optimized to 6.0 using 0.1M NaOH. Incubation was done at 30 ^{o} C for 48 hours, then filtration using filter paper and filter funnel. Exactly 20 mL of the filtrate were deproteinized by adding 5 mL of each 0.1M Zinc sulphate and 0.1M Barium hydroxide (Geisler, *et al*., 2020), diluted with 10 mL of distilled water then filtered. Exactly 5.0 mL each Fehling's solution, were measured and poured into 250 mL Erlenmeyer flask heated to boiling, then methylene blue was added. Titration was done

by drop wise addition of sample solution into a solution whose temperature was (70 - 80 ^{o} C) until the blue colour disappears. All experiments were done in triplicate. The volume and concentration of standard was used to calculate the concentration of the sample, considering the dilution factor.

3.9. Ethanol production and analysis

3.9.1. Experimental procedures

Accurately weighed 20 g of samples were placed in nine Erlenmeyer flask labelled A-Maerua shrub, B-Cassava, C-Sorghum, D-Maize and E-Molasses. Exactly 120 mL of distilled water was measured, poured into the flasks containing samples and stirred to soak completely. The level was marked, then sample were autoclaved at 121^{o} C. Water was added to initial mark.

Exactly 1g of α- amylase enzyme which was in excess and 1 g of yeasts obtained using procedures in Appendix 1, were added to each sample resulting in 5 % inoculums size, then fermentation were undertaken as indicated in Table 3.1 below. To obtain high ethanol concentration batch process SSF was used. The yeasts were labelled as; Isolated yeasts labelled Y1, Y2 and *Saccaromyces cerevisiae* (Y3). Each flask was covered with aluminium foil secured using a rubber band. They were incubated for 48 hrs at 35 ^{o} C these are optimum conditions for fermentation.

Table 3.1: Labels on flasks containing plant samples and yeasts

Plants \ Yeast	Y1	Y2	Y3
Maerua shrub (A)	AY1	AY2	AY3
Cassava (B)	BY1	BY2	BY3
Sorghum (C)	CY1	CY2	CY3
Maize (D)	DY1	DY2	DY3
S. molasses (E)	EY1	EY2	EY3

All experiments were done in triplicate and pH 5.0 because pH (5.0-5.5) minimizes contamination and maximizes ethanol production by yeast (Kanagasabai, Karuppaiya and Viruthagiri, 2019). Sieving was done using tea strainer to remove solid particles then residues were discarded.

Exactly 100 mL of sample filtrates were distilled using well assembled apparatus with thermostatic heater being a source of heat. Temperature was maintained at 85 ^{o}C for ten minutes to extract most of the vapour from the fermentation broth (Negera, 2017). The volume of distillate was measured using a measuring cylinder. The summary of procedures on fermentations and extraction of sample is indicated by the flow chart in Figure 3.1.

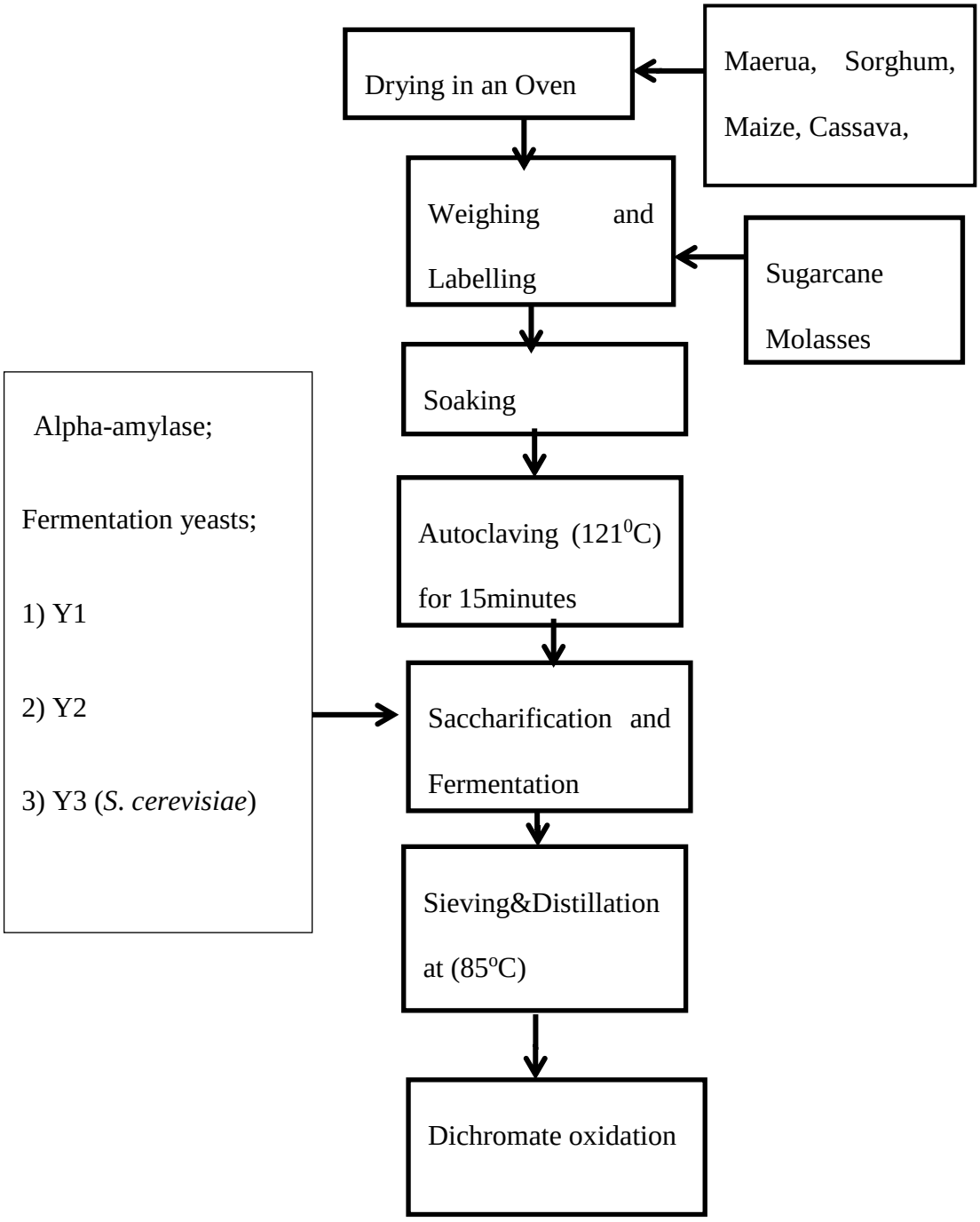


Figure 3.1: Flow chart on procedures of fermentation and extraction of samples

3.9.2. Preparation of standard solutions

Preparation was done by diluting 0.83, 0.73, 0.62, 0.52, 0.42, 0.31, 0.21 and 0.10 mL of absolute ethanol AR (96%) diluted in distilled water to make up 10 mL of solution making 8, 7, 6, 5, 4, 3, 2, 1 % ethanol concentration. At ethanol concentration above 8 % the values did not obey linearity in the correlation graph whose values were measured at 595 nm.

Exactly 2 mL of each solution was added and shaken with equal volume of dichromate reagent then determination of absorbance using spectrophotometer (spectroscan, 30). A calibration curve on absorbance against ethanol concentration (%) v/v was drawn.

3.9.3. Sample oxidation and Spectrophotometric analysis

Exactly 0.5 mL was added to 4.5 mL of water so as to lower the concentration to a measurable concentration of 1% to 8%. Accurately measured 2.0 mL of the resultant solution were shaken for 5 minutes with 2.0 mL, of 0.298 M acidified potassium dichromate solution prepared using procedures in Appendix 2. Then absorbance at 595 nm determined using spectrophotometer (Spectroscan, 30). The concentration was measured against a blank. The results were tabulated and concentration determined. Dichromate oxidation was appropriate in determining alcohol content an idea supported by (Sayyad, Chaudhari, & Panda, 2015).

3.10. Statistical analysis

Descriptive statistics (mean and standard deviations) was used to summarize the optical densities of yeasts, reducing sugar and ethanol concentration. In all cases the level of significance was $p < .05$.

One-way analysis of variance was applied in determining significant difference in optical densities for various yeasts, reducing sugar, ethanol concentration produced by maerua shrub, cassava, sorghum, maize and sugarcane molasses. It was used to test for significant difference of ethanol concentration produced by yeasts.

Statgraphics centurion XVI.I was used to carry out all the statistical analyses. LSD-Fishers least significant difference was used.

CHAPTER FOUR

RESULTS

4.1. Yeast isolation

The finger millet malt sample (FM) had two yeasts which were isolated based on the colony colour. The yeast that had white colony was labelled as Y1, while the one that had creamy colony was labelled Y2.

4.2. Yeast characterization

4.2.1. Yeast morphology

Yeast (Y1) had white colour of the colony, mucoid in nature, raised elevation, smooth margin and butyrous texture as in plate 4.1.

Yeast (Y2) had creamy colony colour, mucoid nature, smooth margin, butyrous texture and raised elevation as seen in plate 4.1.

Saccharomyces cerevisiae (Y3) had white colony on PDA, butyrous texture, mucoid in nature, raised elevation and smooth margin. Plate 4.1 is the image of colony from culture of Y3 as reference yeast.

Plate 4.1: Colonies of yeasts Y1, Y2 and *S.cerevisiae* (Y3) (Source: Author, 2021)

4.2.2. Microscopic characterization

The plate 4.2 show cellular pictures of yeasts as viewed using a light microscope at 100x these represent Y1, Y2 and Y3 respectively. The yeast cells in Y1, Y2 show multilateral budding which is similar to Y3.

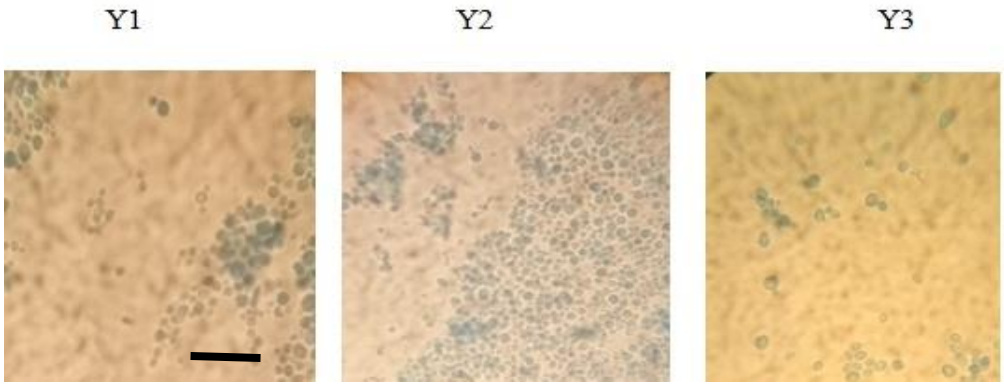


Plate 4.2: Microscopic observation of yeast cells under magnification of 1000 X

Both Y1 and Y2 have unicellular round cells with multilateral budding. Y3 (*Saccharomyces cerevisiae*) has oval shaped unicellular cells and multilateral budding. The two yeast Y1 and Y2 isolated from finger millet malt have microscopic characteristic

similar to Y3 (*Saccharomyces cerevisiae*) therefore these are *Saccharomyces* strain. The yeasts were characterized by a technician from University of Eldoret.

4.3. Physiological characteristics

The yeast physiological characteristics investigated include effect of temperature, ethanol concentration and glucose concentration on yeast growth. The results were presented in Figure 4.1 to 4.3.

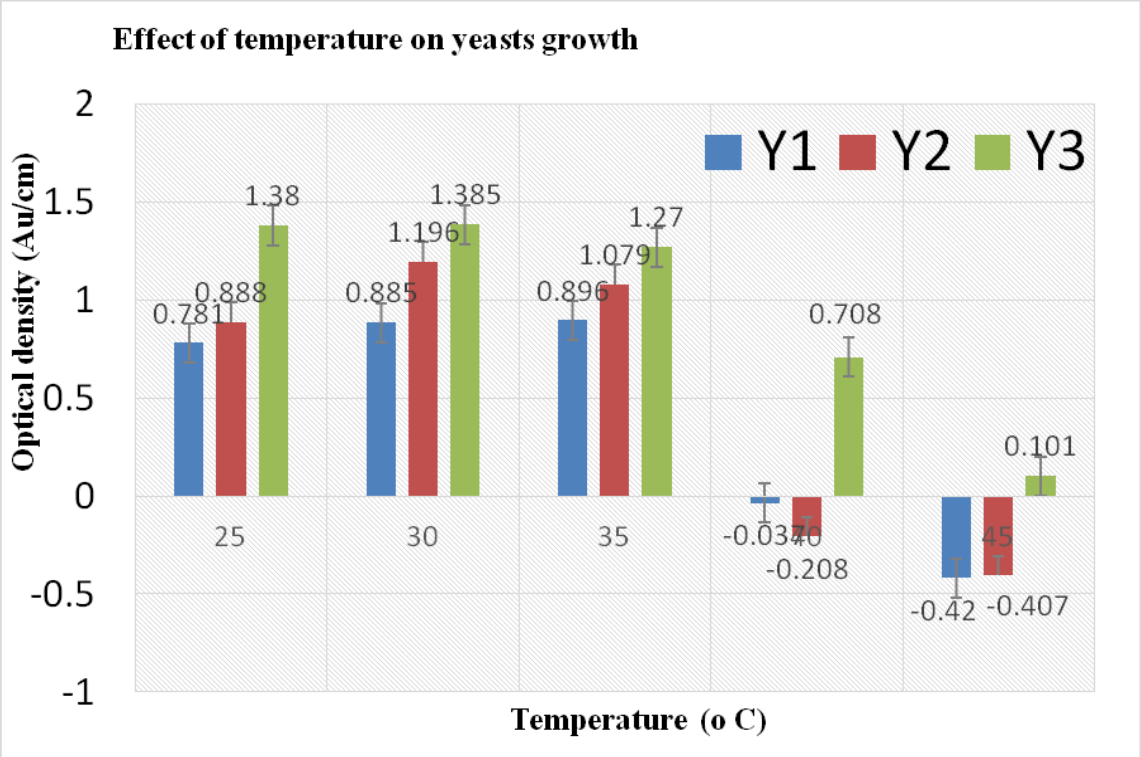


Figure 4.1: Effect of temperature on yeasts growth

The significant differences at $p < .05$ were obtained by comparing mean optical densities of various temperatures. The experiments are in triplicate (N = 3). The values are mean of optical densities (Au/cm). The overall mean of optical densities were used to compare significant difference. Mean values were Y1 (0.4201 ±0.5742a), Y2 (0.5097±0.71a), Y3

(0.9287±0.6056a), p = .467. They have same letter (a) because the difference was insignificant.

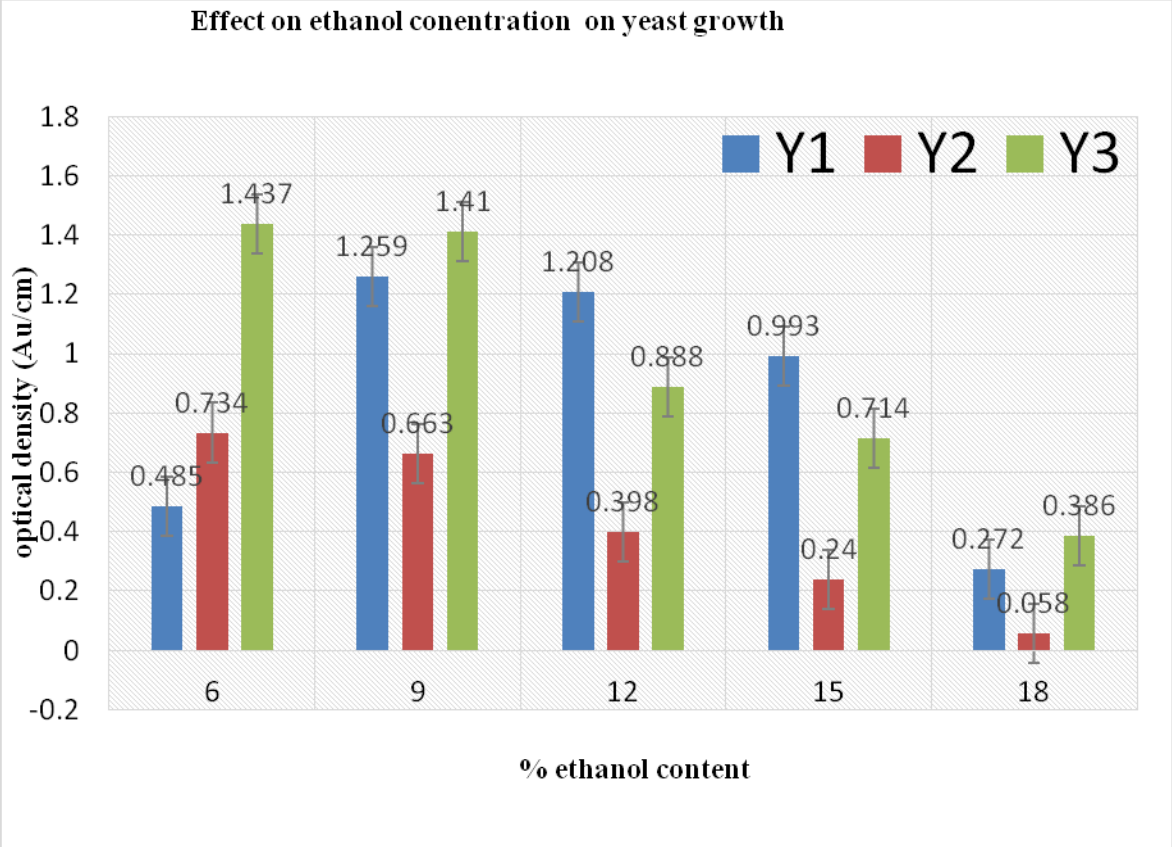


Figure 4.2: Effect of ethanol concentration on yeast growth

The Figure 4.2, show bar graphs of mean optical densities obtained when yeasts were grown for 48 hours in YEPD broth with varying concentration of ethanol. The values of various concentrations were compared for significant difference. Mean values were Y1 (0.8434±0.434a), Y2 (0.4185±0.266a), Y3 (0.9672±0.438a), p = .117. They have same letter (a) because the difference was insignificant. Significant difference of the mean was determined at $p < .05$.

Both yeasts Y2 and Y3 show reduction in optical densities when ethanol concentrations were increased. Y1 indicate some increase in optical from 6% to 9% ethanol concentration. From 9 % to 18% all yeasts indicate a decrease in cellular growth.

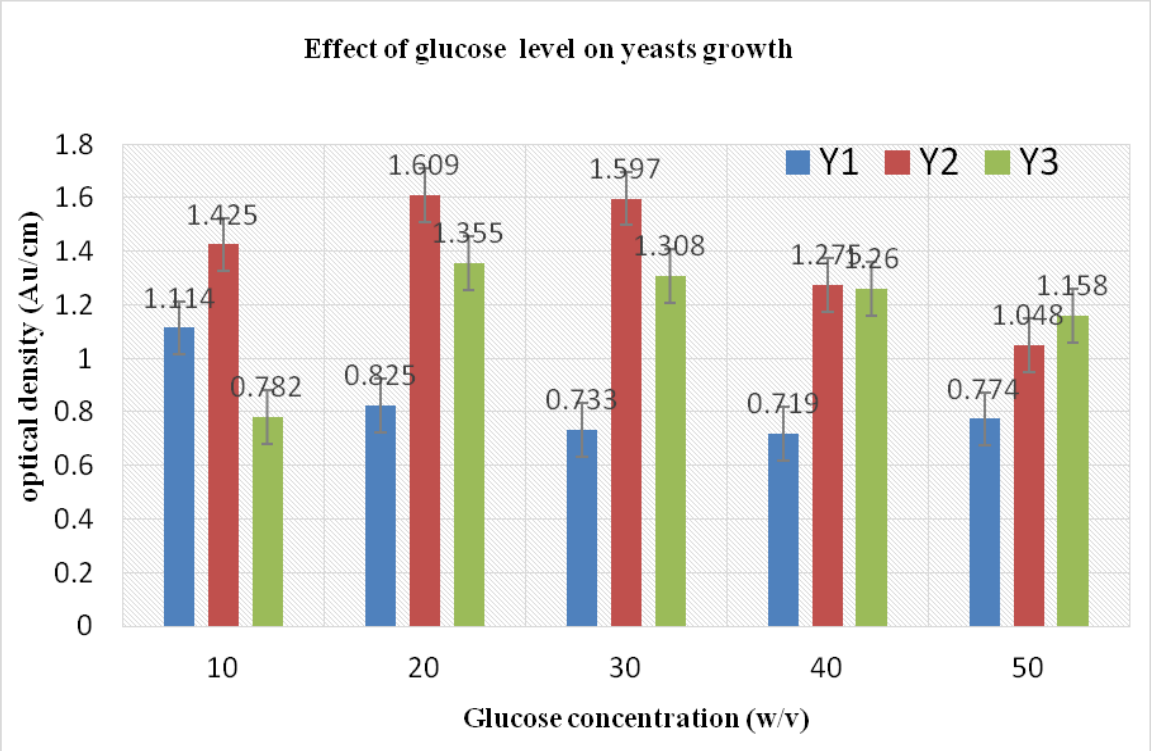


Figure 4.3: Effect of glucose concentration on yeast growth

The Figure 4.3 presents the mean of optical densities with increasing glucose concentration using bar graphs. Mean were compared based on changes of glucose concentration for specific yeast. Mean followed by different letters were significantly different at $p < .05$. The overall mean of yeasts were compared to find significant difference. Mean values were Y1 (0.8329±0.180a), Y2 (1.3907±0.224b), Y3 (1.1726±0.264b), $p = .0045$.

4.4. Reducing sugar concentration

The results of volume (mL) of standard in the Table 4.1 below were extracted from the summary of values from ANOVA table and used in determining glucose concentration.

Table 4.1: Mean volume in mL of standard glucose solution

Standard	Average	SD	Minimum	Maximum	Range
I	11.966	0.208	11.8	12.2	0.4
II	12.000	0.200	11.8	12.2	0.4
III	12.033	0.057	12.0	12.1	0.1
Total	12.00	0.150	11.8	12.2	0.4

No significant difference in mean volume among standard solution (p = .893). Standard was used to determine the concentration of the samples. The formula used was:

$$\boldsymbol{C1\ X\ V1 = C2\ X\ V2} \qquad \textbf{(4)}$$

V 1; standard volume, ***C 1***, standard concentration obtained by calculating mole/L. 10 g of anhydrous glucose per liter ($C_6H_{12}O_6$, RFM = 180). ***C 2*** concentration of sample, ***V 2***; volume of sample obtained from titration (Phwan *et al*., 2019). The values of sample concentration were multiplied by two because the two fold dilution of samples.

The concentrations of reducing sugar (mole/L) from various plants are shown in Figure 4.4 below.

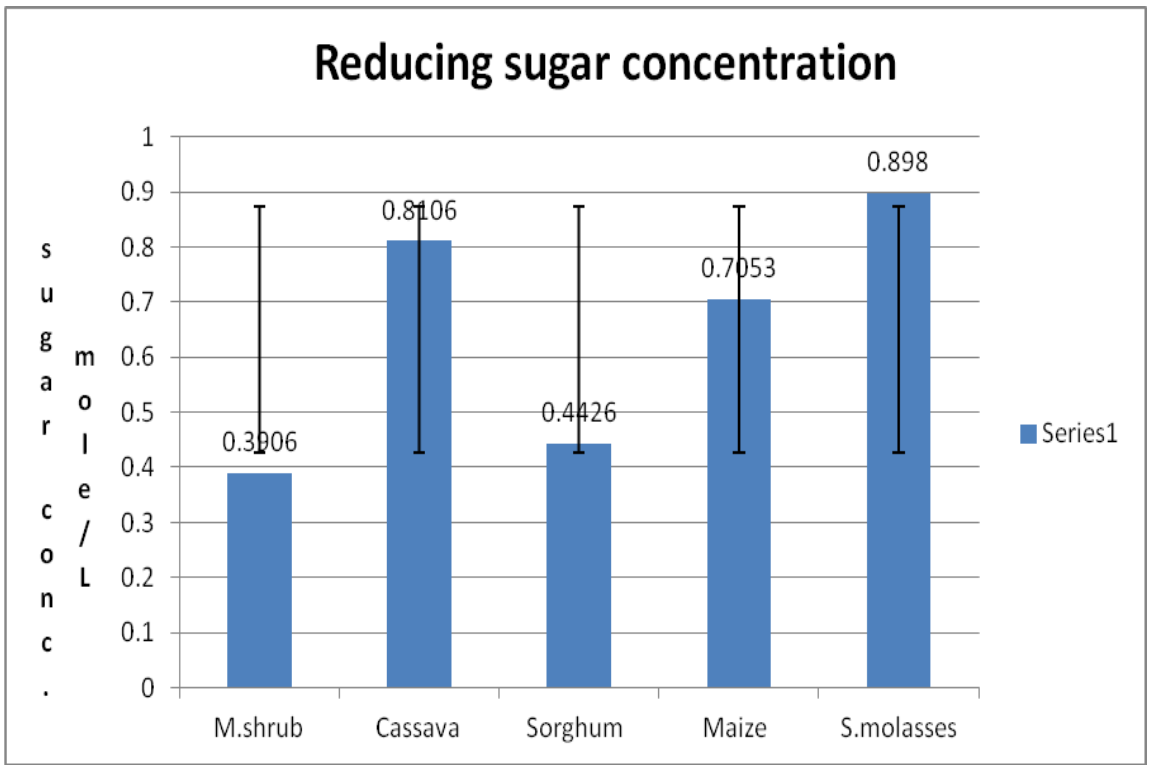


Figure 4.4: Reducing sugar concentration in plant hydrolysates

The Figure 4.4 indicated the reducing sugar concentrations for maerua shrub and sorghum was insignificantly different. Maize, cassava and sugarcane molasses showed significantly different concentration of reducing sugar.

4.5. Data on ethanol yields when yeasts ferment plants

Calibration curve was constructed basing on the results obtained by determining absorbance of the standard solution prepared. Dichromate solution was used as blank. A graph was plotted obtaining a straight line with the formula:

$$Y = 3.3965x - 0.3001 \qquad (5)$$

Where R^2 = .9983 there is a strong correlation between absorbance and ethanol concentration. Concentration of samples was obtained by substituting X with absorbance and considering the volume of distillate. Ethanol concentration in (g/L) were obtained by converting percentage ethanol v/v in 100 mL to a liter then multiplied by density of ethanol (0.789g/ cm^3). The formulae below were used to obtain ethanol productivity.

$$\boldsymbol{Ethanol\ concentration\ (\frac{g}{L})} \textbf{ = (\% v/v) x10 x 0.789} \quad \textbf{(6)}$$

$$\boldsymbol{Ethanol\ productivity = concentration\ \left(\frac{g}{L}\right) \div 48\ hours} \quad \textbf{(7)}$$

Table 4.2: on ethanol concentration and productivity after fermentation

Yeasts / Plants	Y1		Y2		Y3	
	Ethanol Level (g/L)	Ethanol productivity (g/L/h)	Ethanol level (g/L)	Ethanol productivity (g/L/h)	Ethanol level (g/L)	Ethanol productivity (g/L/h)
Maerua shrub	48.299±0.697a	1.006	61.988±0.160b	1.291	48.962±0.206a	1.020
Cassava	53.662±0.024a	1.118	64.052±0.098b	1.334	56.879±0.051a	1.185
Sorghum	53.853±1.237ab	1.122	62.382±2.148b	1.300	51.500±1.617a	1.073
Maize	50.691±0.506a	1.056	66.670±0.227b	1.389	56.263±0.109a	1.172
Sugarcane molasses	49.736±0.146a	1.036	49.978±0.403a	1.041	56.602±0.239a	1.179
F	13.03		42.61		197.6	
p (same)	.00056		.000000		.00000	

NB: Values were determined for significant difference at $p < .05$. Mean denoted using a different letter in a row represent significant difference for yeasts.

Mean ethanol concentration (g/L) and productivity (g/L/h) for plants were, maerua shrub; 53.08, 1.106 (a), cassava; 58.19, 1.212 (a), sorghum; 55.91, 1.165 (a), maize; 57.87, 1.205 (a), sugarcane molasses; 52.11, 1.086 (a), (*p* value = .4239, *F* ratio = 1.03, Df 4, 15) there were no significant difference between values of all plants.

The mean ethanol concentration (g/L) and productivity for each yeast were, Y1; 51.210, 1.0689 (a), Y2; 61.013, 1.271 (b), Y3; 54.041, 1.126 (a), (*p* value = .0273, *F* ratio = 3.96, Df 2, 16). The values with the same letter were not significantly different. Ethanol productivity for Y1 is significantly lower than that of Y2, which is significantly higher than *Saccharomyces cerevisiae* (Y3). Ethanol productivity from Y1 and Y3 were not significantly different.

CHAPTER FIVE

DISCUSSION

5.1. Discussion

It is necessary to investigate ways of lowering the cost of bioethanol production. Non-food crops can potentially provide alternative sugar sources to replace food crops. Reducing the cost of production of bioethanol is also done by using yeasts that produces higher levels of ethanol than commonly used *Saccharomyces cerevisiae.* Therefore, there is need to investigate potential alternative yeasts. The development of high ethanol producing strains is economically important. A high ethanol yield and productivity lowers the distillation cost (Kasavi *et al*., 2012). In ethanol industry, acceptable ethanol concentration from molasses is 11 % in optimized medium industries (Darvishi & Abolhasan, 2019).

5.2. Yeast morphology

The yeast colony morphology was used to characterize the isolated yeasts from finger millet malt. The Y1 exhibited white colour and smooth margin while Y2 show a creamy colour and smooth margin. The yeasts showing creamy or white colony colour are mostly *Saccharomyces* strain (Karki *et al*., 2017). Arachchige, Yoshida and Toyama, (2019) reported creamy colonies to be the characteristic of yeast especially *Saccharomyces* strain. The two yeasts Y1 and Y2 had unicellular cells with multilateral budding patterns, similar to Y3. *S.cervisiae* yeast has unicellular cells with multilateral budding.

5.3. Physiological characterization

The physiological conditions investigated were effect of temperature, ethanol and glucose concentration on yeast growth to determine stress tolerance by yeasts.

5.3.1. Effect of temperature change on growth of yeasts

The graph in Figure 4.1, show that when the temperature increases from 25 to 30 ^{o}C the optical densities also increases for all the yeasts. There were no significant differences between the optical densities of Y1 and Y2, but numerical difference exists. At all temperatures the optical densities of *S. cerevisiae* (Y3) were higher than that of Y1 and Y2.

At temperatures of 30 to 35 ^{o}C there was less difference in their optical densities for all the yeasts. The temperature range was optimum because of significantly high optical densities.

Optical densities of all yeasts significantly decreases as the temperature is increased from 35 to 45 ^{o}C, these strongly indicate high temperature reduces viability as supported by (Deesuth, Laopaiboon, Jaisil, & Laopaiboon, 2012). *S.cerevisiae* (Y3) is more tolerant at 40 ^{o}C compared to both Y1 and Y2.

In the range 40 - 45 ^{o}C all the yeasts show very low optical densities with *S.cerevisiae* (Y3) indicating low growth, while Y1 and Y2 gave negative growth meaning cells died due to high temperature, a point supported by Choudhary *et al*., (2016). The mean of the average temperature tolerance have no significant difference, however, *S.cerevisiae* (Y3)

show highest tolerance compared to the other yeasts in which Y2 is more temperature tolerant than Y1.

5.3.2. Effect of ethanol concentration on growth of yeasts

From the results in Figure 4.2, at the concentration of 6 % (1.4387) ethanol *S.cerevisiae* (Y3) showed higher optical density compared to Y1 (1.2597) and Y2 (0.733). Y1 shows significant increase in cellular growth with increased ethanol level from 6 % to 9 %, unlike Y2 and *S.cerevisiae* (Y3) whose optical densities did not change much.

Increased ethanol content from 9 to 12 %, Y1 gave relatively constant optical densities, while Y2 and *S.cerevisiae* (Y3) showed significant decrease in optical densities. Y1 at 12 % (1.208), 15 % (0.9928) and *S.cerevisiae* (Y3) 12 % (0.888), 15 % (0.714) their optical densities were comparable, however, Y2 showed significant decrease. All the yeasts were tolerant to ethanol concentration of 15 % which is in agreement to the findings by Umen & Okor (2016).

All yeasts showed a sharp reduction in cellular growth when ethanol levels were increased from 15 to 18 %v/v. The high ethanol concentration delays yeast's growth and hence reduction in growth, fermentation and viability of yeast cells (Navarro-Tapia, Nana, Querol, & Pérez-Torrado, 2016).

The optical densities at 18 % v/v ethanol were Y1 (0.272), Y2 (0.058) and *S.cerevisiae* (Y3) (0.386) these showed that all yeasts withstands high ethanol concentration because of growth, although to a lower extend.

Mean optical densities were Y1 (0.8434±0.434), Y2 (0.4185±0.266), *S.cerevisiae* (0.9672±0.438), there were a slight differences in their optical densities. This indicates that Y1 & Y2 are potential yeasts for industrial application. High ethanol producer withstands high ethanol concentration (Kasavi, *et al*., 2012).

5.3.3. Effect of glucose content on growth of yeasts

The results from bar graphs in Figure 4.3 show yeast Y1 with high cellular growth at 10 % w/v then it reduced with increased glucose concentration from 10 % (1.114) to 20 % (0.825) which was a significant difference. At 10 % glucose concentration Y2 gave the highest optical density (1.425) compared to both Y1 (1.114) and Y3 (0.781). *S. cerevisiae* (Y3) at 10 % w/v glucose concentration gave low optical densities which greatly increased when glucose concentration was increased to 20 %.

Between 20 % and 30 % glucose concentration there was no remarkable difference in optical densities of all yeasts, but slight differences exist. Y1 show lower tolerance to glucose levels above 20 % compared to Y2 and *S.cerevisiae* (Y3). Y2 and *S.cerevisiae* (Y3) show highest optical densities at 20 % glucose levels while Y1 is highest at 10 %. The optimum glucose concentration for growth of yeast Y2 and *S.cerevisiae* is 20 %.

Arachchige *et al*., (2019) found all strains yeasts produce significant alcohol concentration at glucose level of 160 g/L which is between 10 to 20% which was the highest in these findings. Ali & Khan (2014) also reported a maximum of 20 % sugar tolerance and ethanol production from 20 % of glucose concentration which decreases when the concentration of sugar is increased.

At 30 % glucose concentration Y1 gave lower growth compared to Y2 and *S.cerevisiae*. An increase in glucose content from 30 % to 40 % causes decreased growth in all yeasts, but Y2 showed remarkable decrease compared to Y1 and Y3. The decrease was attributed to inhibition of growth by the osmotic pressure resulting from high content of sugar of 30 %, this is supported by (Parameswari, Hemalatha, Priyanka & Kishori, 2015).

Further increase in glucose concentration 40 to 50 % resulted in insignificant difference in growth of all the yeasts, this is in agreement to findings by (Negera, 2017). The mean optical density of Y1 for glucose concentration was significantly different compared to optical densities of both Y2 and Y3.

5.4. Reducing sugar concentration

The plant samples were autoclaved at 121 ^{o} C so as to gelatinize starch molecules and lead to sterilization. The treatment of samples with 4 % α-amylase which were in excess was to optimally hydrolyse the starches to reducing sugar. The deproteinizing agents also were in excess to remove all the proteineous reducing matrices that could react with Cu^{2+} functional group therefore resulting to errors.

The mean reducing sugar concentration for maerua shrub from Figure 4.4 was 0.3906 mole/L which was comparable to the value for sorghum 0.4426 mole/L, but both were significantly lower compared to cassava 0.8106 mole/L, maize 0.7054 mole/L and sugar cane molasses 0.8980 mol/L. There were no documented studies on the concentration of reducing sugars from maerua shrub. According to Mavura *et al*., (2008) *M.subcordata* root tuber juice contains polysaccharides 300 mg/mL, amylopectin constituting 70 - 80 %

and amylose form 20-30 %. It is known that polysaccharides can be hydrolysed by amylase enzyme to yield reducing sugar.

Corn, wheat, and potato are known to contain starch approximately 20 % amylose and 80 % amylopectin, these values makes maerua shrub to be similar to starchy plants mentioned. Amylopectin is a water soluble polysaccharides, this property enhances hydrolysis and fermentation of amylopectin. Mavura *et al*., (2008) also found *Maerua subcordata* root tuber juice to contain proteins of 289 mg/mL. These proteins are sources of nitrogen to be used by yeasts as structural and signalling compound.

Some findings on fermentable sugar concentrations of some lignocellulosic feedstock for example sugarcane bagasse, wheat straw, rice straw, wood chips and corn stover were compared to reducing sugar concentration of Maerua shrub. Guilherme, Dantas, Santos, Fernandes and Macedo, (2015) found sugarcane bagasse to yield fermentable sugars of 55.9 g/L when enzyme xylanase was used. Ertas, Han, Jameel, and Chang, (2014) found 30.4 g sugars from 100 g wheat straw. Wood chips of (*Ailanthus excelsa*) lignocellulosic substrate yielded the highest amount of pentose (280 mg/g) and total sugars (285 mg/g) when processed using single step autoclave mediated dilute acid hydrolysis (Sahay & Rana., 2017). When the concentration of reducing sugar for Maerua shrub 0.3906 mole/L (70.308 g/L or 33.78 % g/g or 337.8 mg/g) was compared to sugar concentration of some documented findings for example, sugarcane bagasse, wood chips of (*Ailanthus excelsa*), and wheat straw showed lower sugar concentration.

Enzymatic hydrolysis of hemicellulosic biomass solves the problem of using harsh chemical pretreatment which pollutes the environment, however, lignin prevents enzyme

access to cellulose and hemicellulose therefore requiring pretreatment, a point supported by (Ladeira *et al*., 2020).

When maerua shrub (*Maerua subcordata*) was compared to other sugar sources such as; macroalgae (sea weed) *Sargasum* an invasive species yielded 15.22 g/L of oligomers (del Rio, Dominguez, Viana, Aloia, Lucilia & Gil, 2019), Sea weed *Dilsea carnosa* and *U. lactuca* yielded sugar concentration 125.0 and 360.0 mg/g respectively. *Laminaria digitata* liberated highest glucose concentration of 218.9 mg/g (Kostas *et al*., 2020). Sea weeds *U.lactuca* yielded higher concentration of reducing sugar (360 mg/g glucose) which is higher when compared to Maerua shrub.

Maerua shrub produced reducing sugars (0.3906 mol/L), calculated to obtain (33.74% w/w) which was higher compared to fermentable sugar generated by microalgae (*Chlorococcum* sp.) which was 23.67 % w/w (Rehman & Anal, 2019). Microalga *Scenedesmus sp* gave an average reducing sugar of 23.91 % (Agustini, Hidhayati, & Wibisono, 2019). Fermentable sugar or reducing sugar from various microalgae showed lower concentrations as compared to Maerua shrub. The advantage of maerua shrub is it high starch content that can easily be hydrolysed unlike algae and lignocelluloses which require costly pretreatment methods (Khan, Shin, & Kim, 2018).

Cassava yielded (0.7760 mol/L) reducing sugar when hydrolysed by fungal alpha-amylase enzyme. These results showed cassava as a plant that yields second highest concentration of reducing sugar with significant different to sugarcane molasses. The chemical composition for cassava was found to contain: protein 1.17-3.48 %, fat 0.74-1.49 %, carbohydrates 83.42-87.35 % by (Emmanuel, Clement, Agnes, Chiwona-Karltun

& Drinah, 2012). Cassava root tuber flour had high concentration of carbohydrates this also confirms its high concentration of reducing sugar but lower than that of sugar cane molasses.

The dry sorghum grain flour produced reducing sugar whose concentration was 0.4426 mol /L, converted to (79.668 g/L or 38.3 %). The approximate chemical content: protein 12.25 %, fat 4.24 %, carbohydrate 72.93 % (Mohammed, Ahmed & Babiker, 2011). Most sorghum plants contain tannin, phytic acid and polyphenol which are anti-nutritional factors (Abdelhalim, Kamal & Hassan, 2019). Tannin and phytic acid present in cereals inhibit digestibility and affect nutrient bioavailability for absorption (Feyera, 2020).

Maize flour was found to contain carbohydrates range from 76.85 - 80.31 % (Ogunyemi, Otegbayo & Fagbenro, 2018). These showed that maize had lower carbohydrate concentration than cassava. Reducing sugar concentration from maize meal was lower compared to what was obtained from cassava root tubers. (Kringel, El Halal, Zavareze & Dias, 2020) found starch extraction from roots and tubers was easier than from some cereals due to lower concentration of proteins and lipids.

Cassava and corn have same characteristics as starchy crops, but the main difference is the ratio of amylose to amylopectin. This is because of high amylose: amylopectin in corn starch, cassava starch had highest fermentation (Pradyawong *et al*., 2018). Sugarcane molasses produced appreciable high concentration of reducing sugar (0.898 mole /L) compared to other plant samples. Sugar cane molasses contains free sugars that do not require hydrolysis to be availed, unlike starchy plant products. Sugar cane

molasses contain brix 86.5 %, total sugar 56.0 %, nitrogen 0.61 %, protein 3.81 % (Zohri, Mahmoud, Saddek & Hanafy, 2018).

5.5. Ethanol concentration and productivity by plants

Maerua shrub produced ethanol concentration (48.299±0.697 g/L, 1.006 g/L/h) which was insignificantly different to that of Maize (50.691±0.506 g/L, 1.056 g/L/h) (p = .1) and sugarcane molasses 49.736±0.146 g/L, 1.036 g/L/h, (p = .4109) but cassava 53.662±0.024 g/L, 1.118 g/L/h (p = .0061) and sorghum (53.853±1.237 g/L, 1.118 g/L/h) (p =.0026) were significantly higher when both were fermented using Y1. There was no literature report on fermentation of maerua shrub which could be used to compare with the results. Fermentation of high glucose concentration results in high ethanol concentration and productivity so long as a robust yeast strain is used (Songdech *et al*., 2020). Conditions such as temperature and carbon source should be favourable so as to obtain high concentration of ethanol (Mezenova, Keshtkar, Kulaev, Danshina & Romiani, 2020). This could be the reason why sorghum produced higher concentration of ethanol than maize and sugarcane molasses despite the two having high concentration of reducing sugar than Sorghum.

Saccharomyces cerevisiae, produced maximum ethanol concentration (106 g/L, 4.4g /L/h) in 24 hours of fermentation, results was obtained by (Barcelos, Maeda, Betancur & Pereira, 2011) which was higher than ethanol concentration from sorghum in this investigation, the disparity was caused by high concentration of reducing sugar (250 g/L) because hydrolysis was done using both alpha-amylase and glucoamylase.

Plant feedstock produced ethanol concentration (g/L) and productivity (g/L/h) as follows; cassava (64.052±0.098, 1.334), maize (66.670±0.227, 1.389) sorghum (62.382±2.148, 1.300) and maerua shrub (61.988±0.160, 1.291) which were significantly higher compared to that of sugarcane molasses whose concentration and productivity were (49.978, 1.041) when fermented by Y2. Pradyawong *et al*., (2018) obtained ethanol content from cassava to be (127.9 g/L) which had no significant difference to dent and waxy corn when both were fermented for 72 hours from fermentable sugar concentration of (250 g/L). However, when Y1 and Y3 were used to ferment both maize and cassava, the results showed significant difference. Choi *et al*., (2010) obtained productivity 1.35 g/L/h when cassava starch was fermented using wild type of *Saccharmyces cerevisiae,* the finding agree with the results obtained using Y2.

Cassava produced ethanol concentration and productivity of (56.879 g/L, 1.185 g/L/h) which is significantly higher in comparison to what was produced by maerua shrub, sorghum, maize and sugarcane molasses when fermented by Y3. Cassava produced high reducing sugar concentration which influenced the production of high ethanol concentration this was supported by (Suryawanshi, Khokhar & Patel, 2018). Hariharan Joshy, Sajeevan and Moneyraj, (2020) obtained ethanol concentration that range between (30-35 g/L) when cassava was fermented for (24 h) using *Saccharomyces cerevisiae* with complete conversion of sugar. The productivity from these results was (1.250 - 1.458 g/L/h) which was higher than the results from Y3 (*S. Cerevisiae*). The reason for the difference was the low concentration of sugar that was to be fermented to ethanol by yeast.

Sugarcane molasses produced lower ethanol level compared to maize, cassava and sorghum, the high concentration of the reducing sugar could have interfered with the yeast cellular physiology therefore reducing fermentation efficiency. The lower ethanol concentration from sugarcane molasses could be caused by its lower nitrogen concentration compared to other plants (Zohri *et al*., 2018) and does not contain lipids which are important substances as amino acid. Amino acids provide raw material for energy generation, biosynthesis of structural or defensive compounds against abiotic and biotic stress, while the role of lipids are adaptation to stress, membrane structure and signalling molecule in yeasts (Chen *et al*., 2020).

Maerua shrub should be used in production of cheap bioethanol because it produced high ethanol concentration and productivity in an experiment for example (61.988±0.160, 1.291g/L/h) when fermented by Y2, these results were comparable to the results from maize, cassava, and sorghum.

The production of bioethanol from maerua shrub reduces the cost of bioethanol because it is a plant that is drought resistant, requires less input and grows in marginal lands. Maerua shrub is plant that can grow on marginal lands and produce bioethanol is advantageous because it ensure food security and does not compete with food crops for arable fertile lands.

The cost of raw materials considerably affects the sustainability of bioethanol production (Susmozas *et al*., 2020). It implies that the low cost feedstock such as Maerua shrub contributes lower percentage of the total cost an idea supported by (Pandey *et al*., 2019), the food crops contribute a higher percentage cost because of high demand for food,

therefore causing high bioethanol production cost. Production of bioethanol from sugarcane molasses is also more costly due to its use as food for both humans and animals.

5.6. Effects of yeast strains on ethanol production

Five different plant types were used in fermentation experiment with three types of yeasts. From Table 4.2, the mean ethanol concentration and productivity were high in maerua shrub for Y2 (61.988 g/L ± 0.160, 1.291g/L/h), while Y3 (48.962 ± 0.206, 1.020 g/L/h) and Y1 (48.299 ± 0.697, 1.006 g/L/h) had least concentration of ethanol with a significant difference (p < .0000). Significance difference between Y1 and Y2 (p = .009086), Y2 and Y3 (p = .01258).

From Table 4.2, cassava the mean ethanol concentration and productivity were high for Y2 (64.052 ± 0.098 g/L, 1.334g/L/h), while Y3 (56.879 ± 0.051 g/L, 1.185 g/L/h) and Y1 (53.662 ± 0.024 g/L, 1.118 g/L/h) had the least concentration of ethanol in g/L with a significant difference (P < 0.0000). Significance difference was between Y1 and Y2 (p = .002235) and Y2 and Y3 (p = .04154).

The mean ethanol concentration and productivity were high in sorghum for Y2 (62.382±2.148 g/L, 1.299 g/L/h) then followed by Y1 (53.853 ± 1.237, 1.122 g/L/h). Significance mean difference was between Y2 and Y3 (p = .04154).

For maize the mean ethanol concentration was high for Y2 (66.670 ± 0.227 g/L, 1.388 g/L/h) while Y1 (50.691 ± 0.506 g/L, 1.056 g/L/h) had the least concentration of ethanol

in g/L with a significant difference ($p < .0000$). Significant mean difference was between Y1 and Y2 ($p = .002235$), and Y2 and Y3 ($p = .04154$).

The mean Concentration of ethanol in g/L was high in Y3 for sugarcane molasses (56.602 ± 0.239 g/L, 1.179 g/L/h) insignificantly different than that of Y1 and Y2.

Concentration of ethanol in g/L also differed significantly for yeast across all plant types as presented in Table 4.5. Generally Y2 had highest ethanol concentration and productivity than the other two yeasts, *S. cerevisiae* (Y3) and Y1 whose ethanol concentrations are insignificantly different. Kim *et al*., (2014) obtained highest ethanol contents because the wild yeast utilizes both glucose and galactose. These results show wild yeast that produces high ethanol concentration and productivity in comparison to Y1 and Y2 in Table 4.2. Apart from the differences in yeast fermentation abilities the other factor is the high sugar concentration utilized by *S.cerevisiae* KL17, than sugar used in this study. Y2 produces remarkably higher productivity than *S. cerevisiae* (Y3) and Y1, therefore Y2 significantly makes production to be cost effective by producing higher concentration of ethanol an idea support by (Kasavi *et al*., 2012).

Kechkar *et al*., (2019) obtained yeast with results similar to *S. cerevisiae* and two strains showing better fermentation abilities in harsh environment. Ramos, Duarte, Freire, Dias, Eleutherio and Schwan, (2013) observed four indigenous strains with highest values of fermentation in sugarcane juice compared to traditional yeast. The yeasts obtained in the reports show that indigenous yeasts could have better fermentation performances than industrial yeast and the report in this research is in agreement with the observation.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1. Conclusion

This study showed that the two isolated yeasts from finger millet malt of *Saccharomyces* strain because of colony and cellular characteristics. The consideration of temperature, ethanol and glucose tolerance by yeasts, the finger millet malt contain yeast (Y2) whose characteristics are comparable to those commercial yeast (Y3), while the other yeast (Y1) showed significantly lower tolerance to glucose concentration in comparison to commercial yeast (Y3).

The reducing sugar concentration for maerua shrub is (0.3906 mole/L) which is comparable to sorghum (0.4426 mole/L), but significantly lower compared to cassava (0.7760 mole/L), maize (0.7054 mole/L) and sugar cane molasses (0.8980 mole/L) in which each is significantly different when they are compared (p = .0001).

Maerua shrub is a plant whose mean ethanol productivity is higher than that of sugar cane molasses but lower than that of sorghum, maize and cassava. Mean ethanol concentration (g/L) and productivity (g/L/h) for plants were; maerua shrub (53.08, 1.106), cassava (58.19, 1.212), sorghum (55.91, 1.165), maize (57.87, 1.205), sugarcane molasses (52.11, 1.086). Independent experiments show comparable results when maerua shrub productivity is compared to that of other plants as described in the discussion. Maerua shrub is a novel plant in the production of second generation bioethanol.

Mean ethanol concentration and productivity for each yeast were; Y1 (51.25, 1.068), Y2 (61.02, 1.271), Y3 (54.04, 1.126). Both Y1 and Y2 were isolated from finger millet malt which Y2 gave considerably higher ethanol productivity than *S.cervisiae* (Y3), while the ethanol productivity for Y1 was insignificantly lower than that of *S. cerevisiae* (Y3).

6.2. Recommendations

Basing on the findings from the study yeast Y2 isolated from finger millet malt should be used as fermentation yeast because of its high ethanol productivity compared to *S.cerevisiae*.

Maerua Shrub should be used as sugar source and a second generation bioethanol feedstock because of its efficiency in bioethanol production. Further, the commercial cultivation of this plant by farmers is highly recommended to produce root tubers which can be processed for bioethanol production because the plant produces bioethanol of comparable concentration to food crops, it will improve utilization of marginal, arid and semi-arid lands because of its resistance to drought. It will also replace food crops which are first generation feedstock, hence cheaper bioethanol production and sustained food security in Kenya.

The future studies should investigate on fermentation capabilities of yeasts isolated from samples such as roots, fruits and barks of plants because most of the yeasts are used locally with no or minimal scientific findings. It is also important to research on other non-food plants to produce cheaper bioethanol and biodiesel in Kenya.

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APPENDICES

APPENDIX I: Yeast production

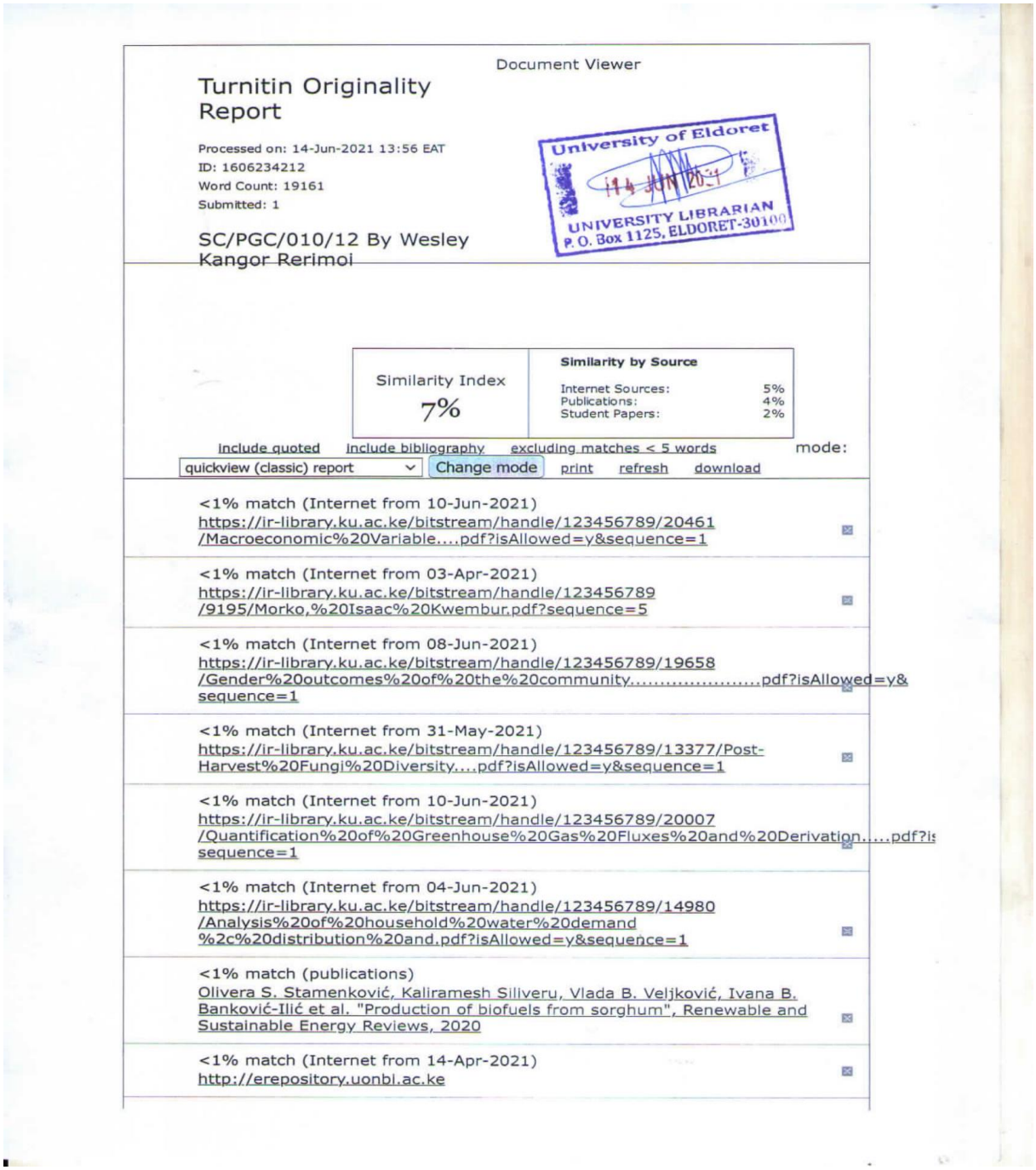
A loop full of yeasts was inoculated into 500 mL autoclaved broth media in 1L conical flasks labelled Y1, Y2 and Y3, sealed, shaken at 150 rpm and 30 ^{o} C, for 48 hours which is optimal conditions for yeast growth.

Yeast strains grow well at pH 3.0 - 6.0, temperature 28-30 ^{o} C. The YEPD broth contained, yeast extract, 1.5 g; peptone, 5 g; dextrose, 5 g ; distilled water 500 mL; the pH of the broth media was 6.0. Yeasts were centrifuged at 5000 rpm obtain yeast while maintaining cellular integrity.

APPENDIX II: Preparation of dichromate reagent

Accurately 40 g potassium dichromate reagent was weighed then placed in a beaker Approximately 200 mL water was measured, added, stirred. Then exactly 270 mL of sulphuric acid (1.84g/cm^3) was cautiously added, resultant solution allowed to cool. The volume was adjusted to 500 mL of the volumetric flask by adding sufficient volume of distilled water to make 0.298 M concentration.

APPENDIX III: Similarity report



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