ISOLATION AND SELECTION OF LOCAL YEAST STRAINS FOR THE PRODUCTION OF SWEET WINE USING PINEAPPLE AND WATERMELON JUICES

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BY

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA, IN PARTIAL FULFILLMENT FOR THE AWARD OF MASTER OF SCIENCE DEGREE IN MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY,

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ZARIA, NIGERIA

DECEMBER, 2017.

DECLARATION

I hereby declare that the work in this Dissertation entitled "Isolation and Selection of Local Yeast Strains for the Production of Sweet Wine Using Pineapple and Watermelon Juices" has been carried out by me in the Department of Microbiology, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

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_____	_____	_____
Name of Student	Signature	Date

CERTIFICATION

This Dissertation entitled "ISOLATION AND SELECTION OF LOCAL YEAST STRAINS FOR THE PRODUCTION OF SWEET WINE USING PINEAPPLE AND WATERMELON JUICES" by Nko Samuel BASSEY meets the regulations governing the award of the degree of Master of Science in Microbiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This piece of work is dedicated to the Lord Almighty for His provision, grace and mercy throughout the course of this work. Also to my parents and siblings for their love and support throughout the course of this work.

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My utmost acknowledgment goes to the Almighty God for his divine love, mercy and protection for seeing me through the successful completion of this study and helping me surmount all the challenges faced throughout this period.

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ABSTRACT

In developing countries like Nigeria 20-30% of fruits produced are wasted due to lack of proper utilization, post-harvest and processing technology. By converting these fruit into value added products like wine is a smart solution for this problem. This study was aimed at the isolation and identification of local yeast strains from spoilt fruits and alcoholic breverages for the production of sweet wines using pineapple and watermelon fruit juices as substrates. The organisms obtained were isolated by spread plating 0.1ml solution obtained from the serial dilution of 1ml of fruit juice obtained from the smashed spoilt fruit and alcoholic breverage. The organisms obtained were characterized culturally, morphologically, biochemically and confirmed using an API Test Kit. The organisms were further screened to obtain the one with the best fermentative property by carrying out the Stress exclusion test, ethanol tolerance test, temperature tolerance test, hydrogen sulfide production test and flocculation test. The wine was then produced by cell purification by washing the yeast cell obtained and preparation of inoculum using 400ml of water and 80g of sugar and these were used for the wine production which lasted for 14 days. During the fermentation the microbiological analysis of the wine was carried out to ascertain the quality of wine produced by identifying the bacteria present in the wine and these were confirmed to be safe for consumption. Finally, sensory evaluation of the wine produced were carried out to ascertain the organoleptic property of the wine produced. A total of seven species of yeast which include *Candida sphaerica, Pichia spp, Candida pelliculosa, Cryptococcus humicola, Candida guilliermondii, Candida famata* and *Kloeckera apiculata* were isolated. *Candida pelliculosa* provided the best fermentative property but since it is potentially pathogenic, an alternative yeast *Kloeckera apiculata* was used for the fermentation. The watermelon juice used as substrate for wine fermentation had initial specific gravity of 1.07g/cm^3 and the fermentation ended at specific gravity of 0.01g/cm^3 while pineapple juice used as substrate

had its specific gravity as 1.10g/cm^3 and fermentation ended at specific gravity of 1.03g/cm^3, the total soluble solid of watermelon juice fermentation was started at 17% and ended at 3.8% while that of pineapple juice fermentation started at 23.6% and ended at 8.2%, these showed the gradual utilization of sugar by *Kloeckera apiculata* in both wines produced. The total titratable acidity in pineapple juice fermentation was started at 0.01% and ended at 0.25% while titratable acidity of watermelon juice fermentation was started at 0.01% and ended at 0.5%. It was observed that titratable acidity increased daily as the days increased. Also, the alcohol content increased daily as the sugar were being utilized because the alcohol in pineapple juice fermentation was started at 0% and ended at 12.9% while the alcohol in watermelon juice fermentation was started at 0% and ended at 7.5%. The temperature decreased gradually during the period of fermentation in pineapple wine from 30-18^{0}C and in watermelon wine from 28-17 ^{0}C. The pH also decreased gradually in pineapple wine from 4.1-3.9 while in watermelon wine from 3.8-3.5. At the end of fermentation the sensory evaluation of the wine showed that the color of the standard wine was preferred to the color of the pineapple and watermelon wine produced, however in terms of taste and aroma the pineapple and watermelon wine produced were preferred to the standard wine because of the presence of glycerol produced by the organism used. Thus, through fermentation, the highly perishable fruits can be converted into a highly nutritious wine which can be made available all year round.

TABLE OF CONTENTS

Cover Page……..i

Fly Leaf……..ii

Title Page……..iii

Declaration……iv

Certification……v

Dedication …….. …….vi

Acknowledgement……vii

Abstract……...viii

Table of Contents……..x

List of Tables……..xiv

List of Figures…….xv

List of Appendices…….xvi

List of Abbreviations……..xvii

1.0 INTRODUCTION……1

1.1 Statement of Research Problem……..4

1.2 Justification of the Study……5

1.3 Aim of the Study……..6

1.4 Objectives of the Study……6

2.0 LITERATURE REVIEW……...…7

2.1 History of Wine……7

2.2 Basic Principles in Wine Production……..9

2.2.1 How Fruit Wine is Made…….…...9

2.2.2 Economic Considerations in Fruit Wine Production……13

2.2.3 Fermentation Systems……..14

2.2.4 Product Recovery……18

2.3 Microorganisms Potentially Useful for Wine Production……..19

2.4 Sources of Yeast for Wine Production…….……26

2.4.1 Natural sources……26

2.4.2 Genetic Improvement of Isolates…….28

2.5 Criteria Used for Selecting Yeast for Wine Production…….28

2.6 Method of Improving Yield in Wine Fermentation……29

2.7 Application /Uses of Wine……..…30

2.7.1 Health Benefits…….30

2.7.2 Fruit Cleaner……..…33

2.7.3 An Anti-Aging effect…..33

2.7.4 Cooking Substitute……...34

2.7.5 Compost Addition……34

2.7.6 Double Down on Stains……34

2.7.7 Reduce the Risk of Osteoporosis……..34

3.0 MATERIALS AND METHODS……...35

3.1 Collection of Samples…….35

3.1.1 Equipments and Sample Preparation……35

3.2 Isolation of Yeast Strains……...35

3.2.1 Characterization of Yeast Strains…….36

3.3 Screening and selection of yeast strains for wine production……38

3.3.1 Stress exclusion Test……38

3.3.2 Ethanol Tolerance Test……..…38

3.3.3 Temperature Tolerance Test……..…39

3.3.4 Hydrogen Sulfide Production Test……...39

3.3.5 Flocculation Test……..39

3.4 Fermentation of Fruit Juice for Wine Production……..39

3.4.1 Cell Purification……...39

3.4.2 Preparation of inoculum……..40

3.4.3 Culture Conditions for Wine Production……..…40

3.5 Microbiological Analysis of Wine Produced……43

3.5.1 Identification of Bacteria Isolates……43

3.5.2 Physicochemical Analysis of Wine……..……49

3.5.3 Proximate Analysis of the Wine……...50

3.5.4 Post Fermentation Treatment……...54

3.6. Sensory Evaluation of the Wine Produced……...55

3.7 Data Analysis……..……56

4.0 RESULTS……57

4.1 Isolation and Identification of Yeasts Strains….....57

4.2 Screening and Selection of Yeast Strain……...61

4.3 Production of Wine from fruit Juice using *Kloeckera apiculata* ……...64

4.3.1 Effect of Specific Gravity on Wine Production……...64

4.3.2 Effect of Total Soluble Solid on Wine Production……...64

4.3.3 Effect of Titratable Acidity on Wine Production……64

4.3.4 Effect of Alcohol on Wine Production……..…64

4.3.5 Effect of Temperature and pH on Wine Production……..…64

4.4 Determination of the Microbiological and Physicochemical Profile of the Wine Produced……...71

4.5 Evaluation of Sensory Quality of the Wine ……75

5.0 DISCUSSION…….79

5.1 Isolation and Identification of Yeast Strains from Spoilt Fruits and Alcoholic Breverage……...79

5.2 Screening and Selection of Yeast Strain with the Best Fermentative Property……..……81

5.3 Production of wine from fruit juice using *Kloeckera apiculata* as fermenting agent…….85

5.4 Microbiological and Physicochemical profile of the wine produced…….90

5.5 Sensory evaluation of wine produced……...93

6.0 CONCLUSION AND RECOMMENDATION…….…...95

6.1 Conclusion…….……..95

6.2 Recommendations……..…97

REFERENCE….....98

APPENDICES……..……106

LIST OF TABLES

Table	Title	Pages
4.1	Cultural, Microscopic and Fermentative Characteristics of Yeasts Isolates….....	58
4.2	API Confirmatory Test for Yeast Isolates……	59
4.3	Sources of Yeasts Isolated, Characterized and Identified……	60
4.4	Screening and Selection of Yeast Strains for Best Fermentative Properties……	62
4.5	Stress Tolerance Test of Isolated Yeasts…..……	63
4.6	Microbiological Profile of Pineapple and watermelon Wine Produce…….	72
4.7	Cultural, Microscopic and Biochemical Characteristics of Bacteria Isolates……...	73
4.8	Confirmed Bacterial Isolates ……...…...	74
4.9	Sources of Bacteria Isolated, Characterized and Identified…….	76
4.10	Proximate Composition of Wines Produced from Pineapple and Watermelon Juices……	77
4.11	Sensory Result of Wines Produced from Pineapple and Watermelon Juices……..…….	78

LIST OF FIGURES

Figure	Title	Page
4.1	Flow Chart for Wine Production……..	42
4.2	Specific gravity pattern during fermentation of watermelon and pineapple wine……..	65
4.3	Pattern of Total Soluble Solid during fermentation of pineapple and watermelon wine……..……	66
4.4	Pattern of Total Titratable Acidity during fermentation of pineapple and watermelon wine……..	67
4.6	Pattern of Alcohol production in pineapple and watermelon wine during fermentation……..…	68
4.6	Temperature of watermelon and pineapple wine during fermentation.....	69
4.7	pH of pineapple and watermelon wine during fermentation…….....	70

LIST OF APPENDICES

APPENDIX **TITLE** **PAGE**

I Production of Pineapple Wine from Pineapple Juice Using *Kloeckera apiculata*……..106

II Production of Watermelon Wine from Watermelon Juice Using *Kloeckera apiculata*…….107

LIST OF ABBREVIATIONS

AAB: Acetic Acid Bacteria

ANOVA: Analysis of variance

API: Analytical profile index

ARA: Arabinose

C.F: Crude Fibre

C.P: Crude Protein

$CaCO_3$: Calcium carbonate

CAT: Catalase test,

CEL: Cellobiose

CFU/Ml: Colony Forming Unit per milliliters

CI; Citrate test

D.M: Dry Matter

DAP: Diammonium phosphate

DE: Diatomaceous earth

ESC: Esculin,

FAO: Food and Agricultural Organization

FRU; Fructose

G: Gas

Gla: Galactose

Gla: Galactose

Glu: Glucose

Glu: glucose

GRM: Gram's reaction test

IN: Indole test

KH_2PO_4: Potasium dihydrogen phosphate

LAC: Lactose

MAL: Maltose

MAN: Mannitol

MAZ: Mannose

MEL: Melebiose

MEZ: Melezitos

mg/l: Milligram per litre

Ml: Milliliters.

MLF: Malolactic Fermentataion

MOT: Motility test

MR: Methyl red

VP: Voges-proskauer

MRS: de Man Rogosa Sharpe agar

MYEPG: Malt yeast extract peptone glucose medium

NA: Nutrient agar

Na_2HPO_4: Sodium phosphate

NaCl: Sodium chloride

NaOH: Sodium hydroxide

ng/L: Nano gram per litre

NR: Nitrate reduction test

PDA: Potato dextrose agar

PDA: Potatoes Dextrose Agar

PPM: Part per million

RAF: Raffinose

RHA: Rhamnose

RIB: Ribose

RPM: Rate per minute

RVD: Rotatory Vacuum Drum

SAL: Salicin

SO_2: Sulphurdioxide

SOR: Sorbitol

SPR: Spore formation test

SUC: Sucrose

TNTC: Too numerous to count

TRE: Trehalose

U: Urease test

XYL: Xylose

YPD: Yeast Peptone Dextrose

YPG: Yeast Peptone Glucose Medium.

CHAPTER ONE

1.0 INTRODUCTION

Wine is a popular drink being enjoyed all over the world. Historians believe that wine started in the Caucasuses and Mesopotamia as early as 6000 BC (Robinson, 2006). Rigveda Amply testified that the wine is perhaps the oldest fermented product known to man. However, the actual birth place of wine is still unknown, although it had been prepared somewhere in 350 BC. (Joshi and Devender, 2005). Wine has been made in India for as many as 5,000 years. It was the early European travallers to the courts of the Mughal emperors Akbar, Jehangir and Shah Jahan in the sixteenth and seventeenth centuries who reported tasting wines from the royal vineyards (Philip, 2000).

Grapes (berries) are the main fruit that has been used for wine production. Though, the suitability of fruits other than grapes have been investigated all over the world, the amount of wine produced from non-grape fruits are insignificant. In many countries, other fruit wines from apple (Spain, France, Belgium, Switzerland and England), plum (Germany) and cashew (India) are in high demand.

Fruit is a structural part of plant that contains seeds, normally fleshy, sweet and edible in the raw states, and examples include: oranges, grapes, strawberries, juniper berries, pineapple, and water melon (Mauseth, 2003). They are ripe ovaries or carpels that contain seed (McGee, 2004). Most fruits are eaten as desserts and they can be processed into liquid products which include fruit juices, wines and other preserves like; marmalade, jams and jellies.

The pineapple and watermelon fruits are popular fruits wildly consumed in Nigeria. They are generally grown in the southern and some eastern parts of the country. Typically, pineapple is flavored and eaten fresh by consumers because of its unique characteristics (Sarah *et al*., 1997). Its flesh is a deep yellow color with good firmness and has a delicious taste

characterized by sweetness and a little acidity. Additionally, it has very intense fruity fragrance and aromatic flavour. Pineapple as a fruit crop has a lot of economic, nutritional, medicinal, and industrial importance (Sarah *et al*., 1997).

On the other hand, watermelon (*Citrullus Lanatus*) is grown for its large edible fruit, which is a special kind of berry with a hard rind and no internal division. Botanically it is called a *pepo*. The fruit has a smooth hard rind which is usually green with dark green stripes or yellow spots on it and a sweet, juicy interior flesh which is usually deep red to pink in color, but sometimes orange, yellow, or white depending on the variety with many seeds, which can be soft and white or hard and black. Watermelon fruit supplies 30 calories of energy and low amounts of essential nutrients. Only vitamin C is present in appreciable content at 10% of the daily value. Watermelon fruit is 91% water, contains 6% sugars (sucrose, fructose and glucose), and is low in fat. Wonderfully delicious and juicy melons are the great source of much-needed water and electrolytes to tame tropical summer temperatures. It quenches thirst while reboosting our body with the anti-oxidant lycopene and vitamin-A (Zohary & Hopf, 2000).

Yeasts are without doubt the most important group of microorganisms exploited by man. No other group of microorganisms has been associated with the progress and wellbeing of humans like yeasts (Anna, 2003). Yeasts are simple eukaryotic and unicellular organisms classified in the kingdom Fungi with over 1,500 species known (Kurtzman & Robnert, 1998). In wine and beer making, yeast is the "ingredient" that converts the simple sugars into ethanol and carbondioxide. The most common species used are *Saccharomyces species.* However, other species are also used. It has been known for a long time that freshly crushed grape juice harbours a diversity of yeast species, principally within the genera *Hanseniaspora* (anamorph *Kloeckera*), *Pichia*, *Candida*, *Metschnikowia*, *Kluyveromyces* and

Saccharomyces. Occasionally, species in other genera such as *Zygosaccharomyces, Saccharomycodes, Torulaspora, Dekkera* and *Schizosaccharomyces* may be present (Graham, 2008). It is also well known that many of these non-*Saccharomyces* species (especially species of *Hanseniaspora, Candida, Pichia and Metschnikowia*) initiate spontaneous alcoholic fermentation of the juice (Graham, 2008).

A typical wine contains ethyl alcohol, sugar, acids, higher alcohols, tannins, aldehydes, esters, amino acids, minerals, vitamins, anthocyanins, and minor constituents like flavouring compounds (Swami *et al.,* 2014). The wines are classified as natural wines (9-14 % alcohol), dessert and appetizer wines (15-21 % alcohol). If fermentation stops before the sugars have all been metabolized by the yeast, the finished product is a **sweet** wine. If all the sugars have been metabolized, the wine is said to be **dry** wine. Dry wine, sweet table wine, specialty wine, champagne, muscat and burgundy wines are natural wines while sweet wine, cherries, vermouth and port wines are regarded as dessert and appetizer wines. (Swami *et al.*, 2014)

Red wine is made from red grapes, which are actually closer to black in colour. There are many different types of red wines. This is considered to be the most classic in the kingdom of wines, mixing the delicious red grapes with a wide range of aromas, from oak to eucalypti, chocolate or even mint hints. The juice from most black grapes is greenish-white; the red colour comes from anthocyan pigments present in the skin of the grape. (Swami *et al.*, 2014).

White wine on the other hand is not exactly white; it is often yellow, gold or straw coloured, depending on whether it includes the skin of the grape or just the juice. White wine can be made by the alcoholic fermentation of the non-coloured pulp of green or gold coloured grapes or from selected juice of red grapes. It is treated so as to maintain a yellow transparent colour in the final product. White wines often taste lighter, crispier and more refreshing than red wine (Swami *et al*., 2014).

According to FAO (1998) fermentation is one of the most ancient and most important food processing technologies which had been neglected by scientists and policy makers especially in traditional fermented products from developing countries. Fermentation is a relatively efficient, low energy preservation process which increases the shelf-life and decreases the need for refrigeration or other form of food preservation technology. It is therefore a highly appropriate technique for use in developing countries and remote areas where access to sophisticated equipment is limited. Fermented fruit wines are popular throughout the world, and in some regions it makes a significant contribution to the diet of millions of individuals (Jolly *et al*., 2003).

Individual yeast strains that carry out fermentation possess different physiological traits. There are several traits that are highly desired in wine strains of *Saccharomyces* (Jolly *et al.*, 2003). The most important characteristic is that the strain be able to complete the fermentation, leaving little or no residual sugar. It is also critical that the strain display a steady rate of fermentation. It is problematic if the rate is too fast as well as too slow. A slow rate of fermentation becomes difficult to distinguish from problem fermentation. If the rate is too fast, the fermentation may reach too high a temperature due to the rate of heat released from metabolism. Rapid fermentations may also lead to increased loss of volatile components (Jolly *et al*., 2003).

1.1 Statement of Research Problem

The pineapple and watermelon industry in Nigeria is faced with lots of challenges ranging from fruit rejections from export markets to inadequate storage and processing facilities, as well as tropical temperature that causes fruit spoilage, coupled with unavailable markets. Also poor handling and transportation of the fruits contribute significantly to high post-harvest losses. According to (Appert, 1997) losses in pineapple fruits harvest could be as high

as 50%. Research institutions and other private companies are trying hard to reduce these losses by processing the fruits into drinks and fresh cut fruits for the local market. Little work however is done on processing the fruits into other products such as wine (Jolly *et al*., 2003).

According to Adams and Moss (1995) maize and cassava dough are fermented and maize, millet, and other cereal grains are brewed into local drinks such as 'pito' and 'brukutu' in Nigeria and in other Sub-Saharan African countries. Very little however is known about fermentation of pineapple, cashew, mango, and orange juices into wine (Au Du., 2010).

The highly perishable nature of fruits especially watermelon and pineapple lead to insecurity in fruit growers and fruit sellers because of the fear of money loss instead of profit making. The high perishability of theses fruits contribute to the seasonality of the fruits thus making it available only during a particular season which is usually very short.

1.2 Justification of the Study

In developing countries like Nigeria 20-30% of fruits produced are wasted due to lack of proper utilization, post-harvest and processing technology. By converting these fruits into value added products like wine is a smart solution for this problem. (Nwachukwu *et al*., 2006). Thus, wine production should be promoted for adding value to local fruits, imported wine reduction, job creation, income generation and rural development.

FAO (2002) and "ComisiónVeracruzana de Comercialización Agropecuaria" (COVECA, 2002) reported that there are several pineapple and watermelon varieties commonly grown in Africa, and for that matter Nigeria, that have favourable pH (4.5-6.5) and sufficient sugar levels for fermentation to occur. Keller *et al*. (2010) also noted that pineapple juice could be converted into wine in the presence of yeast. Thus, through fermentation, the highly perishable pineapple fruit could be converted into a highly nutritious wine which can be made

available all year round. Marketing of this product will ensure security for pineapple growers as wine and other alcoholic beverages will be in high demand throughout the country and the world as a whole. Thus ensuring a secure source of income for both the farmers and the investors. Wine production from other sources other than the traditional fruits has been successfully done on carrot, banana and cashew (Au Du, 2010). A little is done on watermelon and pineapple.

The possibility and the use of pineapple and watermelon for the production of wine will create employment, income generation for farmers and address the post-harvest losses associated with glut on the local market in Nigeria. The findings of this study will provide information with regards to the preservation and storage of pineapple and watermelon in particular and fruits in general. This will ultimately ensure a secured source of income for both the farmers and the investors.

1.3 Aim of the Study

The aim of this study is to isolate and identify local yeast strain for the production of sweet wine from pineapple and watermelon juice.

1.4 Objectives of the Study

The specific objectives of this work were to:

1. Isolate and identify yeast species from fruits and a local alcoholic beverage (Brukutu).
2. Screen and select yeast species with the best fermentative potentials.
3. Produce wine from watermelon and pineapple juices using the selected yeast specie.
4. Determine the microbiological and physicochemical profile of the wine produced
5. Evaluate the sensory quality of the wine produced.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 History of Wine.

The peoples of the Mediterranean began to emerge from barbarism when they learned to cultivate the olive and the vine, according to the Greek historian Thucydides in the fifth century B.C. Wine making is as old as civilization itself. Just as society finds its roots in ancient Mesopotamia, the earliest evidence we have for the cultivation of grapes and the supervised fermentation of their juices dates back to 6000 B.C. in the ancient Middle East. The Egyptians recorded the harvest of grapes on the walls of their tombs; bottles of wine were even buried with pharaohs in order that they might entertain guests in the afterlife (New York Times, 2007).

Wine was also considered a drink of the elite in ancient Greece, and it was a centerpiece of the famous symposia, immortalized by Plato and the poets of the period**.** But it was during the Roman era that wine became popular throughout society. In Roman cities wine bars were set up on almost every street, and the Romans exported wine and wine-making to the rest of Europe. Soon, production and quality of wine in other regions rivaled that of Rome herself: in A.D. 92, Emperor Domitian decreed that all of the vines in the Cahors region (near Bordeaux) be pulled out, ostensibly in favor of the wheat cultivation the empire so desperately needed, but possibly also to quell the competition with Italian wine exports (Jolly *et al*., 2003).

After the fall of Rome, wine continued to be produced in the Byzantine Empire in the eastern Mediterranean. It spread eastward to Central Asia along the Silk Route; grape wine was known in China by the eighth century. But the spread of Islam largely extinguished the wine industry in North Africa and the Middle East. Throughout Europe, wine-making was

primarily the business of monasteries, because of the need for wine in the Christian sacraments (New York Times, 2007). During this period stronger, more full-bodied wines replaced their sweeter ancient predecessors (which usually were mixed with water before drinking). During the Renaissance, the virtues of various wine regions were appreciated by the increasingly sophisticated wine drinkers, and by the 18th century the wine trade soared, especially in France, where Bordeaux became the preeminent producer of fine wines. The development of distinctive strains of wine grapes led to the production of regional wines with easily recognizable characteristics (New York Times, 2007).

In the New World the first successful wine-making occurred in the 19th century. Somewhat surprisingly, Ohio was the first region in America to successfully cultivate grapes for wine, but it was soon eclipsed by wine production in California. About this time grape cultivation first began in earnest in Australia. In the Old World, Champagne was establishing itself as a favorite luxury beverage; and fortified wines such as ports and sherries were becoming increasingly popular, especially in Britain. But despite the growing success of the industry, there was also a catastrophe: late in the century, the phylloxera epidemic destroyed many old European vines, a disaster that affected wine-making for decades. The plague was overcome by grafting cuttings of European varietal vines onto disease-resistant American rootstock (New York Times, 2007).

Today wine-making is a global industry, with most of the countries of the world producing wine. Machines that can harvest huge areas of grape farms by day or night have increased production, and modern viticultural science has ensured that the resulting product meets uniform standards, though sometimes at the expense of quality and flavor. Indeed, there has been a recent trend toward more traditional methods of wine-making such as unfiltered wines that preserve more of the grapes true character (New York Times, 2007).

2.2 Basic Principles in Wine Production

Wines made from fruit other than grapes are frequently called country wines. To understand how to make country wine, it pays to first understand why most commercial wines are made from grapes (Colby, 2013).

Grapes are well suited to this because quality wine grapes contain everything needed to make wine. Good grapes contain enough sugar that creates a preservative level of alcohol when the wine ferments. They also have sufficient acid to balance the sweetness of the wine. Additionally, ideal wine grapes supply just enough tannin to add a bit of astringency — a slight puckering feel in the mouth — which adds to the enjoyment of the beverage. This characteristic is called structure, and many wines (especially reds) are aged in oak barrels to add more tannin structure (Colby, 2013).

The "problem" with making wine from fruit other than grapes is that most fruits do not have the correct ratio of sugar, acid and tannin to make great wine. The straight forward solution to this is to simply add whatever is lacking of those three to the unfermented juice prior to fermentation (Colby, 2013). Nothing in the winemaking process can turn bad fruit into good wine, or even average fruit into good wine. Great wine comes from great fruit, so country wines should not be used as a way to dispose of inedible fruit and expect good results (Colby, 2013).

2.2.1 How Fruit Wine is Made.

Sugar content is measured in units called Brix. Wine grapes contain a higher density of sugar than any other fruit, at about twenty-four Brix when ripe. By comparison, other fruit contains six to eight Brix. From harvest to pressing to inoculation and fermentation, making wine with

fruit is not drastically different from making wine with grapes. The difference comes from the sugar content.

Fruit wine producers need to add sugar so that complete fermentation can take place. Too much sugar and the result would be a syrupy-sweet mess, too little, and the wine won't have enough alcohol content. In addition, tree fruits and berries are naturally more acidic than their vine-grown cousins. This means that fruit winemakers need to add water to the fruit juice before it ferments. Too much water and the wine would be weak and flavourless.

Making fruit wine takes patience, the willingness to oversee the process so that sugars and acids are properly balanced. Many wines lack luster, wines full of residual sweetness, have been produced, which gives fruit wine a bad name. But the most carefully crafted fruit wines are dry and relatively complex — something on par, sweetness-wise, with a Riesling. It takes research, or the services of a good beverage curator, to find a fruit wine that tastes like wine and not Welch (Dharmadhikari *et al*., 2016).

To produce fruit wine of 11-12% alcohol by volume, a sweetening material such as sugar, syrup, or concentrate should be added to raise the sugar content to 20-22°Brix. Acid adjustment is an important consideration in preparing juice for fermentation. In the case of a high acid must, the acid content can be lowered by ameliorating the must with syrup. For acidification, only the acid that is naturally present in a given fruit should be used. For example, in blueberries, citric and malic acids occur naturally and these can be added to increase acidity in blueberry must (Dharmadhikari *et al*., 2016).

Fruit musts are prone to oxidation and microbial spoilage. To prevent these conditions, addition of sulfur dioxide to must in the range of 50-75 ppm is suggested. Nitrogen is an important nutrient for yeast growth. Generally the nitrogen content in many fruits is relatively

low. To ensure vigorous and complete fermentation, the addition of yeast nutrients and diammonium phosphate is recommended (Dharmadhikari *et al*., 2016)

To produce wines with good, fruity aroma, many winemakers ferment fruit must at a relatively low (14.4-15.6°C) temperatures. In the case of apples and cherries, where the fermenting must is juice, the temperature can be regulated by employing jacketed stainless steel tanks equipped with refrigeration. Soft fruits are usually fermented as pulp and temperature regulation during fermentation is, in this case, somewhat difficult. However, effort should be made to keep the fermentation temperature preferably under 23.9-26.7°C (Dharmadhikari *et al*., 2016) Depending on the temperature, the fermentation can last from several days to a few weeks. Fruit wines are usually fermented dry (Dharmadhikari *et al*., 2016).

The young fruit wine is usually cloudy due to the particulate matter that remains in suspension. To clarify the wines, the suspended solids need to be removed. This is achieved by holding the wine in full containers and allowing the solids to naturally settle. The clear wine is racked leaving the lees behind. The process of clarification can be expedited by using various filters and a centrifuge. In general, fruit wines should be clarified as soon as possible. During wine transfers, care should be taken to protect the wine from excess air (oxygen) exposure (Dharmadhikari *et al*., 2016).

During finishing operation, many fruit wines are made into semi-sweet or sweet styles. To achieve this, sugar or concentrate is often used as a sweetening material. To prevent refermentation of the sugar added, sorbic acid is usually added. The finished wine is polish filtered or membrane (0.45 micron) filtered and bottled. Fruit wines are also relatively more susceptible to oxidation, loss of color, and microbial spoilage (Dharmadhikari *et al*., 2016).

Organic acid composition is another key difference in the composition of fruit as compared to grapes. The main acids in the fruits being considered here are malic and citric. Generally these acids tend to give higher pH values, and they are not biologically stable. It is important to note that generally higher pH above 3.6-4.0 encourages the activity of spoilage microbes, and oxidation of wine. It results in poor color, has an impact on wine stability and the action of preservatives, such as sulphurdioxide and sorbic acid (Dharmadhikari *et al*., 2016).

To protect wines from oxidation, judicious use of sulphur dioxide and use of inert gases during wine transfer and storage is recommended. Sulphur dioxide also plays a key role in discouraging malolactic fermentation and microbial spoilage. Its effectiveness is influenced by pH. Periodic adjustment and the maintenance of an adequate amount of free sulphur dioxide in wine is very crucial to making fruit wines. Following rigorous cleaning and sanitizing practices, is a must for fruit wine production although other alternative exist such as pasteurization of the must before the fermentation process. Use of preservatives should not be relied upon as a substitute for sound cleaning and sanitizing measures. Fruit wines are generally produced as sweet wines. They rarely benefit from prolonged aging. They should therefore be produced for early consumption (Dharmadhikari *et al*., 2016).

In order to learn the alcohol concentration of the wine, you need to know the quantity of sugar. The resulting quantity of alcohol in the wine is approximately half of the quantity of the sugar, so assuming that you want to create an assortment with 16% alcohol, then it should originally contain 32°brix sugar (Claire, 2012).

Furthermore, adding sorbate to the wine prior to bottling it is an effective way to ensure that the level of sulfur dioxide is maintained between 30 and 50 PPM (Claire, 2012).

Residual sugar is the sugar from the fruits that is left over after fermentation; more residual sugar makes a sweet wine, and the absence of residual sugar makes a dry wine (Curmudgeon, 2015) Residual sugar can be either sugars that the yeast did not ferment or sugar that the winemaker added after the wine fermented, or both. Alternatively, winemakers often use corn sugar — or dextrose, which is another name for glucose — because yeast ferments it at a higher rate, and therefore reduces the risk of ending up with a sweet wine. Wines also contain very small concentrations of unfermentable sugars — sugars that yeast cannot convert to alcohol, such as pentose, contribute to residual sugar. Although sweetness due to residual sugars may not be detected, glycerol — a minor by-product of alcoholic fermentation, also referred to as glycerine — and ethyl alcohol also contribute sweetness, confusing our taste with actual residual sugar content. A word of caution! Any wine measuring greater than -1.0° Brix — or equivalently having a specific gravity greater than 0.995 — when measured with a hydrometer should be stabilized with potassium sorbate to prevent bottle fermentation. If wine starts refermenting in the bottle, the bottle may explode. If you are making an off-dry or sweet wine, add sorbate at the rate of 10 g/hL (approximately one teaspoon per 20-liter or five-gallon batch) to wine in which sulfite has been added to a concentration of 25–50 mg/L (Pambianchi, 2002).

2.2.2 Economic Considerations in Fruit Wine Production

Definitions for fruit wine vary slightly, but it is generally described as the alcoholic fermentation of the juice of sound, ripe fruit, fruit juices or concentrate, other than juice from fresh grapes. A number of fruit or related species on the Prairies can be used to make fruit wine. These include strawberries, raspberries, black currants, saskatoons, sour cherries (Mongolian and Nanking), wild black cherries (chokecherry), apples/crabapples, plums, red

and white currants, and rhubarb (commonly viewed as a fruit), pineapples and watermelon (Dyck, 2008).

The trends look promising for products such as fruit wines. A 2004 report from Agriculture and Agri-Food Canada (AAFC) on the Canadian wine industry and recent Statistics from Canada reports indicate that there is room for growth in the domestic market. Annual wine consumption in Canada has increased between 1991 and 2004 from 10.06 litres per capita to 13.31 litres per capita, for the population over 15 years of age. However, Canadian per capita wine consumption is very low compared to other major wine-producing countries such as France or Italy, where per capita consumption is between 50 and 56 litres.

With this room for growth, the wine industry has increased capital expenditures by approximately 12 percent annually between 1995 and 2005. Growth in this sector has continued from previous years. The growth is attributed to the industry's ambitious growth plans, and some large wineries are making acquisitions into other lines of alcoholic products such as fruit wines, ciders and hard lemonade (Dyck, 2008). Recent data revealed that from 2000-2004, the value of fruit wine imports has continued to rise at an average rate of 12 per cent. While these figures represent larger scale wineries, this shows growing interest in non-traditional alcoholic beverages. They do not account for the many cottage-sized wineries that mostly focus on domestic sales or selling at the farm gate (Dyck, 2008).

2.2.3 Fermentation Systems

Fermentation processes utilize microorganisms to convert solid or liquid substrates into various products. Commonly consumed fermented product includes bread, cheese sausage, pickled vegetables, cocoa, beer, wine, citric acid, glutamic acid and soy sauce (Chisti, 1999).

Most commercially useful fermentations may be classified as either solid-state or submerged cultures. In solid state fermentation, the microorganism grows on a moist solid with little or no "free" water, although capillary water may be present. Examples of this type of fermemtation are seen in mushroom cultivation, bread making and processing of cocoa. Submerged fermentations may use a dissolved substrate, e.g. sugar solution, or a solid substrate suspended in large amount of water to form a slurry. Submerged fermentation is used for pickling vegetables, producing yoghurt, brewing beer and producing wine and soy sauce.

Solid-state and submerged fermentations may each be subdivided into oxygen- requiring aerobic processes and anaerobic processes that must be conducted in the absence of oxygen. Examples of aerobic fermentation include submerged-culture citric acid production by *Aspergillus niger* and solid state koji fermentations (used in the production of soy sauce). A submerged–culture anaerobic fermentation occurs in yoghurt making (Chisti, 1999).

Fermentation may require only a single species of microorganism to effect the desired chemical change. In this case the substrate may be sterilized, to kill unwanted species prior to inoculation with the desired microorganism. However, most food fermentations are non-sterile. Typically, fermentation used in food processing requires the participation of several microbial species, acting simultaneously or sequentially to give a product with the desired properties, including appearance, aroma, texture and taste. In non- sterile fermentations, the culture environment may be tailored specifically to favor the desired organisms. For example the salt content may be high, the pH may be low, or the water activity may be reduced by additives such as salt or sugar.

Submerged fermentation may be carried out either batchwise, as fed-batch operations or as continous cultures. Batch and fed-batch operations are quite common, continous fermentations being relatively rare.

In batch processing, the fermenter is first filled with the raw material or substrate (carbon source). Then microbes are added and allowed to ferment the raw material under optimum pH and aeration. The product remain in the fermenter until completion of the fermentation. After fermentation, the products are extracted and the fermenter is cleaned and sterilized before the next round. Batch fermentation typically extend over 4-5 days.

In fed batch fermentations, sterile culture medium is added either continuously or periodically to the inoculated fermentation batch. The volume of the fermenting broth increases with each addition of the medium, and the fermenter is harvested after the batch time.

In continuous fermentations, sterile medium is fed continuously into a fermenter and the fermented product is continuously withdrawn, so the fermentation volume remains unchanged. In a "well-mixed" continous fermenter the feed rate of the medium should be such that the dilution rate, i.e. the ratio of the volumetric feed rate to the constant culture volume, remains less than the maximum specific growth rate of the microorganism in the particular medium and at the particular fermentation condition. If the dilution rate exceeds the maximum specific growth rate, the microorganism will be washed out of the fermenter (Chisti, 1999).

Industrial fermentations are mostly batch operations. Typically a pure starter culture (or seed), maintained under carefully controlled conditions, is used to inoculate sterile petri-dishes or liquid medium in the shake flasks. After sufficient growth, the pure culture is used

to inoculate the “seed” fermenter. A culture in rapid exponential growth is normally used for inoculation. Slower-growing organism requires larger inocula, to reduce the total duration of the fermentation. An excessively long fermentation time reduces productivity (amount of product produced per unit time per unit volume of fermenter), and increases cost (Chisti, 1999).

2.2.3.1 Factors influencing fermentation

A fermentation is influenced by numerous factors, including temperature, pH, nature and composition of the medium, dissolved carbondioxide, operational system (e.g. batch, fed batch and continous), feeding with precussors, mixing (cycling through varying environment), and shear rates in the fermenter. Variations in these factors may affect: the rate of fermentation; the product spectrum and yield; the organoleptic properties of the product (appearance, taste, smell and texture); the generation of toxins; nutritional quality and other physico-chemical properties.

The formulation of the fermentation medium affects the yield, rate and product profile. The medium must provide the necessary amounts of carbon, nitrogen, trace elements, and micro nutrients (e.g. vitamins). Specific types of carbon and nitrogen sources may be required, and the carbon: nitrogen ratio may have to be controlled. Concentrations of certain nutrients may have to be varied in a specific way during a fermentation to achieve the desired result. Some trace elements may have to be avoided- for example minute amount of iron reduce yields in citric acid production by *Aspergillus niger*. Additional factors such as cost availability also affect the choice of a medium.

2.2.4 Product Recovery

Recovery of wine and juice from lees is a challenging yet unavoidable task for wineries around the world. The high concentration and variability of the solids makes filtration difficult, however, with the lees comprising up to 10% of a winery's total production volume there is still a high percentage of recoverable wine and juice. Traditionally lees filtration is performed with rotary vacuum drum (RVD) filters or chamber press filters. While these systems can handle the high solids, the open design allows for high oxygen pick up which can negatively impact the product quality. The recovered wine or juice is often downgraded in value and used in lower-tier blends instead of being added back to the original batch (Beverage, 2012). The existing technologies can also require high volumes of filter aids like diatomaceous earth (DE) or perlite which increase the wineries waste generation and disposal requirements, contributing to high operational costs to overcome the drawbacks of rotary vacuum drum (RVD) and chamber press filters, Pall developed the Oenoflow™ HS system.

2.2.4.1 Alternative for Product Recovery

The new system developed by Pall called the Oenoflow™ HS system utilizes microporous membranes that are similar to the hollow fiber membranes that have become the standard in wine clarification applications. The difference with the new Oenoflow HS membranes is that the hollow fibers have a larger internal diameter so that they can process higher solids, up to about 80% by volume. Since filtration is achieved with membranes and without the need for filter aids, operation is more hygienic and does not create waste for environmental landfill. One of the biggest benefits that wineries realize by implementing the Oenoflow HS system is improved filtrate quality, which results in a higher value of the recovered wine or juice. The recovered wine or juice can be blended back to the original batch instead of being used in lower-tier products. Oenoflow HS is a closed system with minimal oxygen pick up, typically

in the range of 0.2 ppm. This is a big improvement when compared to rotary vacuum drum (RVD) filters, which often have oxygen pick up of 4-5 ppm or higher. In addition, the microfiltration membranes provide improved and more consistent filtrate clarity compared to an rotary vacuum drum (RVD) filter or chamber press (Beverage, 2012).

2.3 Microorganisms Potentially Useful for Wine Production

Wine is the product of a complex biological and biochemical interaction between fruits (fruit juice) and different microorganisms (fungi, yeasts, lactic acid bacteria and acetic acid bacteria) and the Mycoviruses and Bacteriophages affecting them (Fleet, 2003). Furthermore, although wine is directly determined by fruit variety, yeasts also affect wine flavour and quality by the production and excretion of metabolites during growth and through autolysis (Fleet, 2003).

2.3.1 The Use of Non-Saccharomyces Yeast

The contribution by the numerous grape-must-associated non-*Saccharomyces* yeasts to wine fermentation has been debated extensively. These yeasts, naturally present in all wine fermentations, are metabolically active and their metabolites can impact on wine quality. Although often seen as a source of microbial spoilage, there is substantial contrary evidence pointing to a positive contribution by these yeasts. Consequently, the impact of non-*Saccharomyces* yeasts on wine fermentations cannot be ignored. They introduce into the process an element of ecological diversity that goes beyond *Saccharomyces species a*nd they require specific research and understanding to prevent any unwanted consequences they might cause or to exploit their beneficial contributions. The role of non-*Saccharomyces* yeasts in wine fermentation is therefore receiving increasing attention by wine microbiologists in Old and New World wine producing countries. Species that have been

investigated for wine production thus far include those from the *Candida, Hanseniaspora, Zygosaccharomyces, Schizosaccharomyces, Torulaspora, Brettanomyces*, *Saccharomycodes, Pichia* and *Williopsis* genera (Jolly *et al*., 2006; Martini *et al.*, 1996)

Kloeckera apiculata is one of the dominant yeasts in several early natural fermentation stages. Besides that, the yeast seems to have some interesting enzymes such as beta-D-glucosidase and beta-D-xylosidase which are key enzymes to release aromatic compounds in winemaking (Kurtzman *et al.,* 2011). Since *Kloeckera apiculata* is negative for maltose fermentation and actually only capable of fermenting glucose, it is very unlikely that a single *Kloeckera apiculata* beer fermentation would work (as mainly maltose is present in wort). *Kloeckera apiculata* however should work very well with Cidre and other natural fruit juices where the most dominant sugar is glucose.

According to Van Zyl and Du Plessis (1961), the following yeasts occurred in highest frequency in South African vineyards: *Kloeckera apiculata, Rhodotorula glutinis, Candida krusei, Candida pulcherrima, Candida laurentii, Cryptococcus albidus*, and *Candida stellata* (*T. bacillaris*). In a more recent, but limited, investigation (Jolly *et al*., 2003), *Kloeckera apiculata, Candida pulcherrima* and *Rhodotorula specie* were still found in dominant numbers but *Kluyveromyces thermotolerans* and *Zygosaccharomyces bailii* were also isolated. Studies by Le Roux. (1973) showed that *Botrytis cinerea* infection of grapes influenced the non-*Saccharomyces* populations – *C. krusei and K. apiculata* increased while *R. glutinis* decreased.

2.3.2 The use of Bacteria

In the 1890s, Müller Thurgau showed that bacteria, not yeast, were required to reduce acidity in wine, by a process we now know as malolactic fermentation (MLF). The importance of malolactic fermentation became evident in the production of Burgundy and wines from California, where it was realized that malolactic fermentation improved wine quality, practices were subsequently employed to be sure of its occurrence. It was not until the late 1960s that the principal bacterium responsible for Malolactic fermentation was characterized. At that time it was identified as *Leuconostoc oenos*, later to be reclassified as *Oenococcus oeni*. Over the last fourty years, *O. oeni's* role in winemaking was studied intensely. In addition, the production of secondary metabolites by *O. oeni* augments the sensory qualities of wine; in other words, malolactic fermentation imparts aroma and flavor attributes to wine. Acetic acid bacteria (AAB) are part of the natural winemaking microflora, however, unless provided with conditions suitable for their growth they just 'hang around' not causing any problems. Give them oxygen and they flourish. Therefore, wine is at high risk of spoilage by acetic acid bacteria during prolonged barrel maturation. Poor management during bottling and subsequent storage of red wine and fruit wine can also give rise to spoilage (Eveline *et al.*, 2009). Two groups of bacteria are particularly significant in wine microbiology—Lactic acid bacteria and Acetic acid bacteria. The pH and ethanol tolerance of species within these groups are main factors that select for their occurrence in winery ecosystems. Within the Lactic acid bacteria, species of *Lactobacillus* and *Pediococcus* are most relevant, along with *Oenococcus oeni* (formerly *Leuconostoc oenos*) which conducts the malolactic fermentation. Within the Acetic acid bacteria, *Acetobacter aceti*, *Acetobacter pasteurianus* and *Gluconobacter oxydans* are commonly isolated, although *Acetobacter liquefaciens* and *Acetobacter hansenii* have recently been found. The malolactic fermentation is an important secondary fermentation that occurs in many wines, generally, about 2–3

weeks after completion of the alcoholic fermentation (Lonvaud-Funel, 1999). Lactic acid bacteria, principally *O. oeni*, are responsible for this fermentation. These bacteria are naturally resident in the wine or commercial strains may be inoculated. Growth of *O. oeni* during this fermentation functions to decrease wine acidity by transforming L-malic acid into L-lactic acid, enhance wine flavor and complexity through production of additional metabolites, and to increase subsequent microbiological stability of the wine by removal of residual nutrients and production of bacteriocins (Fleet, 2001).

2.3.3 Succession of Microorganisms

Generally, species of *Hanseniaspora (Kloeckera), Candida* and *Metschnikowia* initiate the fermentation, and largely originate from the grapes or fruits. Sometimes, species of *Pichia*, *Issatchenkia* and *Kluyveromyces* may also grow at this stage. These yeasts grow to about 10^6–10^7cfu/ml but, by mid-fermentation, begin to decline and die off. At this time, *S.cerevisiae* becomes predominant (10^7–10^8cfu/ml) and continues the fermentation until its completion.

In addition to the successional growth of different yeast species throughout the course of fermentation, there is an underlying successional development of strains within each species. This latter revelation became most evident with the use of molecular techniques that enabled strain differentiation and recognition (Pretorius, 2000; Fleet, 2001).Up to five or more strains of *S. cerevisiae* have been found in one ferment, and similar findings have been reported for some non-*Saccharomyces species* (Fleet, 2001).

Ethanol production by *S. cerevisiae* is considered to be a major factor that governs the growth and influence of non-*Saccharomyces* species during fermentation. Generally, the species of *Hanseniaspora, Candida, Pichia, Kluyveromyces, Metschnikowia and Issatchenkia* found in

fruit juice and grape juice are not tolerant of ethanol concentrations exceeding 5 –7%, and this explains their decline and death as the fermentation progresses beyond the mid-stage (Fleet, 2003). However, low temperatures decrease the sensitivity of these species to ethanol and consequently, wine fermentations conducted at temperatures less than 15 – 20°C may show a greater contribution from *Hanseniaspora* and *Candida species*. On such occasions, these species may equal *S. cerevisiae* as the predominant species at the end of fermentation and, accordingly, would have a greater impact on wine flavor (Fleet, 2003). There are recent reports of some strains of *Candida species* that may have ethanol tolerances similar to *S. cerevisiae* (Fleet, 2003). Strains of *Candida stellata* fall into this category and have been used in co-culture with *S. cerevisiae* to enhance the glycerol content and other flavour characteristics of wines (Fleet, 2003).

Schizosaccharomyces pombe, *Zygosaccharomyces bailii* and *Zygosaccharomyces fermentatiare* well known for their tolerance of high ethanol concentrations (>10%) and occur in winery environments (Fleet, 2003). Surprisingly, they are rarely reported as contributors to grape juice fermentations and the reasons for this require investigation. Possibly, they grow slower than other wine yeasts and, consequently, are out-competed, or they may be inhibited by factors produced by these yeasts. All three species have the ability to utilize malic acid, which is a positive attribute in many winemaking instances. While some strains of these species are known to produce off-flavours, a program of selection and evaluation could reveal strains with desirable flavor attributes.

2.3.4 Strain Tolerance

The increase in ethanol concentration during alcoholic fermentation could also explain the sequential growth of strains within a species. Strains of *S.cerevisiae*, as well as those of other species, vary in their tolerance to ethanol stress (Fleet, 2003). Strains with higher ethanol

tolerance are more likely to dominate at later, rather than earlier stages of fermentation. This behavior has been demonstrated experimentally, along with the interactive effect of fermentation temperature (Torija *et al.*, 2002), and becomes an important consideration in designing mixtures of yeast strains (oligo-strains) for use as cultures to enhance the complexity of wine flavor.

The non-*Saccharomyces* yeasts appear to be less tolerant of very low oxygen availability than *S. cerevisiae*. Removal of residual oxygen from fermentating grape juice by the vigorous growth of *S. cerevisiae* could contribute to the early death of these non-*Saccharomyces* species (Hansen *et al*., 2001). *Non-Saccharomyces* species growing early in the fermentation could utilize amino acids and vitamins, and limit the subsequent growth of strains of *S. cerevisiae*. There are reports that *Kloeckera apiculata* could strip the grape juice of thiamine and other micronutrients, leading to deficient growth of *S. cerevisiae* (Fleet, 2003). However, some non-*Saccharomyces species*, such as *Kloeckera apiculata* and *Candida pulcherrima* are significantly proteolytic and could generate amino acids for use by *S. cerevisiae*. The early death and autolysis of these non-*Saccharomyces* yeasts is another possible source of nutrients for *S. cerevisiae*, and spoilage yeasts. Cell wall polysaccharides, principally mannoproteins, are also released by yeast autolysis and these could combine with tannins and anthocyanins to impact on wine astringency and colour (Fleet, 2003). Although many winemaking variables affect the expression of killer and killer-sensitive phenotypes, there is good evidence that killer interactions may determine species and strain evolution during fermentation. Killer strains of *S. cerevisiae* sometimes predominate at the completion of fermentation, suggesting that they have asserted their killer property and taken over the fermentation. Killer strains have been found within wine isolates of *Candida*, *Pichia* and *Hanseniaspora* and some of these can assert their killer action against wine strains of *S. cerevisiae* (Fleet, 2003).

2.3.4 Detrimental effect of Non-Saccharomyces Yeast

Despite a long held belief among winemakers in Old World wine regions that spontaneous fermentations (comprising mixed cultures of non-*Saccharomyces* and *Saccharomyces* yeasts) produce superior wines compared with pure culture fermentations, earlier authors usually refer to the non-*Saccharomyces* yeasts as spoilage organisms or 'wild yeasts'. This was substantiated by their frequent isolation from stuck fermentations and from spoiled bottles of wine.

Furthermore, although it was known that some non-*Saccharomyces* yeasts could form metabolites, e.g. esters, leading to aromas not always detrimental to wine quality, this was outweighed by the high levels of volatile acids and other undesirable compounds produced. Some yeasts, e.g. *Candida, Pichia* and *Hansenula specie* are capable of forming films on the surface of wine exposed to oxygen. Off-odors, including acetic acid, ethyl acetate and acetaldehyde are also associated with their growth (Granchi *et al.*, 1999). *Brettanomyces specie* and *Dekkera specie* can contribute to 'animal/farmyard/mousy' taints in wines. It has also been reported that *Brettanomyces bruxellensis* can form biogenic amines (Caruso, 2002) that can lead to undesirable physiological effects in sensitive humans. Other non-*Saccharomyces* yeasts such as *Saccharomycodes ludwigii*, more commonly a contaminant of sulphated musts due to its high resistance to Sulphur dioxide, produce large amounts of ethyl acetate and acetaldehyde that negatively affect wine aroma and quality (Ciani , 1998).

Authors of earlier publications also considered non-*Saccharomyces* yeasts to be sensitive to sulphur dioxide in must, and added sulphur dioxide primarily to control their growth and that of spoilage bacteria. Non-*Saccharomyces* yeasts were also known to be poor fermenters of grape must and intolerant to ethanol (Castor *et al.*, 2002), especially in the presence of sulphur dioxide. It was therefore accepted that those non-*Saccharomyces* yeasts not initially

inhibited by the sulphur dioxide died during fermentation due to the combined toxicity of the $S0_2$ and alcohol. Consequently, the non-*Saccharomyces* yeasts were seen to be of little significance in normal wine production and it was recommended that only proven strains of the wine yeast *S. cerevisiae* be used in commercial fermentations.

2.4 Sources of Yeast for Wine Production

Essentially, there are two strategies for obtaining wine yeasts which include (1) Isolation from natural sources and (2) Genetic improvement of natural isolates. Once a prospective isolate has been obtained, it is screened in laboratory trials for essential oenological criteria. Isolates meeting acceptable criteria are then used in micro-scale wine fermentations and the resulting wines are then subjected to sensory evaluation. Strains giving good fermentation criteria and acceptable-quality wines under these conditions are then selected for further development as starter culture preparations.

2.4.1 Natural sources

Generally, wine yeasts for starter culture development have been sourced from two ecological habitats, namely, the vineyard (primarily the grapes or fruits) and spontaneous or natural fermentations that have given wines acceptable or unique quality.

As mentioned already, yeasts are part of the natural microbial communities of grapes and fruits (Fleet *et al.*, 2002). Moreover, there is an attraction that unique strains of yeasts will be associated with particular grape or fruit varieties in specific geographical locations and, through this association, they could introduce significant diversity and regional character or ‘terroir’ into the winemaking process. Lesser numbers are found on winery equipment.

The microflora of grapes and fruits are affected by a number of factors. These include vineyard altitude and aspect, climatic conditions (temperature, rainfall, humidity, maritime influences), grape or fruit variety (cultivar, thickness of grape skin), viticultural practices (fertilization, irrigation, canopy management, use of fungicides, use of elemental sulphur), developmental stage of grapes or fruits, health of fruits and grapes (physical damage to berries, insect pests) and winery waste-disposal practices (Jolly *et al* ., 2006). The manner in which grapes and fruits are sampled (e.g. the berries or bunches) and processed (washing vs. crushing) can also determine what yeasts are isolated (Martini *et al*., 1996), as the number of yeast cells is greater close to the peduncle than it is at the center and lower part of the bunch.

At harvest, grapes and fruits temperature, method of harvest (manual vs. mechanical), method of transport to the cellar (picking cratesibaskets, tipsters), time of transport to the cellar, time lapse before crushing, and sulphite and enzyme addition can all affect yeast populations (Pretorius, 2000). Yeasts found on the surface of grapes and other fruits are introduced into the must at crushing. Other strains found on the surface of cellar equipment can also be transferred to the must (Boulton *et al*., 1996). Populations are further affected by the method of crushing, i.e. pressing whole bunches vs. berries, sulphite addition, enzyme addition, cellar hygiene, types of equipment used, clarification method and temperature control (Pretorius, 2000).

The specific environmental conditions in the must, i.e. high osmotic pressure, presence of $S0_2$, and temperature, all play a role in determining what species can survive and grow. The fermentation rapidly becomes anaerobic and the alcohol levels increase, which further affects yeast populations (Jolly *et al.*, 2006).

2.4.2 Genetic Improvement of Isolates

Through genetic improvement and metabolic engineering technologies, it is now possible to develop wine yeasts with a vast array of specific functionalities. Basically, the desired property is defined (e.g. strain with enhanced glycerol production for yeast; strain with bacteriocin production for bacteria), and then the appropriate genetic tools are applied to construct the strain with that property (Fleet, 2008).

2.5 Criteria Used for Selecting Yeast for Wine Production

Basically, these criteria can be considered under three categories:

(1) Properties that affect the performance of the fermentation process: Fast, vigorous and complete fermentation of grape juice or fruit juice sugars to high ethanol concentrations (48% v/v) are essential requirements of wine yeasts. The yeast should be tolerant of the concentrations of sulphur dioxide added to the juice as an antioxidant and antimicrobial, exhibit uniform dispersion and mixing throughout the fermenting juice, produce minimal foam and sediment quickly from the wine at the end of fermentation. It is most important that the yeast does not give slow, sluggish or stuck fermentations (Fleet, 2008)

(2) Properties that determine wine quality and character: With respect to wine quality and character, it is essential that any yeast produces a balanced array of flavor metabolites, without undesirable excesses of volatiles such as acetic acid, ethyl acetate, hydrogen sulphide and sulphur dioxide. It should not give undesirable autolytic flavor after fermentation. It should not adversely affect wine color or its tannic characteristics. In essence, the yeast must give a wine with a good clean flavor, free of sensory faults, and allow the fruit varietal character to be perceived by the consumer (Fleet, 2008).

(3) Properties associated with the commercial production of wine yeasts: Companies that produce yeasts for the wine industry also have basic needs that must be built into the selection and development process. The cost of production needs to be contained so that the final product is affordable to the wine industry. Consequently, the yeast must be amenable to large-scale cultivation on relatively inexpensive substrates such as molasses. Subsequently, it needs to be tolerant of the stresses of drying, packaging, storage and, finally, rehydration and reactivation by the winemaker (Soubeyrand *et al.*, 2006). These requirements need to be achieved without loss of the essential and desirable winemaking properties (Fleet, 2008).

2.6 Method of Improving Yield in Wine Fermentation

Alcoholic fermentation is conducted as a batch process, where the juice is placed in tanks or barrels, and kept there until the fermentation is completed. Good understanding of the growth and biochemical activities of yeasts in these batch systems, as well as ability to control variables such as temperature, agitation and aeration, have ensured the widespread successful use of this technology and production of better wines (Fleet, 2008).

Utilizing appropriate genetic tools to produce strain of yeast from natural available yeast that would poses the following property will lead to an improve yield in wine making.

(1) Improved fermentation performance (e.g. yeasts with greater efficiency in sugar and nitrogen utilization, increased ethanol tolerance, decreased foam production).

(2) Improved process efficiency (e.g. yeasts with greater production of extracellular enzymes such as proteases, glucanases and pectinases to facilitate wine clarification; yeasts with altered surface properties to enhance cell sedimentation, floatation and floc formation, as needed; and yeasts that conduct combined alcoholic-malolactic fermentations).

(3) Improved control of wine spoilage microorganisms (e.g. yeasts producing lysozyme, bacteriocins and sulphur dioxide that restrict spoilage bacteria).

(4) Improved wine wholesomeness (e.g. yeasts that give less ethanol, decreased formation of ethyl carbamate and biogenic amines, increased production of resveratrol and antioxidants).

(5) Improved wine sensory quality (e.g. yeasts that give increased release of grape terpenoids and volatile thiols, increased glycerol and desirable esters, increased or decreased acidity and optimized impact on grape phenolics) (Fleet, 2008).

2.7 Application /Uses of Wine

2.7.1 Health Benefits

2.7.1.1 Reducing risk of depression

A team from several universities in Spain reported in the journal *BioMed Central Medicine* that drinking wine may reduce the risk of depression. The researchers gathered data on 2,683 men and 2,822 women aged from 55 to 80 years over a seven-year period. The participants had to complete a food frequency questionnaire every year, which included details on their alcohol consumption as well as their mental health (Nordqvist, 2016). The authors found that men and women who drank two to seven glasses of wine per week were less likely to be diagnosed with depression. Even after taking into account lifestyle factors which could influence their findings, the significantly lower risk of developing depression still stood (Nordqvist, 2016).

2.7.1.2 Preventing colon cancer and breast cancer

Scientists from the University of Leicester, UK, reported at the 2nd International Scientific Conference on Resveratrol and Health that regular, moderate red wine consumption can

reduce the rate of bowel tumors by approximately 50% thereby preventing colon cancer (Nordqvist, 2016). Regular consumption of most alcoholic drinks increases the risk of breast cancer. However, red wine intake has the opposite effect, researchers from Cedars-Sinai Medical Center in Los Angeles found in the *Journal of Women's Health*, the scientists explained that chemicals in the skins and seeds of red grapes reduce estrogen levels while raising testosterone in premenopausal women – which results in a lower risk of developing breast cancer. The authors emphasized that it is not just the red wine that has the beneficial compounds, but its raw material – red grape. They suggested that when women are choosing an alcoholic drink to consume, they should consider red wine. They reiterated that they were not encouraging wine over grapes (Nordqvist, 2016). Dutch scientists reported on a study that looked at the effects of resveratrol, red wine, and white wine on lung function.

They found that:

1. Pure resveratrol was good for lung function
2. White wine was also good for lung function
3. Red wine made no difference

A reviewer of the study wrote "Resveratrol may well be just the bystander of something else present in wine. The beneficial effects on lung function are probably related to many compounds present in wine, and not just resveratrol." According to a number of scientific studies, moderate wine drinkers appear to enjoy better lung function, the authors added (Nordqvist, 2016).

In another study, a team from Kaiser Permanente wrote in the journal *Cancer Epidemiology, Biomarkers and Prevention* that red wine consumption may reduce lung cancer risk. Chun Chao, said "An antioxidant component in red wine may be protective of lung cancer,

particularly among smokers. Thus the International Agency for Research on Cancer of the World Health Organization has classified alcohol as a Group 1 carcinogen (Nordqvist, 2016).

2.7.1.3 Preventing Dementia.

A team from Loyola University Medical Center found that moderate red wine intake can reduce the risk of developing dementia. In this study, the researchers gathered and analyzed data from academic papers on red wine since 1977. The studies, which spanned 19 nations, showed a statistically significantly lower risk of dementia among regular, moderate red wine drinkers in 14 countries. The investigators explained that resveratrol reduced the stickiness of blood platelets, which helps keep the blood vessels open and flexible. This helps maintain a good blood supply to the brain (Nordqvist, 2016). Both white and red wines contain resveratrol, but red wine has much more. The skin of red grapes has very high levels of resveratrol. During the manufacturing process of red wine there is prolonged contact with grape skins. Lead investigator, Prof. Edward J. Neafsey, said "We don't recommend that nondrinkers start drinking. But moderate drinking, if it is truly moderate, can be beneficial." Neafsey and colleagues wrote in *The Journal of Neuropsychiatric Disease and Treatment* that moderate red wine drinkers had a 23% lower risk of developing dementia compared to people who rarely or never consumed the alcoholic beverage (Nordqvist, 2016).

2.7.1.4 Protecting from severe sunburn

Wine and grape derivatives can help reduce the damaging effects of UV (ultraviolet) light, scientists from the University of Barcelona in Spain reported in *The Journal of Agricultural Food and Chemistry*. The authors explained that when Ultra Violet rays make contact with human skin, they activate reactive oxygen species (ROS), which oxidize fats, DNA and other large molecules, which in turn stimulate other enzymes that harm skin cells. Flavonoids,

found in wine and grapes, inhibit the formation of the reactive oxygen species in skin cells that are exposed to sunlight (Nordqvist, 2016).

2.7.1.5 Preventing blinding diseases

Red wine can stop the out-of-control blood vessel growth in the eye that causes blindness, researchers at Washington University School of Medicine in St. Louis reported in the *American Journal of Pathology* (Nordqvist, 2016). Diabetic retinopathy and age-related macular degeneration, which is the leading cause of blindness among Americans aged 50+ years, are caused by an overgrowth of blood vessels (angiogenesis) in the eye. The researchers explained that resveratrol is the compound in wine that protects vision. Grapes, blueberries, peanuts, watermelon and some other plants are rich in resveratrol. Therefore it can also be found in fruit wines (Nordqvist, 2016).

2.7.2 Fruit Cleaner

The alcohol and resveratrol, a phytochemical in red wines and fruit wines can kill off bacteria that cause food-borne illness such as *Salmonella* and *E. coli* by just running a little wine over your fruit after rinsing it off with water, dry it off and enjoy. Which means that it is free from germs (Mehmet *et al*., 2015).

2.7.3 An Anti-Aging effect.

It's believed that the powerful antioxidants in the fermented grapes and fruits may form and increase the skin's elasticity. This could be achieved by pouring one glass of wine straight into the bathing water 1-2 times a week and soaking for 30 minutes to get the anti-aging and skin-smoothing benefits desired (Mehmet *et al.,* 2015). Leftover red wines and fruit wines

can be used since its reparative resveratrol and exfoliating tartaric acid soften, disinfect, and regenerate the skin, leaving it looking smoother (Jhaveri, 2014).

2.7.4 Cooking Substitute

Swapping red wine for heavy sauces or oil is a healthy, lighter alternative. A half a cup of oil has 950 calories, but the same amount of red wine and fruit wines only has 98 calories– saving more than 850 calories (Mehmet *et al.,* 2015).

2.7.5 Compost Addition

Usually leftover red wines and fruit wines are poured into a compost bin to activate the bacteria that will turn the compost into fertilizer in the garden (Mehmet *et al*., 2015).

2.7.6 Double Down on Stains

If red wine is spilled, its white brethren can come to your rescue. White wine absorbs the color from red wine stain. Just by letting it soak for a moment and then dab both liquids up (Mehmet *et al*., 2015). White wine is known to help eliminate red wine stains by pouring some over the tainted area, then let it soak for 10 minutes, and rinse with tepid water (Jhaveri, 2014).

2.7.7 Reduce the Risk of Osteoporosis

Resveratrol, the natural compound in wine, is believed to make the human bones better. A recent study cited the phytochemical as a potential treatment for osteoporosis, by helping stimulate bone-forming cells within the body (Mehmet *et al*., 2015).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Collection of Samples

One each of spoilt watermelon (*Citrulus vulgaris*) and spoilt pineapple (*Ananas comosus*) fruits were collected from Samaru market in Zaria metropolis in Kaduna State from which the yeasts used in fermentation were isolated while six clean, firm, healthy and ripe watermelon and pineapple fruits used as substrate for the production of the wine were also collected from the same source and labelled appropriately. All samples obtained were collected aseptically in clean polythene bags and transported to the laboratory.

Burukutu alcoholic drink was purchased in Zaria metropolis in Kaduna State, Nigeria. It was collected aseptically using a clean bowl and transferred aseptically into sterile containers that had already been autoclaved in the laboratory with fitted seal. The containers were then well sealed, labelled and transferred to the laboratory in sterile polythene bags.

3.1.1 Equipment and Sample Preparation

All glass wares were washed with detergent, rinsed severally in clean water, and sterilized in hot air oven at 180°C for 2hrs for growing the yeast cells and production of the wine. The plastic containers, stainless steel trays, kitchen knife and the warring blender that were also used in the research were well sterilized while the work bench were swabbed with cotton wool soaked in absolute ethanol. Pineapple and watermelon fruits used were washed with potassium metabisulphite solution peeled and sliced aseptically with sterile knife.

3.2 Isolation of Yeast Strains

One each of spoilt pineapple and watermelon fruits samples were taken and 10gram of aseptically cut fleshy part of the spoilt fruit sample was homogenized and mixed in sterilized

nine milliliters peptone water then a serial dilution was made up to 10^{-5}, 0.1ml of the last dilution was cultured by spread plate method on Malt yeast extract agar. Also 1milliliter of burukutu alcoholic drink was taken and mixed with sterilized nine milliliters peptone water then a serial of dilution was also made up to 10^{-5}, 0.1 ml of the last dilution was then cultured by spread plate method on Malt yeast extract agar. The inoculated plates were incubated for 48h at 30ºC (Panneerselvam *et al.,* 2011). Suspected yeast colonies were subcultured on malt yeast extract agar plates and incubated at 30ºC for 48h in order to purify it. Purified cultures were maintained on malt yeast extract agar slants and kept at -4ºC for subsequent identification.

3.2.1 Characterization of Yeast Strains

Pure culture of the yeast isolates were characterized using cultural, morphological, biochemical test and confirmed using API test kit (API 20 C identification kit).

3.2.1.1 Cultural characterization of yeast isolates

The cultural characteristics of the yeast isolates were determined using the scheme of Kurtzman & Fell, 2011. Cultural characteristics such as texture (whether mucoid, fluid or viscous, butyrous, friable or membranous), colour, surface appearance (whether glistening or dull, smooth, rough, sectored, folded, ridged or hirsute), elevation (whether flat, depressed in the centre, raised and dome-like or conical) and margin (whether the edge of the colony is entire, undulating, lobed, erose or fringed with hyphae or pseudohyphae) were observed.

3.2.1.2 Morphological characterization of yeast isolates

A single colony of yeast isolate was mixed in a drop of distilled water placed on glass slide and smeared and allowed to dry off. The smear was then stained using diluted methylene blue dye, air dried and observed under light microscope at x10, x40 and x100 objective with oil immersion (Noroul *et al*., 2013). The shape and size of the vegetative cells were observed such as globose, subglobose, ellipsoidal, ovoidal, cylindrical, elongated, apiculate, lunate and

triangular also the mode of conidia formation, position and site of bud formation were also observed.

3.2.1.3 Biochemical characterization of yeast isolates

3.2.1.3.1 Carbon Assimilation Test.

A basal medium containing 0.5% (w/v) yeast extract and 0.75% (w/v) peptone was used with bromothymol blue as indicator. Aliquot of 9ml of the medium was dispensed into test tubes containing durham tubes. The tubes were plugged with cotton wool and sterilized at 121°C for 15minutes. After cooling, an aliquot of 2.0ml of filter sterilized solutions (6% w/v) of glucose, sucrose, fructose, maltose, lactose, galactose and raffinose were added into the different test tubes aseptically after which 0.1ml yeast suspension of the washed yeast cells was added. Uninoculated tubes were used as control. The tubes were incubated at 30°C for 48h and observed for colour change and gas production in the durham tubes. The Durham tubes were placed in the media to trap the carbon dioxide released. The fermentation medium was green in color and turned yellow (acidic) or blue (alkaline) if the yeast cells have the ability to ferment the respective sugars (Jimoh *et al*., 2012).

3.2.1.3.2 Analytical Profile Index confirmation of yeast isolates using API 20C Test Kit.

This test kit consists of a single-use disposable plastic strip with 32 wells containing substrates for 29 assimilation tests (carbohydrates, organic acids, and amino acids), one susceptibility test (cycloheximide), one colorimetric test (esculin), and a negative control. The yeast identification procedures were conducted in accordance with the manufacturer's instructions. A portion of yeast isolate from pure colonies was aseptically transferred from a freshly inoculated stock culture to sterile distilled water to prepare a suspension with a final turbidity equivalent to McFarland standard two. Five drops of this suspension was dispensed to an ampule of C medium provided by the manufacturer and homogenized to prepare an even dispersion of inoculum. After homogenizing, the inoculum suspension was used to

inoculate the wells in the strip, the lid of the strip was replaced, and the system was incubated at 30°C for 48 h. The strips were visually examined, and growth determined to be positive or negative based upon the presence or absence of turbidity in the wells. The result was then transformed into numerical bio codes, and the isolates were identified through the use of the API 20C Analytical Profile Index.

3.3 Screening and Selection of Yeast Strain for Wine Production.

3.3.1 Stress Exclusion Test.

Stress exclusion test was conducted as described by Thais *et al*. (2006). The stress exclusion test was overall carried out for 15 days by inoculating onto different media. The ability to grow under different stress conditions was tested by growing yeast isolates on Yeast Peptone Glucose (YPG) (1 g/ml yeast extract, 1 g/ml peptone, 2 g/ml glucose and 2 g/ml agar) medium and incubated at 30ºC for 3 days. A single colony was then transferred and continuously grown on YPG medium and incubated at 37^{o}C for another 3 days, before further subculture of the isolated yeast colony on YPG medium containing 8% (v/v) ethanol and incubated at 30ºC for 3 days. A single isolated colony on YPG with 8% ethanol was further subcultured on YPG supplemented with 20% (w/v) glucose and incubated under the same conditions as above. Finally the yeast cells were transferred to YP (1 g/ml yeast extract, 1 g/ml peptone) medium supplemented with 2% (w/v) sucrose and 8% (v/v) ethanol and incubated under the same conditions as above. Strain survival of yeast under various stress conditions provide useful information on its ability to grow and carry out fermentation as impaired yeast (Querol *et al*., 2003).

3.3.2 Ethanol Tolerance Test

The ability of the isolated yeast strains to grow in higher ethanol concentrations medium was tested by growing them in yeast peptone glucose broth containing 3 different concentration of

ethanol, 10% , 13% and 15% (v/v), respectively and incubated at 30ºC for 48 h (Thais *et al*., 2006). The ethanol stress was conducted to observe its tolerance to ethanol.

3.3.3 Temperature Tolerance Test

The ability of the yeast to grow at higher temperatures was determined by plating the yeast isolates onto yeast peptone glucose agar medium and incubated at 3 different temperatures, 25°C, 37°C and 45ºC for 48h (Thais *et al*., 2006).

3.3.4 Hydrogen Sulfide Production Test

The ability of the yeast to produce hydrogen sulphide (H_2S) was examined by growing the yeast isolates on lead acetate medium (4 g/ml glucose, 0.5 g/ml yeast extract, 0.3 g/ml peptone, 0.02 g/ml ammonium sulfate, 0.1 g/ml lead acetate and 2 g/ml agar) and incubated 30ºC for 10 days (Ono, 1991).

3.3.5 Flocculation Test

In this test, a wire loop was used to touch the colony of interest and this was inoculated in 10 ml of yeast peptone glucose broth and incubated at 30□C for 3 days. After incubation, tubes were agitated to observe the flocculation formed (Thais *et al*., 2006). Flocculation indicates their economic effect on production of yeast biomass and ensures a high cell density and large volume of harvested cells and also display it ability to raise the ethanol productivity during the fermentation process.

3.4 Fermentation of Fruit Juice for Wine Production.

3.4.1 Cell Purification.

Ten (10 ml) of yeast peptone dextrose (YPD) broth (1% yeast extract, 2% peptone and 2% dextrose) was inoculated with a single colony of the selected yeast strain and incubated overnight at 30°C. After incubation, 1 ml of the yeast culture was introduced into 200 ml YPD medium. The yeast cells were allowed to grow at a temperature of 30°C at 50rpm for

three to four hours on a shaker. Then, after determining the yeast cell growth in the medium, using the heamocytometer the cells were centrifuged at 5000 rpm for 5 min with a g-force of 2200. The pellets formed in the centrifuge tubes were re-suspended in 30 ml of sterile distilled water. The starter was then inoculated at 3.0 x 10^8 cells per ml yeast obtained using the heamocytometer (Byaruagaba-Bazirake *et al*., 2013).

3.4.2 Preparation of Inoculum

Preparation of starter or inoculum was performed by using 400 ml water and 80 g of sugar (Andri *et al*., 2012). After which the medium and all materials were autoclaved at 121°C for 15 min while the fruit juice was pasteurized. After the medium was allowed to cool, the identified and purified yeast (3.0 x 10^8 cells per ml volume) was then transferred into the medium containing water and sugar only (Andri *et al*., 2012) and left to stand for 10min.

3.4.3 Culture Conditions for Wine Production

One each of sound, healthy and ripe pineapple and watermelon fruits was selected, washed and aseptically peeled off using presterilized knife. The juice was obtained by blending the sliced fruits in a sterilized blender. The juice extracted (4Litres) was pasteurized at 85–90ºC for 5min (Panneerselvam & Maragatham, 2011). After cooling the juice, the pH of the fruit juice was adjusted to 4.5 by addition of calcium carbonate (($CaCO_3$) food grade) and malic acid food grade. Then, the required amount of sugar was added to adjust the final total soluble solids to 24ºBrix. Diammonium phosphate (DAP) was also added to the juice at an amount of 0.05% w/v prior to fermentation to supplement the nitrogen required in alcoholic fermentation (Andri *et al*., 2012). Also thiamine which is a very important source of nutrient for the growth of *Kloeckera specie* was added to the juice prior to fermentation. In the fermentation medium, bacteria activity was suppressed by pasteurizing the fruit juice prior to fermentation. Then the inoculum containing 3.0x10^8 cells/ml was then added to the cooled fruits juice (3.5litres each) and left to ferment. After 6 days of fermentation, it was filtered

through muslin cloth and the filtrate was kept for secondary fermentation which lasted for 8 days making a total of 14 days of fermentation in transparent rubber containers with air tight covers/seal. Yeasts population, pH, Total soluble solid, Total titratable acidity, Alcohol content, were determined throughout the experiment.

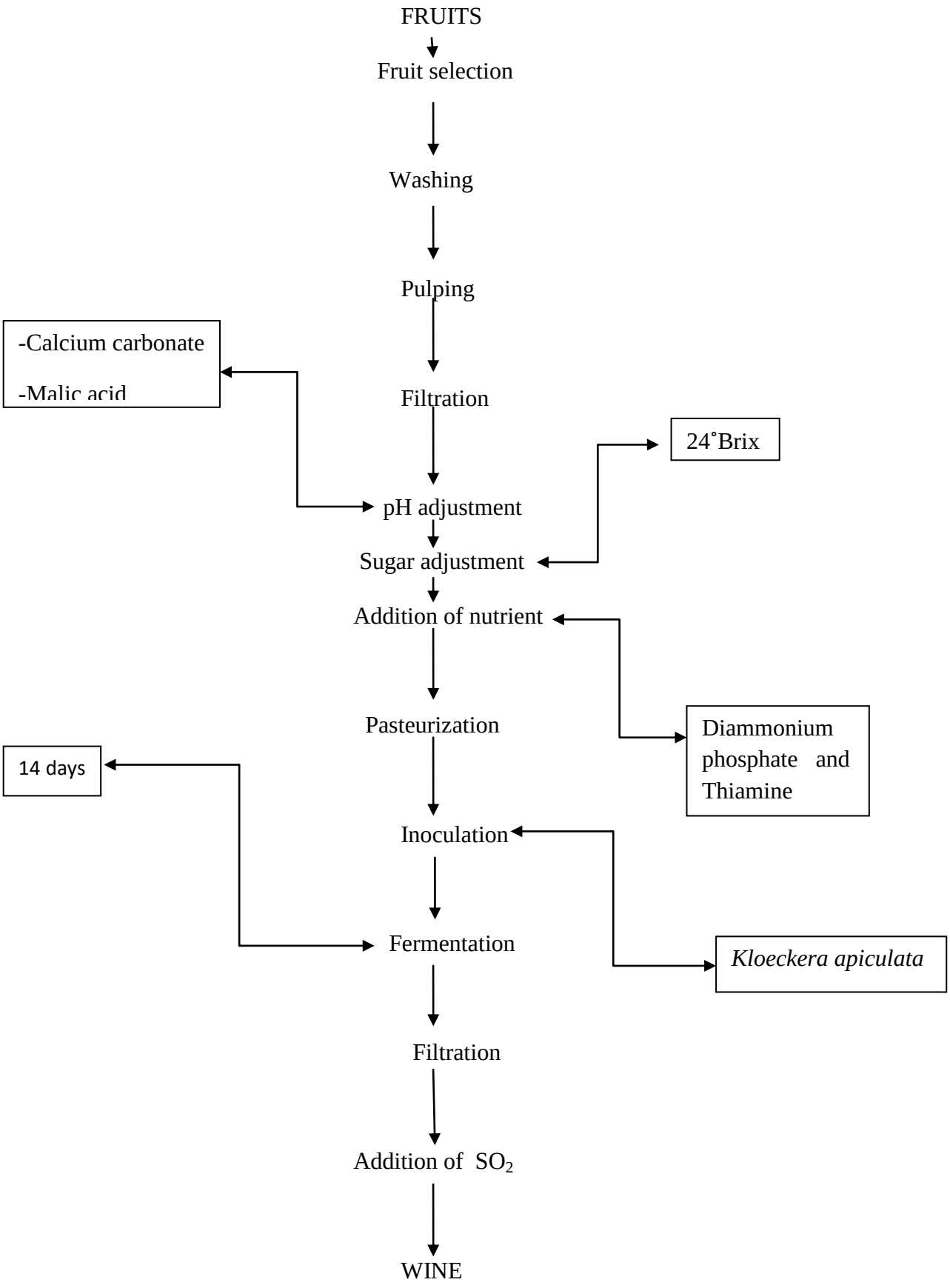


Fig 1: Flow Chart for Wine Production

3.5 Microbiological Analysis of Wine Produced.

At every 24h, sample of the wine was aseptically withdrawn from each fermenter and 1ml of the wine sample was serially diluted in 0.1% peptone water. The yeasts, mould and bacteria present were enumerated and isolated by spread inoculation of 0.1 ml of the 10^{-6} dilution onto plates of nutrient agar (NA) and incubated at 30°C for 24 h for bacterial growth and potato dextrose agar (PDA) incubated at 28°C for 72 h for molds and yeast growth. Resultant colonies were enumerated with the aid of a colony counter, purified by streaking technique on freshly prepared nutrient agar (NA) and potato dextrose agar (PDA) and identified by a standard protocol (Ajay & Sanjay, 2010).

3.5.1 Identification of Bacteria Isolates.

3.5.1.1 Cultural and Microscopic Identification of Bacteria Isolates.

3.5.1.1.1 Growth on de Man's Rogosa Sharpe agar

Since most bacteria associated with wine are lactic acid bacteria, the isolated colonies were inoculated on de Man Rogosa Sharpe agar which is a selective media for the growth of lactic acid bacteria. The media was prepared by suspending 66.73g of the media in 1litre of distilled water and then boiled according to manufacturer's instruction. The media was then dispensed into tubes and autoclaved at 121°C for 15min and allowed to cool. After cooling the isolated bacteria were streaked on the prepared media and incubated in a microaerophilic environment which was constructed using a stainless steel for 72h (Jason *et al.,* 2004).

3.5.1.1.2 Gram staining

To detect the fundamental difference in cell wall composition of the bacteria, a smear from a pure culture of the bacteria isolate was made by placing a drop of distilled water on a sterile glass slide and using a sterile loop to touch the isolated colony of bacteria and mix well in the water drop on the slide and then allowed to dry and then heat fixed by passing the slide three times over a flame. The prepared slide was then flooded with crystal violet for 60 second and

washed off with running tap water. The slide was then flooded with gram's iodine for 60 second and washed off with running tap water. The bacterial cells on the slide was decolorized with 95% alcohol and 5% acetone solution until the solvent on the slide flows colorless from the slide approximately 5-10seconds and rinsed immediately with running water. Then it was counter stained with safranine for 30 second then rinsed with tap water and allowed to air dry(Jason *et al.,* 2004). When viewed under the microscope, cells that appeared pink in color were recorded as Gram negative while purple or dark blue cells were recorded as Gram positive.

3.5.1.1.3 Spore Formation Test

A smear from a pure culture of the bacteria isolate was made by placing a drop of distilled water on a sterile glass slide and using a sterile wire loop to touch the isolated colony of bacteria and mix well in the water drop on the slide and then allowed to dry and then heat fixed by passing the slide three times over a flame. The glass slide was then flooded with malachite green solution for 3 min then washed under a flowing tap. Safranine solution was then applied for 30sec then the glass slide was washed, air dried and examined using oil emersion. Spores stained green while vegetative cells stained pink (Jason *et al.*, 2004).

3.5.1.1.4 Temperature Test

The ability of the bacteria to grow at different temperatures was determined by plating the bacteria isolated onto nutrient agar medium and incubated at 3 different temperature regimes which were 15°C, 30°C and 45ºC for 48h (Jason *et al.,* 2004). Growth was determined by noting the number of colonies formed.

3.5.1.1.5 Motility Test

To test the ability of the bacteria to move by itself, nutrient agar medium was prepared in a test tube and the test bacteria was inoculated with a straight wire loop by making a single stab down the center of the tube to about half the depth of the medium and then Incubated at

37°C. Test tubes were examined at intervals. Results were read by holding the tube up to the light and looking at the stab line to determine motility. Non-motile bacteria generally gave growths that were confined to the stab-line and had sharply defined margins leaving the surrounding medium clearly transparent while the motile Bacteria typically gave diffuse, hazy growths that spread throughout the medium rendering it slightly opaque (Jason *et al.*, 2010).

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3.5.1.2 Biochemical Identification of Bacteria Isolates.

3.5.1.2.1 Catalase Test.

The ability of the bacteria to produce enzyme to detoxify hydrogen peroxide by breaking it down into water and oxygen gas was determined by placing a drop of normal saline on a clean glass slide then a sterilized and cooled wire loop was used to pick up a small amount of the bacteria culture not more than 24h from the nutrient agar plate and placed on the normal saline on the glass slide to make a smooth suspension. Then a Pasteur pipette was used to place a drop of hydrogen peroxide on the smear and observed for the appearance of gas bubbles. The bubbles resulting from production of oxygen gas indicated a catalase positive result (Sagar *et al.,* 2015).

3.5.1.2.2 Urease Test

The ability of the bacteria to hydrolyze urea with the release of ammonia and carbon dioxide was tested by taking a colony of the test bacteria of not more than 24h and placing it in a test tube containing Urea test broth (20g urea, 9.5g Na_2HPO_4, 9.1g KH_2PO_4, 0.1g yeast extract and 0.01g phenol red) and incubated for 24h at 37°C. Bright pink color was recorded as a positive result while no color change was regarded as a negative result (Sagar *et al.,* 2015).

3.5.1.2.3 Methyl Red- Voges Proskauer Test (MR-VP Test).

The ability of the bacteria to utilize glucose and convert it to a stable acid like lactic acid, acetic acid or formic acid as end product was tested by aseptically inoculating the MRVP medium (1litre of deionized water, 7g peptone, 5g glucose, 5g dipotassium phosphate) with the test bacteria of not more than 24h culture and incubated at 37°C for 48h. After incubation, a sterile pipette was used to remove two – 1ml aliquots and placed into two small test tubes separately. One tube was used for the methyl red test and the other was used for the Voges-Proskauer test. Five drops of methyl red 0.02 %(0.1 g of methyl red, 300 ml of ethyl alcohol, 95% and 200ml distilled water) was added to one test tube and the result was read immediately for methyl red test. While to the second test tube for the Voges-Proskauer test, 15 drops of Voges-Proskauer A, reagent was added and mixed well to aerate the sample. Followed by 5 drops of Voges-Proskauer B, reagent in the test tube and then it was well mixed by shaking to aerate the sample. The result was read after 15 min. A distinct red color was recorded as positive for methyl red test and a yellow color as negative. While for Voges-Proskauer test a positive test is indicated by a pink-red color that developed within 5 minutes (Sagar *et al.*, 2015).

3.5.1.2.4 Citrate Test

In other to determine the ability of the test bacteria to utilize sodium citrate as its only carbon source and inorganic ammonia phosphate as it fixed nitrogen source, the test bacteria was aseptically inoculated on Simmon's Citrate agar slant (5g sodium chloride, 2g sodium citrate (dehydrate), 1g ammonium dihydrogen phosphate, 1g dipotassium phosphate, 0.2g magnesium sulfate (heptahydrate),0.8g bromothymol blue, 15g agar,1000ml deionized water) and incubated at 37°C for 3days. Blue color indicated a positive test while green color indicated negative test (Sagar *et al*., 2015).

3.5.1.2.5 Nitrate Reduction Test.

The ability of the bacteria to produce the enzymes nitrate reductase and nitrite reductase was tested by Inoculating nitrate broth with pure culture of the test bacteria aseptically and incubated at 37°C for 24h. After then one dropfull of sulfanilic acid and one dropfull of α-naphthylamine was added. At this point, a color change to red indicated a positive nitrate reduction test and the test could be stopped at this point but absence of color change indicated the absence of nitrite and the test was proceeded by adding a very small amount of zinc (a toothpick full) to each broth. Therefore at this point, a color change to red indicated a negative nitrate reduction test because this meant that nitrate must have been present and must have been reduced to form nitrite. No color change means that no nitrate was present and thus no color change at this point indicated a positive result.

3.5.1.2.6 Indole Test

The ability of the bacteria to split amino acid tryptophan into indole and pyruvic acid using the enzyme called tryptophanase was tested by aseptically inoculating the pure isolated test bacteria in tryptone broth and incubated overnight at 37°C. Then few drops of Kovac's reagent were carefully added. The Formation of red ring at the top was considered as positive result while no color change after addition of reagent was considered as negative result (Sagar *et al*., 2015).

3.5.1.3 Confirmatory Test

3.5.1.3.1 Sugar Fermentation

The ability of the bacteria to produce acid when they ferment carbonhydrate was tested by aseptically preparing Phenol Red Broth(10g Trypticase,5g Sodium Chloride,1g Beef extract,7.2 ml of 0.25% phenol red solution,10g Carbohydrate source, 1000ml distilled water) followed by the transfer of 5ml of the phenol red broth into different test tubes for the different sugars. Durham tubes were inserted in each test tube to detect gas production and

the test tubes were sealed with cotton wool and autoclaved at 121°C for 15 min to sterilize. The different sugar solutions (1% glucose, 1% galactose,1% fructose, 1% sucrose,1% arabinose, 1% maltose, 1% lactose, 1% arabinose, 1% cellobiose, 1% mannitol, 1% mannose, 1% meleboise, 1% raffinose, 1% ribose, 1% salicin, 1% melezitose, 1% rhamnose, 1% sorbitol, 1% trehalose, 1% xylose) were prepared separately and filter sterilized using membrane filter. The sugar solution were then added to the broth base and mixed aseptically**.** This was followed by aseptically inoculating each test tube with the pure isolate of test bacteria using a sterile wire loop and Incubated at 37°C for 24h. Yellow coloration indicated a positive result while red coloration indicated a negative result. On the other hand presence of bubble in the durham tube indicated positive result for gas production while absence of gas in the durham tube showed that it was negative for gas production.

3.5.1.3.2 Esculin Test

The ability of the bacteria to hydrolyze esculin in the presence of bile was tested by using a sterile wire loop to pick one colony of the isolated bacteria from a 24h culture and have it inoculated onto the surface of bile esculin agar medium containing ferric ammonium citrate and incubated at 37°C for 24h. The result was observed. Blackening of more than half of the agar slant indicated a positive result while no blackening of the medium was recorded as negative.

3.5.1.3.3 Sodium Chloride Test

This test was used to distinguish *Enterococcus species* from the group D Streptococci, *Streptococcus bovis* and *Streptococcus lactis* based on the ability of the bacteria to grow in the presence of variable amount of Sodium chloride (NaCl). This test was conducted by dissolving 3.8g of tryptic soy broth and 6.5g of sodium chloride in 100ml distilled water then dispensing it into different test tubes, sealed and autoclaved. After cooling sterile wire

loop was used to aseptically pick up the bacteria isolated and transferred into the sterile medium and incubated at 37°C for 24h. Growth of the organism indicated positive result.

3.5.1.3.4 Test for Production of Ammonia from Arginine.

A loopful of the test bacteria was aseptically taken from a 4 day old L-arginine culture and placed into 0.5ml of distilled water. Then one drop of Nessler's reagent was added and result was read as positive reaction for presence of ammonia if a colour ranging from a pale yellow to a dark brown precipitate was produced Martin *et al*. (2003).

3.5.2 Physicochemical Analysis of Wine

3.5.2.1 Total Soluble Solids (TSS)

The Total Soluble Solid was determined using a hand hydrometer. The readings were expressed in ºBrix. It was obtained by aseptically collecting a 60ml of the wine and placed in a sterile 100ml measuring cylinder then the hydrometer was aseptically suspended in the wine and the reading was taken (Musyimi *et al*., 2013).

3.5.2.2 Total Titratable Acidity(TTA)

Pineapple and watermelon wine were titrated against 0.1M NaOH using phenolphthalein as indicator, the results were expressed as percentage of malic acid. The volume of NaOH used in the titration was noted (Wilker *et al*., 2003) using the formulae

Malic acid (g/100 ml) = [(V) (N) (75) (100)] / [(1000) (v)]

Where

V = ml of NaOH solution used for titration,

N = Normality of sodium hydroxide solution, (0.1 was used)

v= volume of wine sample (5 ml was used)

The predominant acid in pineapple is citric acid and malic acid while watermelon contains predominantly malic acid (Daniel *et al*., 1996).

3.5.2.3 Ambient Temperatures of wine (^{o} c)

Daily ambient temperature of the wine was taken throughout the experimental period. Average daily temperatures were obtained using a thermometer and recorded in degree Celcius (°C) (Wilker *et al*., 2003).

3.5.2.4 Alcoholic Content

The alcoholic percentage levels (%/ vol.) in the wine was determined using an alcohol hydrometer by aseptically collecting a little portion of the wine and place it in a sterile 100ml measuring cylinder then the hydrometer was suspended in the wine and the reading was taken. Readings were taken for 14 days during fermentation and mean alcohol level (%/ vol.) was calculated and recorded (Wilker *et al*., 2003).

3.5.2.5 pH of wine

The pH value of the wine was determined using a pH meter. This was obtained by dipping the pH meter aseptically in a little portion of the wine aseptically collected from the fermentation vessel into a sterile beaker. Readings were taken for 14 days during fermentation. The pH of the juice was adjusted by addition of calcium carbonate (($CaCO_3$) food grade) and malic acid respectively, prior to fermentation.

3.5.3 Proximate Analysis of the Wine

The proximate analysis of the wine was carried out to determine the moisture content, ash content, protein content, lipid content and carbohydrate contents and the results were compared to that of a standard pineapple wine.

3.5.3.1 The Moisture Content

The wine was titrated with the Karl Fischer reagent. The water reacted stoichiometrically with the Karl Fischer reagent. The volume of Karl Fischer reagent required to reach the

endpoint of the titration was directly related to the amount of water in the sample (Butter, 2000).

3.5.3.2 The Ash Content

Twenty (20) ml of the wine was pippeted into a previously tared platinum dish (original weight p_o g) and evaporated on the boiling water-bath, the residue on the hot-plate was heated at 200°C until carbonization began. When no more fumes were produced, the dish was placed in an electric muffle furnace maintained at 525 ± 25°C. After 15 min of carbonization, the dish was removed from the furnace, and 5 ml of distilled water was added, then it was evaporated on the water-bath, and again the residue was heated to 525°C for 10 min (Butter, 2000). For wines with high sugar content, it is advantageous to add a few drops of pure vegetable oil to the extract before the first ashing to prevent excessive foaming. After cooling in the desicator, the dish was weighed (p_1 g). The weight of the ash in the sample (20 ml) was then calculated as

$p = (p_1 — p_O)$ g

The weight P of the ash in grams per liter was given to two decimal places by the expression:

$P = 50$ p.

3.5.3.3. The Protein Content

Ten (10ml) of each sample was pippeted into a Buchi digestion tube and each tube with the sample number was marked using ink. Five millilitres of water (for reagent blank) was pippetted into another Buchi digestion tube then this tube was marked as reagent blank. The Buchi tubes with sample numbers were then reduced to syrup on a steam bath. A catalyst tablet and 10 ml conc. sulfuric acid were added and digested at 80% power using Buchi digestor connected to an aspirator, until thick, white fumes became clear; it was then digested for 30 minutes more. Total digestion time was approximately 90 minutes. Then it was cooled to room temperature before proceeding. Receiving (titration) beakers for the distillation were

prepared by pipetting 10.0 ml of 0.1molar sulfuric acid into each beaker. Then 10 ml of distilled water was then added to each beaker to cover the tip of the distillation tube. The digestion tube was placed on Buchi distillation apparatus and hydrolysed with 75 ml of water, followed by 75 ml of 25% aq. NaOH and then distilled into receiving beaker containing the 10.0 ml of 0.1molar sulfuric acid for 3 minutes. Titrate was distillated to pH 6.0 with 0.1M NaOH.

Protein g = $[(V_a \times N_a) - (V_b \times N_b)] \times 14.007 \times 6.25 \times V_c\ 1000 \times V_s$

Where:

V_a = Volume of 0.1molar sulfuric acid.

N_a = Normality of 0.1molar sulfuric acid.

V_b = Volume of 0.1molar sodium hydroxide used in the titration.

N_b = Normality of 0.1molar sodium hydroxide.

V_c = Volume serving in ml; 5 fluid ounce serving = 148 ml.

V_s = Volume of sample, blank or standard pippetted.

3.5.3.4 The Lipid Content

The wine temperature was first of all adjusted to 38°C using a thermometer and a standard pipette was used to pipette 17.6 ml of wine into each of three Babcock bottles. After the pipette had been emptied, the last drop of wine from the pipette tip was blown into the bottle and temperature was adjusted to 22°C. 17 ml of sulfuric acid (specific gravity 1.82–1.83) was dispensed and carefully added into the test bottle, with mixing during and between additions, care was taken to wash all traces of wine into the bulb of the bottle. Time for complete acid addition did not exceed 20sec. The wine and acid were then mixed thoroughly. Bottles were placed in centrifuge and heated to 60°C making sure that bottles were counter balanced and that the heater of the centrifuge was on. The bottles were centrifuged for 5 min after the proper speeds was reached, the centrifuge was stopped and hot water (60°C) was added until

the liquid level fell within 0.6 cm of the neck of the bottle. The water was carefully permitted to flow down the side of the bottle. Then the bottles were again centrifuged for 2 min. The centrifuge was stopped and enough hot water (60°C) was added to bring the liquid column near the top graduation of the scale. Thereafter, the bottles were centrifuged for 1 min then the bottles were removed from the centrifuge and placed in a heated (57°C) water bath deep enough to permit the fat column to be below the water level of the water bath. The bottles were allowed to remain at least 5 min before reading. The samples were removed from the water bath one at a time, and the outside of the bottle were dried quickly. Glymol (red reader) was added to top up fat layer and immediately a divider was used to measure the fat column to the nearest 0.05%, while holding the bottle in a vertical position at eye level. It was measured from the highest point of the upper meniscus to the bottom of the lower meniscus. All tests in which the readings were indistinct or uncertain were rejected. The reading of each test was recorded and the mean % fat and the standard deviation were determined (Butter, 2000).

3.5.3.5. The Carbohydrate Content

The glucose standard solution (100 mg glucose/L) and deionized distilled water were used. Aliquots of the glucose standard was pipetted into clean test tubes (duplicates for each concentration) such that the tubes contained 0–100ml of glucose (1000ml mechanical pipettor was used to pipette samples), in a total volume of 2 ml. These tubes were used to create a standard curve, with values of 0–100mg glucose/2 ml. The 0mg glucose/2 ml sample was used to prepare the reagent blank. Mg Glucose/2 ml 0 20 40 60 80 100 ml glucose stock solution 0 0.2 0.4 0.6 0.8 1.0 ml deionized distilled water 2.0 1.8 1.6 1.4 1.2 1.0 3 The wine kept at room temperature about 100 ml was poured into a 500-ml Erlenmeyer flask and gently shaked and continued gently shaking until no observable carbon dioxide bubbles was seen. So the samples tested would contain 20–100mg glucose/2 ml, after dilution, 1.0 ml of

sample was pippeted into a test tube and 1.0 ml of deionized distilled water was added to each diluted sample and analyzed in duplicate. Then 1.0 ml of wine was pippetted into a 1000-ml volumetric flask, and diluted to volume with deionized distilled water. The flask was sealed with Parafilm and mixed well. To each tubes containing a total volume of 2 ml, 0.05 ml 80% phenol (200 ml mechanical pipettor was used) was added and properly mixed. To each tube, 5.0 ml H_2SO_4 was added and mix properly on a Vortex test tube mixer. Tubes were allowed to stand for 10 min and then placed in a 25°C bath for 10 min (i.e., to cool them to room temperature). The test tubes were properly shake again before reading the absorbance by wearing gloves to pour samples from test tubes into cuvettes, cuvettes was not rinsed with water between samples. Then the spectrophotometer was zeroed with the standard curve sample that contained 0mg glucose/2 ml (i.e., blank) was retained in one cuvette for later use. Absorbance of all other samples was read at 490 nm. Standard curve tubes from low to high concentration (i.e., 20mg/2 ml up to 100mg/2 ml) was read, and then wine sample too was read. To be sure that the outside of the cuvettes were free of moisture and smudges, the outside of the cuvette was wiped with a clean paper wipe prior to inserting it into the spectrophotometer for reading and one of the duplicate tubes from a standard curve sample with an absorbance reading of 0.5–0.8 was used to determine the absorbance spectra from 450 to 550 nm by reading the tube at 10 nm intervals. The spectrophotometer was zeroed with the blank at each 10 nm interval (Butter, 2000).

3.5.4. Post Fermentation Treatment

3.5.4.1. Sulphur Addition

Sulphur dioxide in the form of potassium metabisulfite (58% SO_2) was added to sterilize the wine completely at the end of the fermentation process. SO_2 was substituted with pasteurization at the beginning of the fermentation process because *Kloeckera apiculata* which is the organism used for the production of the wine is usually affected by sulphur

dioxide. Also thiamine a yeast nutrient preferred by *Kloeckera species* was used in large quantity in this fermentation because it reduced the effect of sulphur dioxide on *Kloeckera species* prior to the fermentation (Wilker *et al*., 2003).

3.5.4.2. Racking

Racking is the process of moving the wine from one container to another, leaving behind sediments. The wine was racked several times. The first racking of the wine was after the wine had settled a day after filtering, after small solids had settled out of solution and then after five days. The wine was transferred to another container. Racking was done at room temperature because at a higher temperature it reduces oxygen pickup (Wilker *et al*., 2003).

3.5.4.3. Clarification and Stabilization of White Wine.

Clarification and stabilization are the processes by which insoluble matter suspended in the wine is removed before bottling. The best method is that of natural clarification that is the sedimentation of yeast cells at the end of the fermentation and this is what was used in this wine production. Clarification could also be achieved by the addition of bentonite at a rate of 2 grams/3.785 litres. This usually clear most white wines and provides a degree of protein stability (Wilker *et al*., 2003).

3.6 Sensory Evaluation of the Wine Produced.

Ten people were made to taste the wine to determine the organoleptic properties of the wine and this was achieved by, filling the glass 1/3 or less full and swirl the wine around to get the molecules up in the air then each individual was made to Sniff the wine and savor the aroma and bouquet (odours in wine derived from processing and aging). Each individual was made to taste the wine by taking some in their mouth and rolling it around their tongue during which they would note the sweet, sour and bitter tastes and the balance and body of the wine (Panneerselvam and Maragatham, 2011).

3.7 Data Analysis

The results obtained were analysed using one way ANOVA without replication at ($p \leq 0.05$) because it tests if the value of a single variable differs significantly among three or more levels of a factor.

CHAPTER FOUR
4.0 RESULTS

4.1 Isolation and Identification of Yeasts Strains.

In this study, seven yeast isolates were obtained altogether. Two yeast isolates each were obtained from burukutu alcoholic drink and spoilt pineapple, while three different yeasts were isolated from spoilt watermelon. The yeasts isolated were identified and confirmed using API Test kit to be *Candida sphaerica, Pichia specie, Candida pelliculosa, Cryptococcus humicola, Candida guilliermondii, Candida famata* and *Kloeckera apiculata* as shown below.

The different yeasts isolated from burukutu alcoholic drink, spoilt pineapple and spoilt watermelon were characterized primarily based on their macroscopic and microscopic appearance. On malt yeast extract agar plates the yeast colonies isolated were observed to be whitish to creamy in color, with smooth surface appearance, entire margin and most of their elevation were convex as shown in Table 4.1. Sugar fermentation test was further carried out on all the yeasts isolated and the result showed that some were able to assimilate glucose but not all were able to ferment it with production of gas as shown in Table 4.1. Out of all the yeasts isolated *Kloeckera apiculata* with isolate code SWW7 was found to have one of the best fermentative properties, as it could both ferment and assimilate glucose and is non pathogenic as compared to others in the Table 4.1.

The yeasts identified were confirmed using API Test kit by testing their reaction to different sugars that is their ability to produce turbidity when compared to the control as shown in Table 4.2. The identified yeasts include *Candida sphaerica* and *Pichia specie* which were isolated from burukutu alcoholic drink followed by *Candida pelliculosa* and *Cryptococcus humicola* from spoilt pineapple and finally *Candida guilliermondii, Candida famata* and *Kloeckera apiculata* from spoilt watermelon as shown in Table 4.3.

Table 4.1: Cultural, Microscopic and Fermentative Characteristics of Yeasts Isolates.

Isolates Codes	Growth on MYEPG	Microscopic Characteristics	Sugar Fermentation							Inference
			Glu	Suc	Fru	Mal	Lac	Gla	Raf	
BKT 1	The surface appearance is smooth, creamy in color, it elevation is raised and has an entire margin	Spherical cell without budding and pseudohyphae is absent.	+	+	-	-	+	+	-	*Candida sphaerica*
BKT 2	Mucoid, whitish, smooth colonies with entire margin and elevation is raised.	Small elongated budding yeast cells, pseudohyphae is present	+G	-	+	-	-	-	-	*Pichia specie*
SWW 3	Creamy,mucoid,smooth, with entire margin that is conical	Spherical budding cells. Pseudohyphae is present and branched.	+G	+G	+	+	-	±	+	*Candida guilliermondii*
SWW 4	Rough, white, with undulating margin. It has a convex elevation and the cells are spherical	Ovoid budding cell, no pseudo hyphae is present.	±G	±G	+	±G	-	±G	+	*Candida famata*
SPP 5	Smooth, mucoid, creamy colony with entire margin, the cells are raised	Ovoid budding yeast cells, pseudohyphae is present.	+G	+G	+	+	-	±	+	*Candida pelliculosa*
SPP 6	Smooth, mucoid, creamy colony with entire margin and raised cells.	Ovoid budding cells. Pseudohyphae is present.	+	+	+	+	-	+	±	*Cryptococcus humicola*
SWW7	White, smooth, mucoid colony that is slightly raised at the centre	Spherical budding cells pseudohyphae is absent.	+G	±	+	-	-	+	+	*Kloeckera apiculata*

Key: **MYEPG**; Malt yeast extract peptone glucose medium, **Glu**; glucose, **Suc**; sucrose, **Fru**; fructose, **Mal**; maltose, **Lac**; lactose,

Gla; galactose, **Raf**; Raffinose.**+**; positive, **-**; negative, **G**; gas, **±**; weak.

Table 4.2: API Confirmatory Test for Yeast Isolates.

Sugars	**BKT 1**	**BKT 2**	**SWW3**	**SWW4**	**SPP 5**	**SPP 6**	**SWW 7**
Control	-	-	-	-	-	-	-
D-glucose	+	+	+	+	+	+	+
Glycerol	-	-	-	+	+	+	-
Calcium 2-keto-gluconate	-	+	+	+	-	+	+
L –arabinose	-	+	-	-	-	+	-
D –xylose	+	+	-	-	+	+	-
Adonitol	-	-	+	+	-	+	-
Xylitol	-	-	+	-	+	+	-
D –galactose	+	+	+	+	-	+	-
Inositol	-	-	-	+	-	+	-
D –sorbitol	+	-	+	+	+	+	-
Methyl–Ad-glucopyranoside	-	+	+	-	+	+	-
N –Acetyl-Glucosamine	-	+	+	+	-	+	-
D-celiobiose	-	-	+	+	+	+	-
D-lactose	-	-	+	+	-	+	-
D-maltose	+	+	+	+	+	+	-
D-saccharose (sucrose)	+	+	+	+	+	+	-
D- trehalose	+	+	+	+	+	+	-
D-melezitose	-	-	+	+	+	+	-
D-raffinose	+	-	+	+	+	+	-
Inference	*Candida sphaerica*	*Pichia spp*	*Candida guilliermondii*	*Candida famata*	*Candida pelliculosa*	*Cryptococcus humicola*	*Kloeckera apiculata*

Key:+;positive,-;negative

Table 4.3: Sources of Yeasts Isolated, Characterized and Identified.

Sample Type	No. of yeasts isolated	Specific yeasts isolated	Codes used for each yeast
Burukutu drink.	2	*Candida sphaerica*	BKT 1
		Pichia spp	BKT 2
Spoilt Pineapple	2	*Candida pelliculosa*	SPP 5
		Cryptococcus humicola	SPP 6
Spoilt watermelon	3	*Candida guilliermondii*	SWW 3
		Candida famata	SWW 4
		Kloeckera apiculata	SWW 7

Key: **BKT 1**- *Candida sphaerica,* **BKT 2** - *Pichia spp,* **SPP 5**- *Candida pelliculosa,* **SPP 6**- *Cryptococcus humicola,***SWW 3**- *Candida guilliermondii,***SWW4**- *Candida famata* **SWW 7**- *Kloeckera apiculata*

4.2 Screening and Selection of Yeast Strain

The seven yeast isolated were further screened to select the yeast with the best fermentative property by determining their flocculation abilities, ethanol tolerance at different percentage, hydrogen sulphide tolerance, temperature tolerance and stress tolerance as shown in Table 4.4 *Candida sphaerica, candida famata* and *Kloeckera apiculata* displayed the highest flocculation abilities while *Pichia Specie* and *Cryptococcus humicola* did not grow at all in the medium and therefore did not form any flocculant. In the presence of 15% ethanol only *Candida famata* and *Candida pelliculosa* survived with little growth. At 13% ethanol all yeast isolated grew moderately except *Cryptococcus humicola* which could not grow at all. At 10% ethanol *Cryptococcus humicola* still did not grow while *Candida guilliermondii*, *Candida famata, Candida pelliculosa* and *kloeckera apiculata* had the highest growth. Hydrogen sulphide test showed that only *Candida famata* and *Candida pelliculosa* can survive the presence of hydrogen sulphide. All yeast isolated grew very well at 25°C except *Pichia specie* which displayed a very poor growth at that temperature. At 30°C *Candida guilliermondii, Candida pelliculosa* and *kloeckera apiculata* had the highest growth while others grew moderately. At 37°C *Candida guilliermondii* and *Candida pelliculosa* had the highest growth while others grew moderately. At 45°C only *Candida pelliculosa* grew.

Stress tolerance test in Table 4.5 showed that all the seven yeast isolated survived the first and second stage of stress tolerance test and they organisms grew very well during the first and second stage except for *Pichia specie*. In the third stage of stress tolerance test all yeast isolated grew moderately except *Candida pelliculosa* which grew exceptionally well. No yeast isolated survived the fourth stage of stress tolerance except *Candida pelliculosa*.

Table 4.4: Screening and Selection of Yeast Strains for Best Fermentative Properties

Test	**BKT 1**	**BKT 2**	**SWW3**	**SWW4**	**SPP 5**	**SPP 6**	**SWW 7**
Flocculation test	+++	+	++	+++	++	+	+++
Ethanol 15%	-	-	-	+	+	-	-
Ethanol 13%	+	+	+	+	+	-	+
Ethanol 10%	+	+	++	++	++	-	++
Hydrogen sulphide test	-	-	-	+	+	-	-
Temp. 25°C	++	+	++	++	++	++	+++
Temp. 30°C	+	+	++	+	++	+	++
Temp. 37°C	+	+	++	+	++	+	+
Temp. 45°C	-	-	-	-	+++	-	-
Yeast species	*Candida sphaerica*	*Pichia spp*	*Candida guilliermondii*	*Candida famata*	*Candida pelliculosa*	*Cryptococcus humicola*	*Kloeckera apiculata*

Key: **-** : Not turbid/ no growth, **+:** It is turbid but no floculant was formed/Growth is observed, **++:** Floculant are formed/Moderate growth,**+++:** Floculant are densely formed/ Highly populated.

Table 4.5: Stress Tolerance Test of Isolated Yeasts.

Test	**BKT 1**	**BKT 2**	**SWW3**	**SWW4**	**SPP 5**	**SPP 6**	**SWW 7**
YPG at 30°C for 3 days.	+++	++	+++	+++	+++	+++	+++
Further subcultured at 37°C for 3 days	+++	+++	+++	+++	+++	+++	+++
Further subcultured on YPG+8% ethanol at 30°C for 3 days	+	+	+	+	++	+	+
Further subcultured onYPG+20% glucose	-	-	-	-	+	-	-
Yeast species	*Candida sphaerica*	*Pichia spp*	*Candida guilliermondii*	*Candida famata*	*Candida pelliculosa*	*Cryptococcus humicola*	*Kloeckera apiculata*

Key: **-** : Not turbid/ no growth, **+:** Growth is observed, **++:** Moderate growth, **+++:** Highly populated, **YPG**: Yeast Peptone Glucose medium.

4.3 Production of Wine from fruit Juice using *Kloeckera apiculata*

4.3.1 Effect of Specific Gravity on Wine Production.

The specific gravity was noted throughout the wine production and it was observed that as time increased that is as the days of fermentation increased the specific gravity decreased in both pineapple and watermelon wine produced as shown in Figure 4.1. However, the pineapple wine gave the highest specific gravity compared to the watermelon wine produced as shown in appendix I and II.

4.3.2 Effect of Total Soluble Solid on Wine Production.

The total soluble solid was also noted and it was found out that as the days of fermentation increased, the total soluble solids decreased in both watermelon and pineapple wine as shown in Figure 4.2. However, it was higher in pineapple wine as compared to watermelon wine produced as shown in appendix I and II

4.3.3 Effect of Titratable Acidity on Wine Production.

The titratable acidity of the wine increased as the days of fermentation increased in both pineapple and watermelon wine as shown in Figure 4.3. The highest titratable acidity occurred in watermelon wine as shown in appendix I and II

4.3.4 Effect of Alcohol on Wine Production.

The amount of alcohol produced was investigated using a hydrometer and the amount of alcohol produced was observed to increase as fermentation increased in both watermelon and pineapple wine produced as shown in Figure 44. However, the alcohol produced was highest in pineapple wine compared to watermelon wine as shown in appendix I and II

4.3.5 Effect of Temperature and pH on Wine Production

As the days increased the pH decreased consistently while the temperature was found not to follow any known pattern in both pineapple and watermelon wine produced.

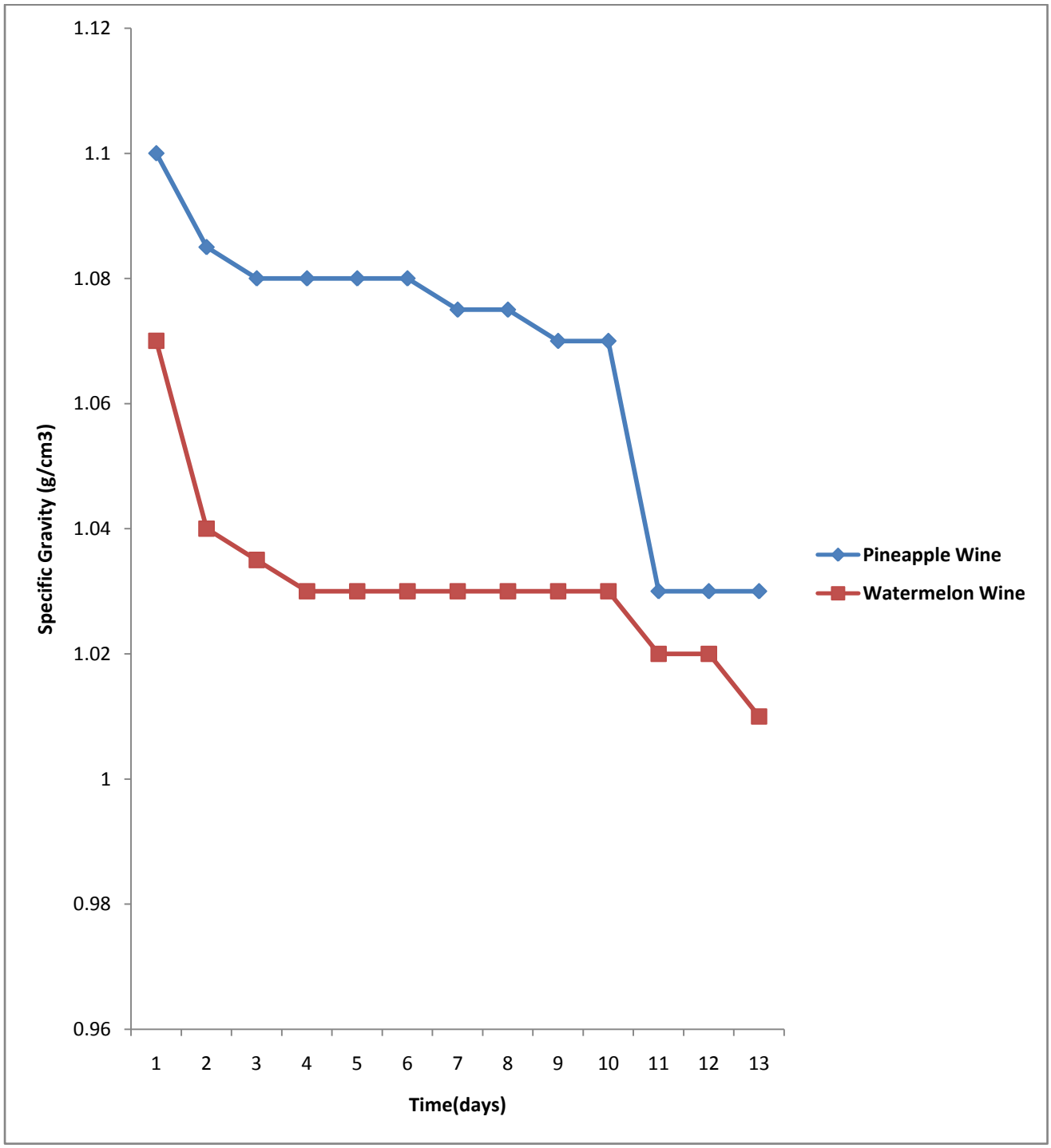


Fig 4.2: Specific gravity pattern during fermentation of watermelon and pineapple wine.

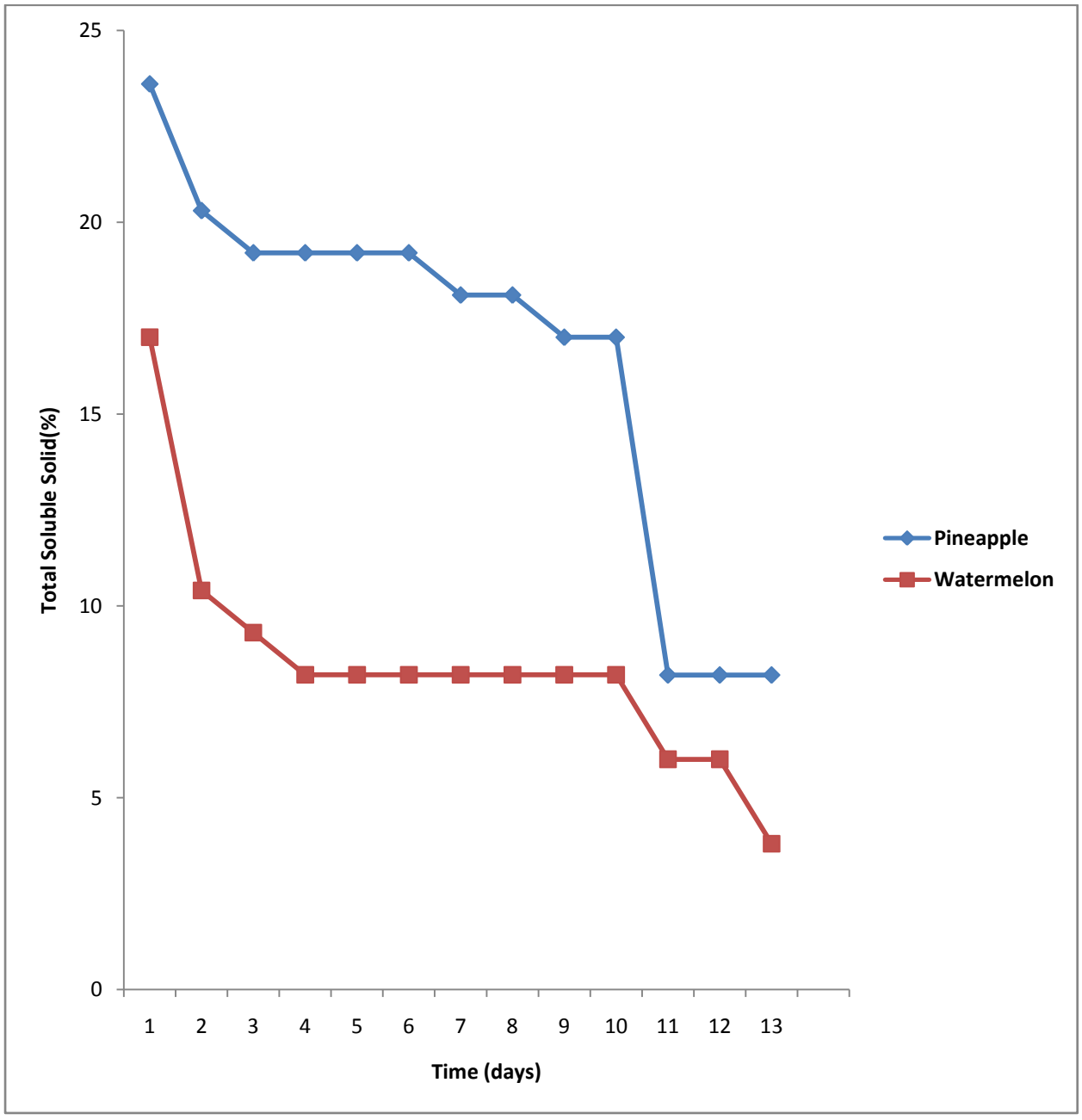


Fig 4.3: Pattern of Total soluble solid during fermentation of pineapple and watermelon wine.

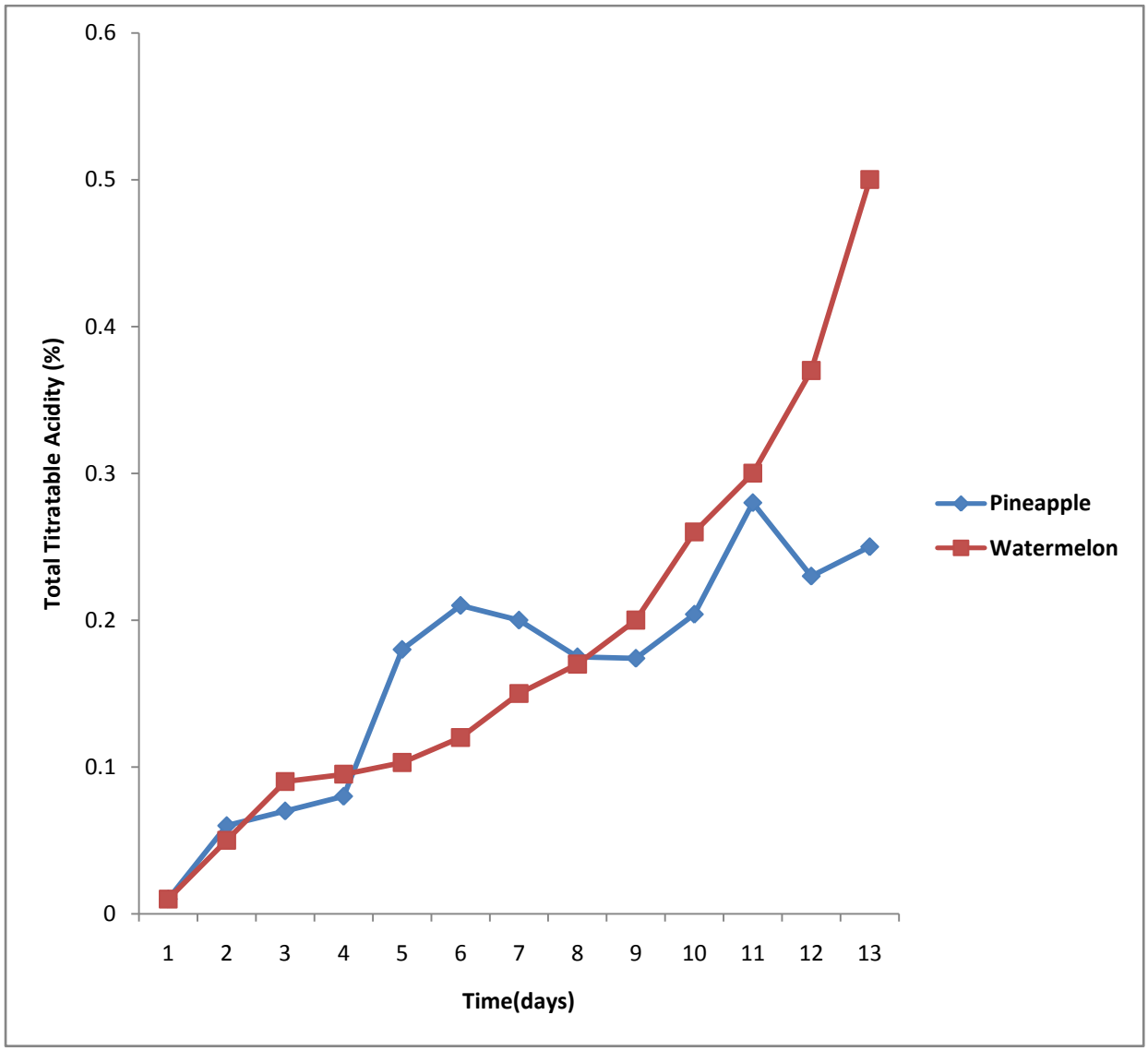


Fig 4.4: Pattern of Total titratable acidity during fermentation of pineapple and watermelon wine.

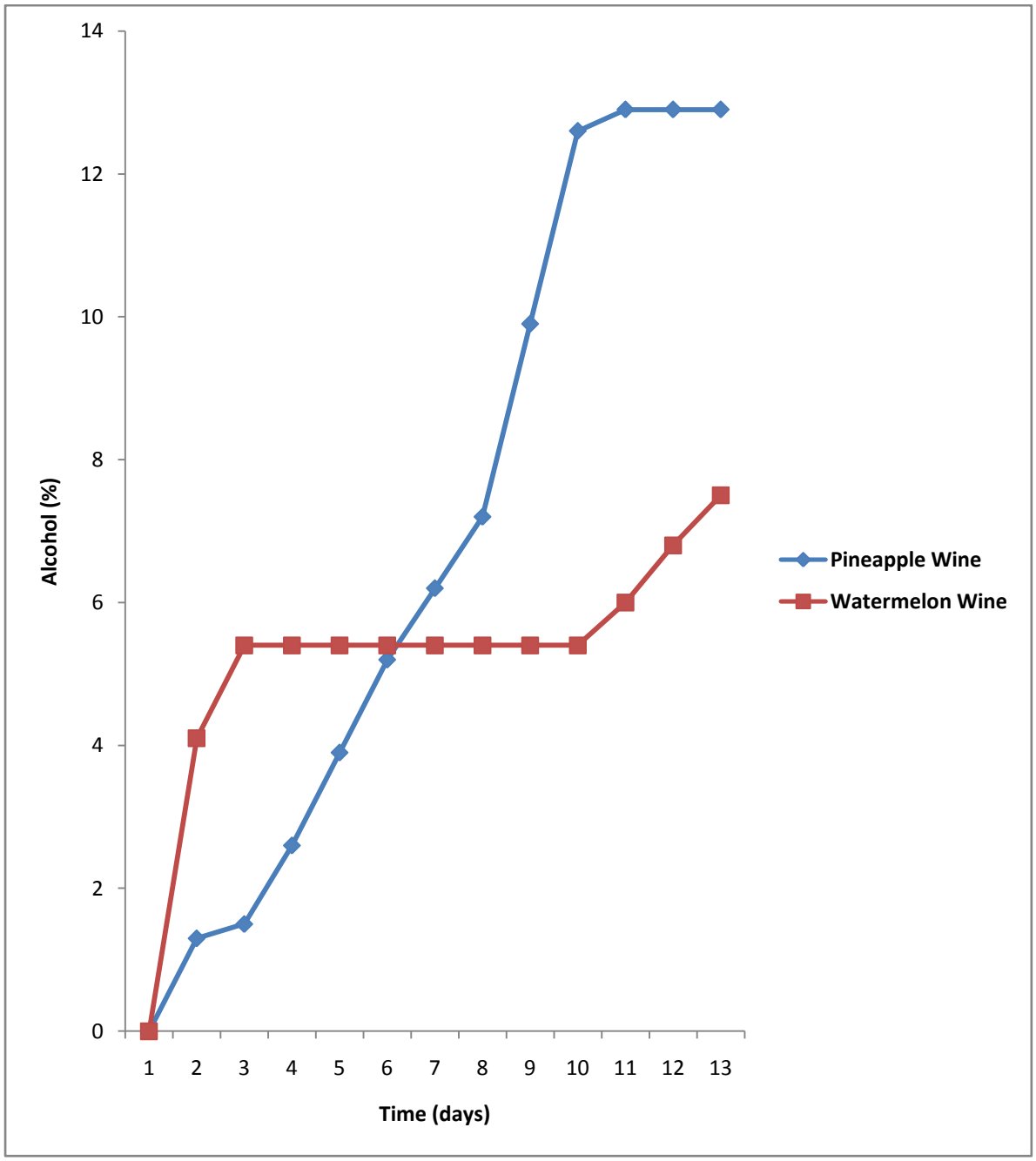


Fig 4.5: Pattern of Alcohol production in pineapple and watermelon wine during fermentation.

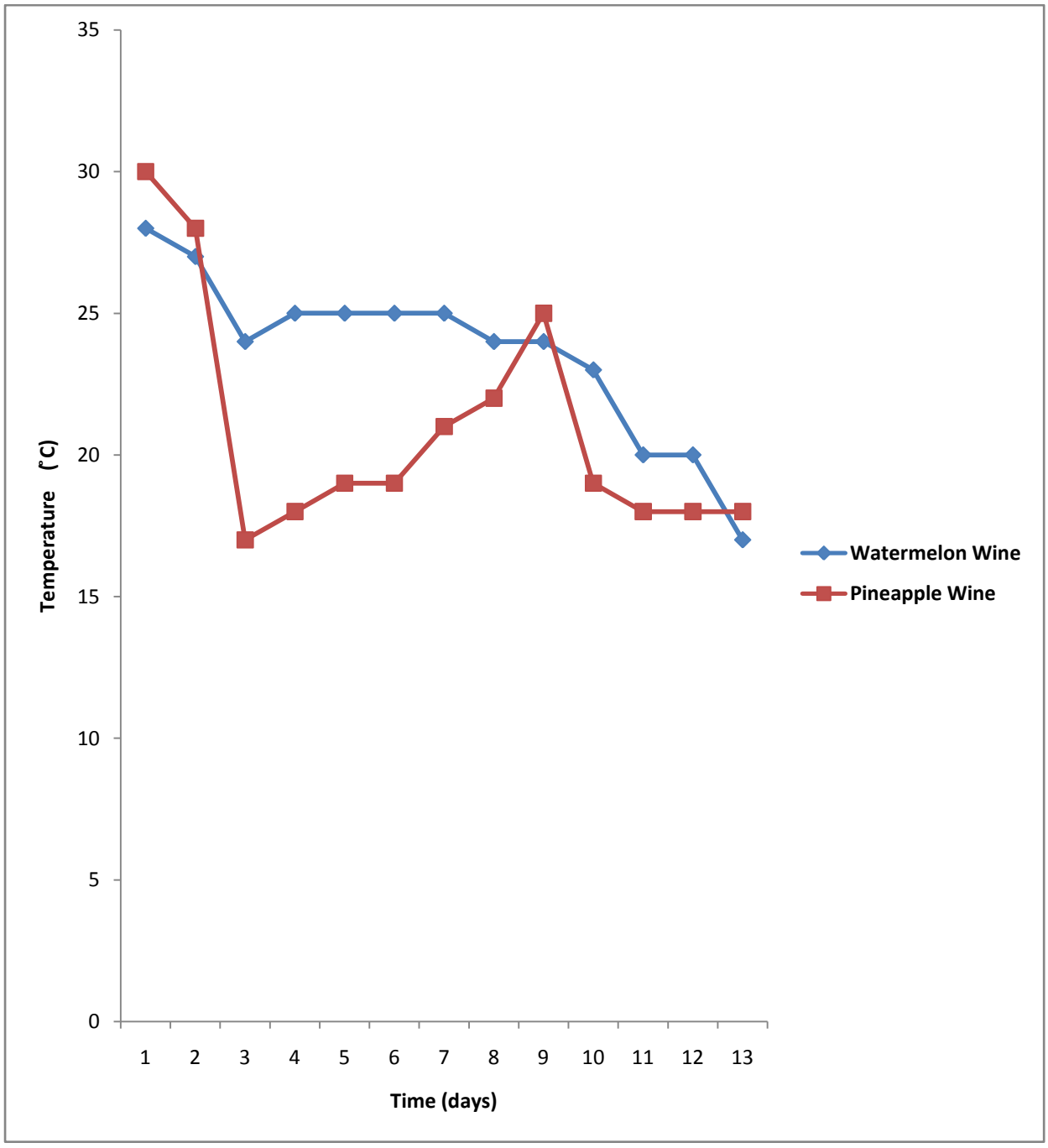


Fig 4.6: Temperature of watermelon and pineapple wine during fermentation.

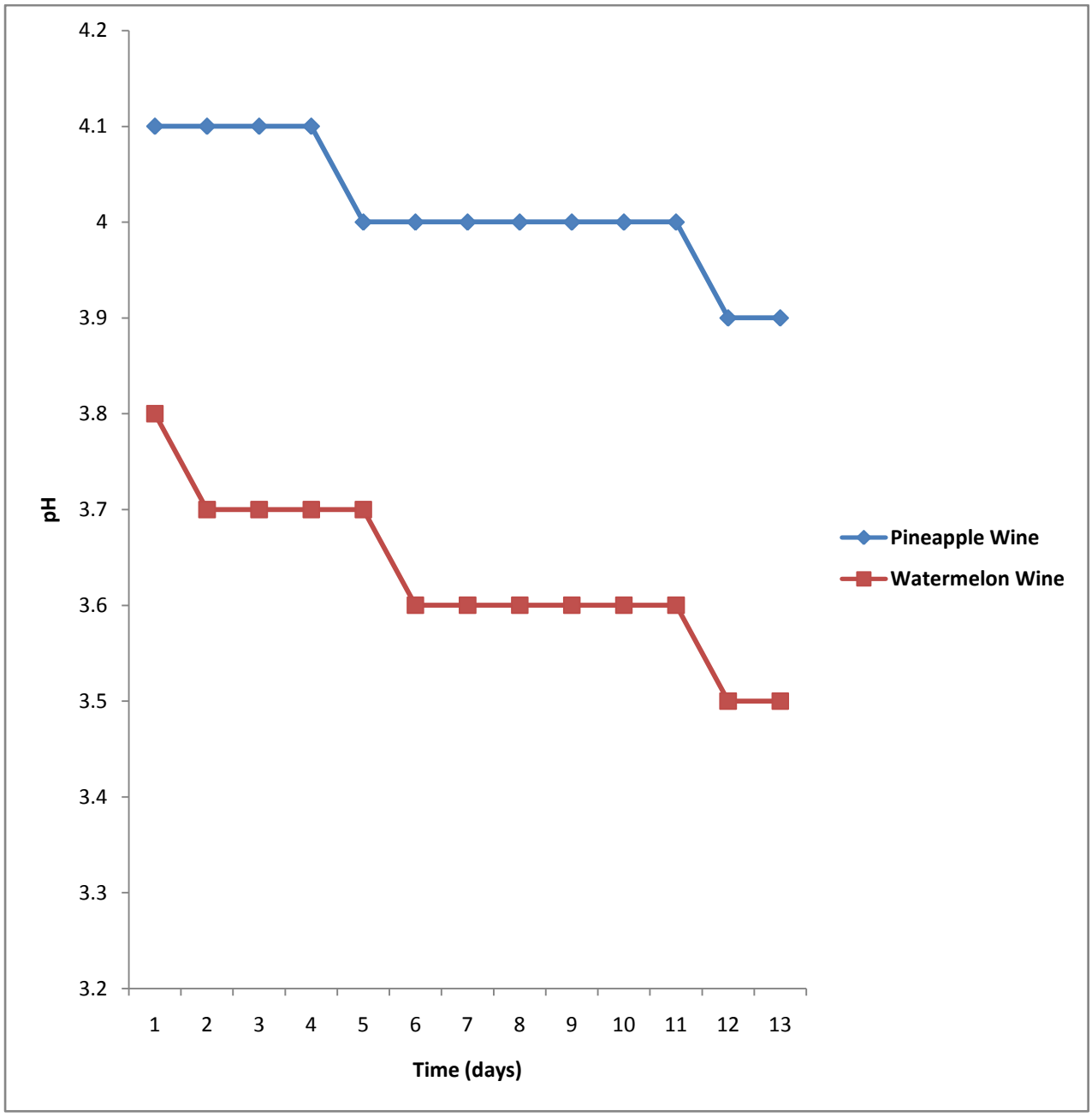


Fig 4.7: pH of pineapple and watermelon wine during fermentation.

4.4 Determination of the Microbiological and Physicochemical Profile of the Wine Produced.

Microbiological analysis of the wine was carried out during the period of fermentation to ensure that the wine was safe for consumption. The volume of inocula used was 0.1ml at a dilution factor of 10^{-2}, it was observed that in both pineapple and watermelon wine the number of colonies on the Potatoes dextrose agar plate increased daily as the days increased but on the tenth day the number of colonies started decreasing. On the nutrient agar plate the number of colonies was observed to increase daily also on the tenth day it also started decreasing as shown on Table 4.6. The total yeast count on the potatoes dextrose agar plates also increased as the days increased and started decreasing on the tenth day in both pineapple and watermelon wine. Also on the nutrient agar the total bacteria count in both pineapple and watermelon wine was found to increase consistently as the days increased and then on the tenth day it started decreasing in number. The bacteria obtained were further investigated and found out to be *Lactobacillus plantarum*, *Lactobacillus brevis* and *Pediococcus specie* as shown on Table 4.7 and it was observed that on de Man Rogosa Sharpe agar two colonies which were *Lactobacillus species* appeared whitish while *Pediococcus specie* appeared creamy. All the three bacteria isolates obtained were gram positive rod cells, non-spore formers, non-motile and could all grow at 15°C and 30°C while at 45°C only *Pediococcus specie* could survive. The biochemical test carried out showed that all the three bacteria obtained are catalase negative, methyl red negative, Voges- proskauer negative and indole negative. *Lactobacillus plantarum* and *Pediococcus specie* tested positive for urease test and nitrate reduction test and only *Lactobacillus brevis* tested positive for citrate test. The sugar fermentation test showed that only *Lactobacillus plantarum* could utilize lactose and sucrose sugars while *Lactobacillus brevis* alone could utilize sugar arabinose while all the bacteria isolated could utilize glucose and fructose sugar as shown

Table 4.6: Microbiological Profile of Pineapple and Watermelon Wine Produced at Dilution Factor of 10^{-2} and 0.1 Volume of Inocula.

Time (Days)	Number of colonies on PDA from Pineapple wine	Number of colonies on NA from Pineapple wine	Total yeast count (CFU/Ml) on PDA from Pineapple wine	Total bacterial count (CFU/Ml) on NA from Pineapple wine	Number of colonies on PDA from watermelon wine	Number of colonies on NA from watermelon wine	Total yeast count on PDA. (CFU/ML) from watermelon wine	Total bacterial count on NA. (CFU/ML) from watermelon wine
1	60	50	6	5	80	90	8	9
2	70	72	7	7.2	150	200	15	20
3	95	98	9.5	9.8	250	240	25	24
4	175	200	17.5	20	300	315	30	31.5
5	300	450	30	45	TNTC	TNTC	TNTC	TNTC
6	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
7	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
8	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
9	TNTC	TNTC	TNTC	TNTC	70	90	7	9
10	100	125	10	12.5	65	80	6.5	8
11	70	80	7	8	50	60	5	6
12	50	60	5	6	30	50	3	5
13	30	40	3	4	10	30	1	3

Key: **NA**; Nutrient Agar, **PDA**; Potatoes Dextrose Agar, **CFU/ML**; Colony Forming Unit per milliliters, **ML**; Milliliters, **TNTC**; Too numerous to count.

Table 4.7: Cultural, Microscopic and Biochemical Characteristics of Bacteria Isolates.

Isolates code's	Growth on MRS	GRM	SPR	MOT	Temp. Test			Biochemical Tests							Sugar Fermentation							Inference
					15°c	30°c	45°	Cat	U	MR	VP	NR	CI	IN	Glu	Gla	Fru	Suc	Ara	Mal	Lac	
PWW1	Round whitish, convex, colonies with entire margin.	+ve rods	-	-	+	+	-	-	+	-	-	+	-	-	+	+	+	+	-	+	+	*Lactobacillus plantarum*
WWW2	Round opaque, smooth, convex colonies with entire margins.	+ve rods	-	-	+	+	-	-	-	-	-	-	+	-	+	-	+	-	+	+	-	*Lactobacillus brevis*
WWW3	Round, creamy colonies	+ve spherical cells	-	-	+	+	+	-	+	-	-	+	-	-	+	+	+	-	-	-	-	*Pediococcus specie*

Key: **MRS**; de Man Rogosa Sharpe agar, **GRM**; Gram's reaction test, **SPR**; Spore formation test, **MOT**; Motility test, **CAT**; Catalase test, **U**; Urease test, **MR**; Methyl red, **VP**; Voges-proskauer, **NR**; Nitrate reduction test, **CI**; Citrate test, **IN;** Indole test, **Glu**; Glucose, **Gla**; Galactose, **Fru**; Fructose, **Suc**; Sucrose, **Ara**; Arabinose, **Mal**; Maltose, **Lac**; Lactose.

Table 4.8: Confirmed Bacterial Isolates

Bacteria isolates	**NH_3 from arginine**	**NaCl (%)**			**ARA**	**CEL**	**MAN**	**MAZ**	**MEL**	**RAF**	**RIB**	**SAL**	**LAC**	**MEZ**	**RHA**	**SOR**	**XYL**	**TRE**	**ESC**	**Inference**
		2	**4**	**6.5**																
PWW 1	-	+	+	-	-	+	+	+	+	+	+	+	-	+	+	+	-	+	+	*Lactobacillus plantarum*
WWW2	+	+	-	-	+	-	-	-	-	-	+	-	+	-	-	-	+	-	+	*Lactobacillus brevis*
WWW3	-	-	+	+	+	+	-	+	-	-	+	+	-	-	+	-	+	+	-	*Pediococcus specie*

Key: **ARA**; Arabinose, **CEL**; Cellobiose, **MAN**; Mannitol, **MAZ;** Mannose, **MEL**; Melebiose, **RAF**; Raffinose, **RIB;** Ribose, **SAL;** Salicin, **LAC;** Lactose, **MEZ**; Melezitos, **RHA**; Rhamnose, **SOR**;Sorbitol, **XYL**; Xylose, **TRE**; Trehalose, **ESC**; Esculin, -; negative, +; positive.

The bacteria isolates obtained were confirmed to be *Lactobacillus plantarum*, *Lactobacillus brevis* and *Pediococcus specie* by further testing their abilities to utilize more sugars as shown in Table 4.8 and it was observed that only *Lactobacillus brevis* could produce ammonia from arginine and only *Pediococcus specie* could survive in presence of 6.5% NaCl. Table 4.9 showed that *Lactobacillus plantarum* was obtained from pineapple wine while *Lactobacillus brevis* and *Pediococcus specie* were obtained from watermelon wine.

Proximate composition of the wines produced from pineapple and watermelon was also carried out in comparison to that of a standard pineapple wine as shown in Table 4.10 and the result showed that watermelon wine produced had the highest moisture content followed by the standard pineapple wine purchased. Protein was highest in watermelon wine produced followed by pineapple wine produced while the standard pineapple wine purchased had the lowest protein content. The pineapple wine produced had the highest fat content followed by the watermelon wine produced while fat was completely lacking in the standard pineapple wine obtained. Also the standard pineapple wine purchased had the highest carbonhydrate content compared to the pineapple and watermelon wine produced as shown on table 4.10

4.5 Evaluation of Sensory Quality of the Wine

Sensory analysis of the wine produced was carried to determine the quality of the wine produced as shown on Table 4.11. The colour of the standard pineapple obtained was preferred to that of the pineapple and watermelon wine produced. The taste of the pineapple wine produced was preferred to that of the standard pineapple wine obtained. The aroma of the pineapple and watermelon wine produced was preferred over that of the standard pineapple wine obtained. The overall acceptability of the wines showed that the pineapple wine produced was mostly preferred

Table 4.9: **Sources of Bacteria Isolated, Characterized and Identified.**

Sample Type	**Nos of Bacteria Isolated**	**SpecificBacteria Isolated**	**Codes used for each Bacteria**
Pineapple wine	1	*Lactobacillus plantarum*	PWW 1
Watermelon wine	2	*Lactobacillus brevis*	WWW 2
		Pediococcus spp	WWW 3

KEY: PWW 1- *Lactobacillus plantarum*,**WWW 2-** *Lactobacillus brevis*,
WWW 3- *Pediococcus spp*.

Table4.10: Proximate Composition of Wines Produced from Pineapple and Watermelon Juices.

Wines Types	Dry Matter (%)	Moisture content (%)	C.P (%)	C.F (%)	Fat (%)	Ash (%)	Carbonhydrate content (%)
Pineapple wine	12.06	87.94	0.63	0.00	0.04	17.14	82.19
Watermelon wine	8.21	91.79	1.56	0.00	0.02	17.39	81.03
Standard pineapple wine	8.66	91.34	0.31	0.00	0.00	0.00	99.69

Key: **D.M**; Dry Matter, **Moisture content**; 100- D.M, **C.P**; Crude Protein, **C.F**; Crude Fibre.

Table 4.11: Sensory Result of Wines Produced from Pineapple and Watermelon Juices.

Parameter	**Standard wine**					**Pineapple produced wine**					**Watermelon produced. wine**				
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
Color	-	-	-	2	8	-	-	1	3	6	-	1	2	3	4
Taste	-	-	3	2	5	-	-	1	2	7	-	1	-	3	6
Aroma	-	-	1	7	2	-	-	-	3	7	-	-	3	2	5
Overall acceptability	-	-	-	4	6	-	-	-	2	8	-	-	1	2	7

Key: **A**; dislike extremely, **B**; dislike very much, **C**; no preference, **D**; like slightly, **E**; like very much.

CHAPTER FIVE
5.0 DISCUSSION

5.1 Isolation and Identification of Yeast Strains from Spoilt Fruits and Alcoholic Breverage.

In this study, a total of three sample types which are burukutu alcoholic drink, spoilt pineapple fruit and spoilt watermelon fruit were opted for the isolation of yeast other than *Saccharomyces*, because a lot of work has been done on *Saccharomyces.* Seven (7), yeasts were obtained all together. Two (2) been isolated from burukutu drink, two (2), from spoilt pineapple and three (3) from spoilt watermelon. This might be due to richness in the nutritional composition of the fruits and alcoholic drink. This agrees with the work of Onuorah, *et al*. (2013) who isolated fungi such as *Candida specie, Pichia specie*, *Fusarium specie, Rhizopus specie* and *Aspergillus specie* from spoilt pineapple fruit and stated that high moisture content of pineapple fruit is a limiting factor in their preservation. Effiuvwevwere and Oyelade (2000) also reported that *Aspergillus specie* and *Candida specie* are responsible for the rottening of pineapple fruit. Tournas *et al*. (2006) isolated *Pichia specie*, *Candida specie,* and *Rhodotorula specie* from pineapple and watermelon fruits.

In this study yeasts isolated included *Candida sphaerica* and *Pichia specie from* burukutu alcoholic drink, *Candida pelliculosa*, and *Cryptococcus humicola* from spoilt pineapple and *Candida guilliermondii, Candida famata* and *Kloeckera apiculata* from spoilt watermelon. In this study, *Candida specie* was predominant. This may be due to nutritional variability of the specie and their ability to tolerate presence of high alcohol. This agrees with the work of (Kindu, 2015) who stated that *Candida specie* occurred more in spoilt fruits due to their ability to tolerate acidic environments.

The isolates obtained were subcultured on malt yeast extract agar and the colonies formed varied in colour from white to cream, they had smooth surface appearance, entire margin and raised colonies except for *Candida famata* that had rough, whitish colony with undulating margin and convex elevation. From the result above, *Candida sphaerica*, *Candida famata* and *Kloeckera apiculata* lacked pseudohyphae. This can be attributed to the fact that septated hyphae or pseudohyphae, most likely evolved at a later point in time which means that early fungi (those that existed first) lacked pseudohyphae and its formation is known to promote virulence. Therefore these organisms are one of the early organisms that occurred and are less virulent Richard *et al*. (1994)

All the yeast smeared and observed appeared spherical to ovoid in shape in presence of methylene blue stain. This is because the actual shape of yeasts are spherical to ovoid in shape and methylene blue stain only provides a platform to see them more clearly as cells that are non-viable are stained dark blue while cells that are viable remain clear of the stain. This agrees with the work of Alaa *et al*. (2015) who stated that methylene blue stains the yeast cell darker and makes them visible to the eyes.

All the yeasts isolated were able to ferment glucose with gas production except for *Candida sphaerica* and *Cryptococcus humicola*. The yeasts isolated were confirmed using API Test kit for the fermentation of the various sugars.

5.2 Screening and Selection of Yeast Strain with the Best Fermentative Property.

All the seven yeast isolate obtained, characterized and identified were screened by carrying out different tests on the individual yeast to select the yeast with the best fermentative property for wine production. Tests carried out include flocculation test, ethanol tolerance test, hydrogen sulphide test, temperature tolerance test and stress tolerance test.

Flocculation is the term used to describe the property expressed by certain yeasts, when the spontaneously aggregate to form flocs with sediment in the culture. In this research, during the screening of the yeast to identify the one with the best fermentative property, it was observed that *Candida sphaerica*, *Candida famata* and *Kloeckera apiculata* had the best flocculation ability. This agrees with the work of Kocher and Pooja (2011) who stated that some strains such as *Kloeckera apiculata* had the ability to flocculate. Also Farias and Manca (2003) also stated that *Kloeckera apiculata* poses intense cell interactions in malt yeast peptone glucose medium and that optimum flocculate is observed at pH 4.5 in presence of calcium. He further stated that the electron microscopy shows how the cells are attached to each other along their sides by fine hair like threads. One of the significance of flocculation is that the use of selected pure yeast inocula of known ability is preferred to wine elaboration, therefore the indigenous flora must be avoided and the flocculation of *Kloeckera apiculata* could be an economic method to do it. This means that in wine production, since there is need to obtain consistent wine flavor and predictable quality, the use of selected pure yeast inocula of known ability is preferred and therefore, the indigenous flora must be avoided. The flocculation phenomenum is an important and economic method to do it. Also flocculation indicates their economic effect on production of

yeast biomass and ensures a high cell density and large volume of harvested cells and also display it ability to raise the ethanol productivity during the fermentation process.

Only *Candida famata* and *Candida pelliculosa* were able to grow in presence of 15% ethanol concentration at normal temperature, while all the yeasts isolated grew in presence of 13% ethanol at normal temperature except *Cryptococcus humicola* which could not even grow at 10% ethanol. This work agrees with the work of Ludmila *et al*. (2012) who stated that *Cryptococcus humicola* cells grown on glucose and ethanol died at 0.05mg/ml and 0.2mg/ml level of cellobiose lipid, respectively. *Candida guilliermondii,famata, pelliculosa* and *Kloeckera apiculata* grew well in the presence of 10% ethanol. This agrees with the work of Kindu (2015) who stated that Candida occurred more in spoilt fruits due to their ability to tolerate the presence of acidic environment. Also, Bilbao *et al*. (1997) stated that the growth of *Kloeckera apiculata* was enhanced at lower temperature in higher concentration of ethanol. High concentration of alcohol is reported to be toxic to yeast by inhibiting the cell growth due to the destruction of the cell membrane of the yeast. Therefore *Cryptococcus humicola* was not able to grow at all in ethanol because the cell wall of the yeast may have been destroyed by alcohol which led to the death of the organism.

The ability of the organisms to produce hydrogen sulphide was tested by growing the organisms on lead acetate medium and the result showed that only *Candida famata* and *Candida pelliculosa* were able to produce hydrogen sulphide on lead acetate medium, which means that hydrogen sulphide is not toxic or harmful to these two organisms since they can produce it that is they can survive in presence of hydrogen sulphide. Hydrogen sulphide production by yeast is a notable spoilage reaction but the extent of its production varies significantly among the strains within

each species. Thus some strains within specie could be significant spoilage organism while the other strains may not be detrimental. The remaining organisms that could not produce hydrogen sulphide showed that they can be affected or harmed by the presence of hydrogen sulphide. Sulphur in form of sulphur dioxide is normally added to the wine to kill unwanted microorganisms but since *Kloeckera apiculata* cannot survive the presence of hydrogen sulphide, therefore hydrogen sulphide was not used in the initial process of this wine production but rather an alternative which is pasteurization of the fruit juice was used to sterilize the medium before adding the inoculum. However it was used at the end of the fermentation to completely sterilize the wine and kill all living microorganism present in the wine.

During the temperature tolerance test it was observed that as temperature increased, the growth of *Kloeckera apiculata, Cryptococcus humicola, Candida famata and Candida sphaerica* decreased while the growth of *Candida pelliculosa* increased in number as temperature increased. *Pichia specie* and *Candida guilliermondi* remain constant in number as temperature increased. This means that *Kloeckera apiculata, Cryptococcus humicola, Candida famata and Candida sphaerica* prefer low temperature for their growth and are referred to as psychrophiles while *Pichia specie* grew very slow at moderate temperature and are therefore slow growers. However, *Pichia specie* and *Candida guilliermondi* grew well at moderate temperature and are referred to as mesophiles. *Candida pelliculosa* grew very well at 45°C and therefore it is referred to as a thermophile. Fermentation conducted under low temperature enables a rise in production and aroma retention, which favour an improvement in aromatic profile of the wine. This is because fermentation alters yeast nitrogen transport and metabolism thus hampering the coordination between carbon and nitrogen metabolism at low temperature. On the other hand,

high temperature may disrupt enzyme and membrane functions, resulting in stuck fermentation. Fermentation at higher temperature may have adverse effect on the wine in stunning the yeasts to inactivity and even 'boiling off' some of the flavour of the wine.

In the stress tolerance test all the organisms' survived upto the third stage of stress tolerance but only *Candida pelliculosa* survived the fourth stage of stress tolerance test. Strain survival of yeast under stressful condition provides useful information on their ability to grow and carry out fermentation as impaired yeast. This is because yeast growth during fermentation usually does not occur in optimal condition and are continuously exposed to several stress especially osmotic and ethanol stress. But since all isolates could survive to the third stage of stress tolerance test, this indicates that the can survive most of the conditions they will be confronted with in the fermentation vessel. This agrees with the work of Lee *et al*. (2011) who stated that *Candida specie* and *Pichia specie* could survive alcohol and osmotic stress and are alcohol tolerant yeast.

Therefore, from the above results, it has been observed that *Candida pelliculosa* is the best organism of choice from the seven yeasts isolated and screened for the fermentation processing of wine but it has a problem in that it is potentially pathogenic and therefore cannot be used for wine production. However, *Kloeckera apiculata*, is non-pathogenic, has a high flocculation ability, can withstand presence of 13% ethanol, grows best at 25°C and cannot produce hydrogen sulphide on lead acetate medium but can survive the stress tolerance test upto the third stage and has good properties. It was further discovered that all the organisms identified were implicated for pathogenity except for *Kloeckera apiculata* which was found to be non-pathogenic. Since wine must be consumed and pathogenic strain would be harmful to health of the individuals consuming the wine so all the isolates were eliminated and the only non-pathogenic organism

which is *Kloeckera apiculata* which was identified with 95.2% confidence level using API test kit was used for the wine production. *Candida species* thereby rarely trigger infection in healthy people, but take advantage of a locally or systematically impaired immune system to proliferate in the host and cause disease termed "Candidiasis" and therefore is not suitable for consumption in wine. However, wild yeasts such as *Candida* and *Pichia* can sometimes be used in wine production but are unpredictable and may introduce less desirable trait to the wine this can contribute to spoilage of the wine. *Pichia specie* isolated in this work has very poor fermentative properties. From the tests carried out, it is a slow grower and cannot form flocculants which determines the number of cell produced. This agrees with the work of Melanie and Lane (2011) which gave the reasons for the poor fermentative ability of the organism.

5.3 Production of wine from fruit juice using *Kloeckera apiculata* as fermenting agent.

In the production of pineapple and watermelon wine the fermentation was carried out for 13 days during which the sugars available were used up. The fermentation in pineapple wine was started with a sugar level of 23.6°Brix at a specific gravity of 1.10g/cm^3 so that the complete utilization of the sugar will give an alcohol level of 13.6% alcohol in pineapple wine and the watermelon wine was started with a sugar level of 17°Brix at specific gravity of 1.07g/cm^3 so that complete utilization of the sugar will give alcohol level of 9.5%. During the fermentation, (Fig 4.1) it was observed that as the days of fermentation increased the specific gravity decreased gradually in both wines and remained constant for a long period of time before decreasing further. This is because *Kloeckera apiculata* requires a long time for uptake and release of specific amino acid during fermentation Gump *et al., (*2010). The specific gravity decreased daily as the sugars were

been utilized. The lowest specific gravity was observed on the last day in the pineapple fermentation and also in the watermelon fermentation and this was used as a marker to know the level of residual sugar remaining in the wine and the alcohol level of the wine. This result is due to microbial metabolism of available sugar in the wine. This result agrees with the work of Uraih (2003), Awe (2011) and Huseyin *et al.,* (2006) who observed a decrease in specific gravity as the days increased. The specific gravity values of both pineapple and watermelon wines were observed to differ, statistically, these differences were not observed to be significant across the days ($p \leq 0.05$). However, the decrease was almost equivalent in pineapple and watermelon wine which indicates the presence of almost equal succession of microorganism in both wines produced.

It was also observed that as the days increased, the total soluble solid decreased in both pineapple and watermelon wine (Fig 4.2). The total soluble solids of the pineapple wine was measured at 23.6°Brix to give a calculated alcohol level of 13.6% but the final alcohol level obtained was 12.9% while in watermelon wine they total soluble solid was measured at 17°Brix to give a calculated alcohol level of 9.5% but the actual alcohol level obtained at the end of the fermentation was 7.5% which means that not all the sugars was completely utilized in both wines. This is attributed to the fact that 90-95% of total soluble solid is sugar and not all is converted to ethanol. Margalit, (1997) stated that about 5% of the sugar is consumed to produce by- products (glycerol, succinic acid, lactic acid, 2,3-butanediol and acetic acid), about 2.5% is consumed by the yeast as carbon source and about 0.5% are left over as unfermented residual sugar. In the pineapple wine produced, the total soluble solid decreased consistently which indicate active utilization of the sugars by the micro-organisms and production of alcohol.

Also non-*Saccharomyces* yeasts are reported to lack the ability to complete fermentation due to their low ethanol tolerance but are effective in improving wine quality (Toro and Vaquez, 2002; Clement-Jimenze *et al.,* 2004). In watermelon wine, it was observed that in the first three days, the total soluble solid decreased because the population of yeast increased after which the total soluble solid remained constant till the tenth day of fermentation and further decreased. This agrees with the work of Chanprasartsuk *et al.,* (2012a) who used *Hanseniaspora uvarum* as a single culture and mixed culture and noticed that after the second day of fermentation, the total soluble solid remained stable throughout the fermentation period but yet there was continous increase in alcohol content. This may be due to the slow utilization of the sugars by the yeast. The rate of decrease of total soluble solid was consistent in both wines which agree with the fact that they contain almost equal number of microorganisms.

The Total titratable acidity was observed to increase consistently as the days increased throughout the period of fermentation for both watermelon and pineapple wine. This is due to the microbial succession that is evident with concomitant production of intermediate acid from alcohol initially produced during yeast metabolism. It was further observed that as the titratable acid concentration increased daily, the pH of the wine decreased. This is attributed to yeast metabolism and also shows the fermentation during the fermentation stages which is crucial to wine production. Acidity plays a vital role in determining the wine quality by aiding the fermentation process and enhancing the overall characteristics and balance of the wine. Lack of acidity will mean a poor fermentation. This agrees with the work of Awe, (2011) who stated that the total titratable acidity increased as the days increased. In this work, the total titratable acid concentration was observed to increase from initial concentration of 0.01% to a final

concentration range of 0.5% for watermelon wine and 0.01% to 0.25% for pineapple wine. The lowest titratable acidity was observed in pineapple wine which means that the pineapple wine taste more acidic than the watermelon wine because the titratable acidity relates more to the 'acid taste' of a wine while the pH relates more to things like microbial stability and susceptibility to mould and bacterial spoilage. The titratable acidity is always less than the total acidity, because not all of the hydrogen ions expected from the acids are found during the determination of titratable acidity. Despite the observed differences in titratable acidity concentration of the fruit wines, these differences statistically were not observed to be significant across the days ($p \leq 0.05$). This is in agreement with the result of Chikala *et al.* (2010) who stated that there was no significant difference between the values he obtained in presence of the tested yeast strains.

This present study reveals low pH values in the fruit wines produced throughout the fermentation period, as the pH decreased throughout the period of fermentation (Fig 4.6). pH which is the logarithm of the concentration of free protons in the wine produced, and is usually expressed with a positive sign. The pH obtained for the final product which range between 4.1-3.5 for both watermelon and pineapple wine falls within the acidity level of sweet and dry wines. Usually, acidity of wines lies between pH 3 and 7; higher acidity are sometimes encountered with fortified and sparkling wines. Decrease in pH value could be due to microbial succession from yeast to lactic acid bacteria resulting in the production of more acid as is evident in malolactic acid fermentation. In order to supplement the sugar content of the must, glucose was part of the additives. Reports have shown that the major problem associated with the use of tropical fruits in wine production is their low sugar content Alobo and Offonry, (2009).

Remarkable amount of alcohol was produced from the fruit wines during fermentation with the test yeast strains. The alcohol content of both wines increased gradually as the days increased indicating that the sugars were been utilized by the yeast, confirming the conversion of sugars into alcohol. As much as 80% of alcohol was produced during the aerobic fermentation stage which is consistent with previous submission that about 70% of fermentation activities occur during the aerobic fermentation phase. The final alcohol content of the pineapple wine was 12.9% while that of watermelon was7.5% which ranks among good table wine. Steady increase in alcohol content was observed in the fruit wines throughout the period of fermentation. The higher alcohol level was observed in pineapple wine and lower alcohol level was observed in watermelon wine. This is attributed to the fact that pineapple wine contained higher amount of sugar compared to the watermelon wine. Although, the values of alcohol content in the fruit wines were observed to be different during the different fermentation days, these differences were not observed to be significant statistically ($p \leq 0.05$). This trend is irrespective of the fruit wine. The variation in alcohol levels is usually due to difference in their optimal physico-chemical conditions such as temperature and pH. Alcoholic fermentation leads to a series of by products in addition to ethanol. Some of the byproduct include carbonyl compounds, alcohols, esters, acids and acetyls, all of them influencing the quality of the finished product. The composition and concentration level of the byproducts can vary widely (ng/L to hundreds of mg/l)(Plutowska and Wardencki, 2008 Darte *et al*., 2010). The yeast produced glucanolytic and xylanolytic enzymatic activities which influenced alcoholic fermentation.

Temperature did not follow any specific trend in both watermelon and pineapple wine. It has been reported that temperature affect the gene expression in yeast (Staci, 2003). The optimum

growth temperature for *Kloeckera apiculata* is 17-25°C. However, the fermentation was carried out at 17-30°C thus favouring *Kloeckera apiculata* in total alcohol production. Temperature affect the sensitivity of the yeast to alcohol concentration, growth rate of the microorganism, rate of fermentation, viability and length of lag phase and temperature changes could be due to microbial metabolism of available nutrients to produce alcohol and other fermentation products with the resultant generation of heat. This is why the temperature was started at room temperature then decreased using ice block as the alcohol content of the wine increased, since *Kloeckera apiculata* cannot survive in alcohol at high temperature. This result agrees with the reports of Robinson, (2006) and Okafor, (2007) who stated that *Kloeckera apiculata* could survive high alcohol content at low temperature.

5.4 Microbiological and Physicochemical profile of the wine produced.

Throughout the period of fermentation, quality control of the wine produce was carried out to determine the microbial standard of the wine. The increase in total yeast count during aerobic fermentation can be attributed to the presence of utilizable sugar and yeast nutrient. Daily aeration aided rapid multiplication of the yeast cells (Berry, 2000). In both pineapple and watermelon wine the number of micro-organisms increased significantly then started reducing in the first week of anaerobic fermentation. This may be due to increase in alcohol content of the wine. During the anaerobic fermentation stage, yeast was noted only in the first week. This is likely because during this stage alcohol levels had increased in the fermenting medium and lead to the death of yeasts and also depletion of nutrient has taken place which also lead to the death of lactic acid bacteria present in the medium. During anaerobic growth the yeast utilized

intermediate product like acetalydehydes as hydrogen acceptors for alcohol production (Prescott *et al*., 2008).

The three bacteria species identified and confirmed during the fermentation are non-pathogenic bacteria and therefore the do not constitute any treat to health. They are associated with fruits and locally fermented drinks this is in agreement with the work of Awe, (2011) Moulds could not be isolated due to low level of oxygen as the fermenters were air tight and also it could as well be due to the enhanced level of acid produced by *Kloeckera apiculata* and *Lactobacillus* species. The lactic acid bacteria added sour taste to the wine and also decreased the pH of the wine Amoa-Awua *et al*., (2007). These bacteria control the growth of undesirable microorganisms such as Enterobacteria by the production of hydrogen peroxide Eveline *et al*. (2009). The fruit juice provided all the nutrients and favorable conditions necessary for microbial fermentation and also thiamine a type of yeast nutrient which is very necessary for the growth of *Kloeckera apiculata* was added to the medium because it stimulate the yeast growth, speed up fermentation and reduces the production of sulphur dioxide binding compounds. Inspite of the variation in microbial count in the various fermented substrates, a high microbial count was noted, this observation may be due to high water activities which lead to enhancement in the microbial succession in different substrates. Also glucose was used in the fermentation instead of sucrose since *Kloeckera apiculata* metabolize or ferment glucose at higher rate than sucrose. Malolactic fermentation impacted desirable flavor which was "buttery" to the wine during maturation.

Proximate analysis as seen in Table 4.10 above, showed that the test fruits are poor sources of protein with watermelon wine having the highest percentage of crude protein of 1.56%. Proteins

are major source of *energy*, as well as containing essential amino-acids, such as lysine, tryptophan, methionine, leucine, isoleucine and valine, which are essential to human health, but which the body cannot synthesize. Proteins are also the major structural components of many natural foods, often determining their overall texture. But wines are generally poor sources of proteins. The highest moisture content was also observed in watermelon wine which was 91.79% the high moisture content of the wine can be associated with the fruits and this account for their high perishable nature and short shelf life under normal storage conditions while the lowest moisture content was observed in pineapple wine produced. Watermelon wine had the highest percentage of protein and moisture content which means that it has a shorter shelf life than pineapple wine. The carbonhydrate content is higher in pineapple wine than in watermelon wine as compared to the standard pineapple wine which has a carbonhydrate content of 99.69%. The reasonable amount of carbonhydrate gives account of their high calorie value. The percentage ash content was higher in watermelon wine (17.39%) than in pineapple wine (17.14%) and completely absent in standard pineapple wine. Ash content is of paramount importance since it is the inorganic residue remaining after the water and the organic matter have been removed which provide a measure of the total amount of minerals within a food. This means that the watermelon wine contains more nutrient than pineapple wine produced. The fat content was higher in pineapple wine with a value of 0.04% than in watermelon wine with a value of 0.02%. Lipids are a major source of energy and provide essential lipid nutrients. Nevertheless, over-consumption of certain lipid components can be detrimental to our health. In many foods the lipid component plays a major role in determining the overall physical characteristics, such as flavor, texture, mouth feel and appearance. For this reason, it is difficult to develop low-fat alternatives of

many foods; because once the fat is removed some of the most important physical characteristics are lost. The crude fibre was completely absent in both pineapple wine and watermelon wine and in the standard pineapple wine purchased.

5.5 Sensory evaluation of wine produced.

The sensory analysis of the wine is important in determining the quality of the wine produced. It revolve around the taste, aroma and colour of the wine produced. It was observed that the colour of the standard pineapple wine was mostly preferred followed by that of pineapple wine produced as shown in Table 4.11 above. This is due to the fact that more sophisticated equipment was used in clearing the standard pineapple wine in the winery. Also, cell wall polysaccharides, principally manoproteins which were released by yeast during autolysis combined with tannin and anthocyanine to impact the wine astringency and colour.

In the comparison of the taste and aroma of the wines, the pineapple and watermelon wines produced were preferred to the taste of the standard pineapple wine. This is because glycerol is a wine constituent which contribute to the sweetness, viscousity and smoothness of the wine produced and it is produced only by *Kloeckera apiculata* (Chanprasartsuk *et al*., 2010b) Secondary metabolites produced by *Kloeckera apiculata* augment the sensory qualities of the wine produced. *Kloeckera apiculata* have interesting enzymes such as beta-D-glucosidase and beta-D-xylosidase which are key enzymes to release aromatic compound in wine making. Reduction of acid in wine by bacteria is known as malolactic fermentation. It improves wine quality where employed. Malolactic fermentation impacts flavor and aroma to the wine and this was performed by the *Lactobacillus plantarum, brevis* and *Pediococcus* species present in the

wine. Malolactic bacteria decreased acidity in the wines by transforming L-malic acid to L-lactic acid, enhanced wine flavor and complexity through production of additional metabolites and increased stability of the wine by removal of residual nutrients and production of bacteriocin that prevent the growth of other bacteria in the wine. This is in agreement with the work done by Romano *et al*. (1997a) who stated that, wine makers and wine researchers have come to realize that also non-*Saccharomyces* yeasts contribute in significant measure to the flavour and quality of wine than previously thought.

CHAPTER SIX
6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

The overall conclusions that could be drawn from the present study are;

That a total of seven yeasts were isolated, identified and characterized from spoilt fruits and alcoholic beverage to be *Candida sphaerica, Pichia spp, Candida pelliculosa, Cryptococcus humicola, Candida guilliermondii, Candida famata* and *Kloeckera apiculata.*

That the yeast with the best fermentative property was *Candida pelliculosa* but since it is pathogenic *Kloeckera apiculata* which is non-pathogenic and also had good fermentative properties was selected for the wine production.

That pineapple and watermelon wine were produced separately using *Kloeckera apiculata* as fermenting agent during which the different parameters were noted daily and they include pH (4.1-3.9 for pineapple wine and 3.8-3.5 for watermelon wine), Total soluble solids which decreased gradually (17.0-3.8 for watermelon wine and 23.6-8.2 for pineapple wine), Total titratable acidity (0.01-0.25 for pineapple wine and 0.01-0.5 for watermelon wine), Alcohol content (0-12.9 for pineapple wine and 0-7.5for watermelon wine) were observed statistically not to be significant across the days ($p \leq 0.05$) despite the overall difference in values of each parameter.

That the microbiological analysis of the wine produced revealed the presence of *Lactobacillus plantarum, Lactobacillus brevis* and *Pediococcus specie* which are lactic acid bacteria and are non-pathogenic and thus the test result declared the wine safe for consumption.

The wine produced using *Kloeckera apiculata* was preferred in taste and aroma compared to the standard pineapple wine produced in the winery however not in colour.

6.2 Recommendations

1. Molecular analyses such as genome sequencing should be carried out on the strains of *Kloeckera species* as this will be of great industrial biotechnological importance.
2. Other organisms as well as other fruits should also be tried for their potentials to serve respectively as fermenting agents and substrates for alcohol production
3. Rapid and inexpensive recovery methods for wine so produced should be established to save time, increase recovery rate and purity.
4. Nigerian government should encourage the establishment of local industries for wine production from fruits so as to reduce importation as well as help to reduce unemployment.

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Appendix I: Properties of Pineapple Wine from Pineapple Juice Produced Using *Kloeckera apiculata.*

Time of fermentation (days)	Specific gravity (g/cm^3)	Total soluble solids (%)	Total titrable acidity (%)	Potential Alcohol content (%)	Alcohol content (%)	Temp.(°c)	pH
1	1.10	23.6	0.01	13.6	0	30	4.1
2	1.085	20.3	0.06	11.5	1.3	28	4.1
3	1.08	19.2	0.07	10.9	1.5	17	4.1
4	1.08	19.2	0.08	10.9	2.6	18	4.1
5	1.08	19.2	0.18	10.9	3.9	19	4
6	1.08	19.2	0.21	10.9	5.2	19	4
7	1.075	18.1	0.20	10.2	6.2	21	4
8	1.075	18.1	0.175	10.2	7.2	22	4
9	1.07	17.0	0.174	9.5	9.9	25	4
10	1.07	17.0	0.204	9.5	12.6	19	4
11	1.03	8.2	0.28	4.1	12.9	18	4
12	1.03	8.2	0.23	4.1	12.9	18	3.9
13	1.03	8.2	0.25	4.1	12.9	18	3.9

Appendix II: Properties of Watermelon Wine from Watermelon Juice Produced Using *Kloeckera apiculata.*

Time of fermentation (Days)	Specific gravity (g/cm^3)	Total soluble solids (%)	Total titratable acidity (%)	Potential Alcohol content (%)	Actual Alcohol content (%)	Temp.(°c)	pH
1	1.07	17.0	0.01	9.5	0	28	3.8
2	1.04	10.4	0.05	5.4	4.1	27	3.7
3	1.035	9.3	0.09	4.8	5.4	24	3.7
4	1.03	8.2	0.095	4.1	5.4	25	3.7
5	1.03	8.2	0.103	4.1	5.4	25	3.7
6	1.03	8.2	0.12	4.1	5.4	25	3.6
7	1.03	8.2	0.15	4.1	5.4	25	3.6
8	1.03	8.2	0.17	4.1	5.4	24	3.6
9	1.03	8.2	0.2	4.1	5.4	24	3.6
10	1.03	8.2	0.26	4.1	5.4	23	3.6
11	1.02	6	0.3	5.4	6	20	3.6
12	1.02	6	0.37	2.7	6.8	20	3.5
13	1.01	3.8	0.5	1.4	7.5	17	3.5