PRODUCTION OF WATERMELON-CARROT FERMENTED BEVERAGE USING *SACCHAROMYCES CEREVISIAE* ISOLATED FROM LOCALLY FERMENTED ALCOHOLIC BEVERAGES

BY

OKWOR, Chioma Rita
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ABSTRACT

Fermented fruit beverage is a beverage produced by subjecting fruit juice to fermentation using yeast like *Saccharomyces* cerevisiae. The study was based on the production of fermented fruit beverage (watermelon and carrot) using *Saccharomyces cerevisiae* isolated from locally fermented alcoholic beverages (Palmwine, *burukutu* and *pito*). The yeast isolated was identified using microscopy, sugar utilization test, ethanol tolerance and molecular identification. This was inoculated into the must and allowed to undergo primary and secondary fermentation for 4 and 7 days respectively. The concentration of the yeast was adjusted to 0.5 McFarland standard. The isolates assimilated glucose, fructose and sucrose with the exception of xylose. The ethanol tolerance was high at 5 – 10 % and low at 15 – 20 %. During fermentation the temperature of the Must ranged from 28.03 ± 0.01 to 32.80 ± 0.39 ºC, pH 3.55 ± 0.01 to 5.02 ± 0.16, total soluble solid 0.15 ± 0.00 to 7.05 ± 0.42 mg/L. Specific gravity ranged from 0.97 ± 0.00 to 1.06 ± 0.001 kg/m^3. The final alcohol concentration of the mixed wine was 7.3 ± 0.06 to 9.05 ± 0.07 %. The proximate analysis showed a statistical difference in carbohydrate, moisture and crude protein ($p < 0.05$) while there was no statistical difference in fat and ash ($p > 0.05$). Sensory evaluation showed preference for watermelon and carrot wine> watermelon > carrot. This study suggests that alcoholic fruit beverage can be produced from watermelon-carrot using *Saccharomyces cerevisiae* isolated from palmwine.

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<center>**CHAPTER ONE**</center>

1.0   **INTRODUCTION**

1.1   Background to the Study

Alcoholic fruit beverages are produced by fermentation of fruit juice using yeast like *Saccharomyces cerevisiae*. Grapes have preference over other fruits owing to their natural chemical balance which speeds up their fermentation process without the addition of sugars, acids, enzymes, or other nutrients (Ogodo *et al*., 2015). However, a wide variety of fruits which differ in shape, colour, taste and nutritive value are widely used for production of fermented beverages (Jagtap and Bapat, 2015). Fruits used for the production of fruit wines in different parts of the world include: apples, berries, cherries, wild apricots, pears, kiwifruit, plums, peaches, strawberries, currants, bananas, pineapples, cashew nuts, pomegranates, lemons, tangerines, oranges, dates, and figs (Joshi, 2009; Joshi *et al*., 2017). Home-made wines have been produced with variety of fruits; apple, banana, cashew, watermelon, orange, plum, strawberry, guava, cherries, pawpaw, cucumber using S. *cerevisiae* which converts the sugar contents of the substrate to alcohol, organic acids and esters (Duarte *et al*., 2010; Isitua and Ibeh, 2010). A combination of one or two of these fruits can be used in mixed fruit wine production. For instance, Ogodo *et al*. (2015) reported the production of mixed fruit wines from pawpaw, banana and watermelon using *Saccharomyces cerevisiae* isolated from palm wine.

Saccharomyces cerevisiae has been an essential component of human civilization because of its extensive use in food and beverage fermentation with high commercial significance. It is involved in the production of many fermented

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beverages, such as wine, beer and cider, distilled beverages, such as rum, vodka, whisky, brandy (Hittinger *et al.*, 2018). This is because of its ability to ferment sugar in fruit juice with the production of alcohol, carbon (IV) oxide and under anaerobic condition. It can also grow rapidly in sugar and produce high yield of biomass anaerobically (Olowonibi, 2017). Fermentation can take place either from a spontaneous development of the raw material microflora, or from the addition of a pure yeast culture (Hittinger *et al.*, 2018).

1.2   Statement of the Research Problem

Nigeria is one of the African countries blessed with abundant fruits and vegetables which are mostly sold raw across markets in towns and villages. Most of these fruits get spoilt as a result of under consumption and preservation. However, despite the availability of the fruits all year round, Poor handling and transportation system, high temperature, inadequate storage and processing facilities contribute significantly to high post-harvest losses and consequently financial loss in Nigeria.

Previous studies on watermelon focused mainly on wine production using *Saccharomyces cerevisiae* isolated from either *burukutu, palm wine* or *pito*. This research is based on screening for yeast isolate with the best fermenting potential from the above alcoholic beverages and also production of fermented fruit beverage.

1.3   Aim and Objectives of the study

The aim of this study was to produce fermented carrot-watermelon (mixed) beverage using *Saccharomyces cerevisiae* isolated from locally fermented alcoholic beverages.

The objectives of the study were to:

i. Isolate and identify *Saccharomyces cerevisiae* from locally fermented alcoholic beverages (palm wine, *burukutu* and *pito*).

ii. Produce fermented beverage from watermelon and carrot juice (mixed) using the best yeast species from the locally fermented alcoholic beverages (*palm wine*, *burukutu* and *pito*).

iii. Determine the physiochemical properties of the beverage produced.

iv. Evaluate the sensory qualities of the beverage produced

1.4 Justification for the Study

Watermelon and carrot have full array of nutrients, carbohydrates, sugar, soluble and insoluble fibre, sodium, vitamins (A, C, B1, B6), minerals, fatty acids, amino acids and carotenoid which are of great nutritional and health benefit to humans (Charoensiri *et al.,* 2009).

In developing countries like Nigeria, 20-30 % of fruits produced are wasted due to lack of proper utilization, post- harvest and processing technology. Fermentation into value added product is a vehicle of choice to this problem (Nwachukwu *et al*., 2006). Thus, fermented fruit beverage should be promoted to extend the shelf life and add values to local fruits, reduce dependence on imported wine, and create employment opportunities.

Maize and other cereal grains are brewed into local drinks such as *pito*, *burukutu, kunu zaki* in Nigeria and in other Sub-Saharan African countries, very little is known about fermentation of pineapple, cashew, mango, water melon, carrot and orange juices into alcoholic fruit beverages (Audu, 2010). Production of carrot-watermelon will increase the number of fermented drinks as well as nutrient intake.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Watermelon and Carrot Plants

Watermelon (*Citrullus lanatus*) is a fruit that belongs to the family of cucumbitacea. The fruit is round with reddish mesocarp having a lot of seed and mostly common in the south Asia. There are various species with different coloured endocarps; red, yellow and orange flesh. In Nigeria, watermelon is fermented, blended and consumed as juice, nectar, fruit cocktails and can also be used as an appetizer or snacks depending on how it is prepared (Inuwa *et al*., 2011). It serves as a good source of vitamins and Phytochemicals that have chemo-preventive effects against cancer (Enukainure *et al*., 2010). The seeds are also reported to possess medicinal properties and are used to treat chronic or acute eczema. It contains high levels of proteins, lipids and is a rich source of carbohydrate and fibre. Furthermore, they are used as a domestic remedy for urinary tract infection, hepatic congestion, catarrh, worm remedy, abnormal blood pressure (Amadi *et al*. 2003). Watermelon contains large amount of beta carotene and are significant sources of lycopene.

Carrot is the most important crop of *Apiaceae* family. Member of this family have small, mostly white, 5-parted flowers arranged in umbrella-like inflorescence called umbel (Essing, 2013). It has various cultivars, ranging from white, yellow, orange, red, purple, or very dark purple. Carrot was first used for medical purposes and gradually as food (Carlos and Dias, 2014). It is an important source of bioactive compounds with beneficial effect on the consumer's health as

carcinogenic, antioxidant, immune boosting properties, as well as the pro-vitamin activity of some carotenoids (Fiedor and Burda, 2014). Its antioxidants property is a function of β-carotene. Worldwide consumption of carrot has steadily increased in recent years due to its nutritional benefits.

2.2   History of Fermentation

Fermentation is one of the oldest forms of food preservation technologies in the world. Indigenous fermented foods such as bread, cheese, and wine have been produced and consumed for thousands of years and are strongly linked to culture and tradition especially in rural households and village communities. However, the development of fermentation technologies was lost in the midst of history. Anthropologists have postulated that it was the production of alcohol that motivated primitive people to settle down and become agriculturists. Some people believed that the consumption of fermented food is pre-human (Hornsey, 2012). The first fermented foods consumed probably were fermented fruits. Hunter-gatherers would have consumed fresh fruits but in times of scarcity would have eaten rotten and fermented fruits. Repeated consumption would have led to the development of the taste for fermented fruits. It is recorded that fermented drinks were being produced over 7000 years ago in Babylon, 5000 years ago in Egypt, 4000 years ago in Mexico, and 3500 years ago in Sudan.

2.3   Fermentation Processes

Fermentation is a biochemical process which involves the conversion of sugars into products such as alcohols, organic acids, carbon dioxide and flavouring compounds   mediated mainly by the yeasts and their enzymes (Nout, 2009). Fermentation helps to control microbial growth, improve taste, aroma, shelf life,

texture, nutritional value of foods, among other attractive properties (Nout, 2009). Fermentation process may lead to the detoxification and destruction of undesirable constituents present in raw foods such as phytates, tannins and polyphenols as well as lactose removal (Schaasfsma, 2008). For example, cocoa beans are not edible due to their bitter and astringent taste, but after being fermented, they become the basic raw material for the production of chocolate. The production of good quality fermented foods depends on the presence, growth and metabolism of different microorganisms. Thus, microorganisms confer differentiated characteristics on food through products derived from their own metabolism (Hutkins, 2006). The production of organic acids, alcohols and volatile compounds is directly related to the characteristics of the final product (Djeni *et al*., 2011).The biological activity of the microorganisms during the process can also result in the production of several metabolites capable of suppressing the growth and survival of undesirable microorganisms (Ross *et al.*, 2002). The yeast *S. cerevisiae* has been widely used in fermentation processes and is closely associated with the food and fermented beverages production for human consumption, such as bread, chocolate, wine and beer (Scott and Sullivan 2008). In addition, these yeasts combine many desirable industrial properties, including high fermentation capacity, flocculation, ethanol tolerance, osmotic pressure, tolerance to low pH. Therefore, they do not promote problems regarding the oxygenation of large volumes in the fermentation industry (Stewart, 2014). It is estimated that there may be more than 5,000 common varieties of fermented foods and beverages being consumed in the world by billions of people (Tamang *et al.,* 2016).

2.4   Factors affecting Fermentation Processes

2.4.1   Effect of temperature on fermentation

Internal temperature of the most is the most crucial factor considered by winemakers during fermentation. The biochemical process of fermentation itself creates a lot of residual heat which can alter the ideal temperature range of must for the wine (Fundira *et al.,* 2012). Thus, fermentation is an exothermic process. Yeasts are active in a very broad temperature ranging from 0 ^{0}C to 50 ^{0}C, with an optimum temperature range of 20 ^{o}C to 30 ^{o}C.   The temperature of fermentation is usually from 25 ^{o}C to 30 ^{o}C, this makes yeast an important microorganism for fermentation. However, in winemaking, the temperature must not exceed 29.4 ^{o}C for red wines or 15.3 ^{o}C for white wines. Otherwise, the growth of yeast cells is inhibited. Therefore, a lower temperature is ideal as it increases the production of esters, other aromatic compounds, and alcohol itself. This makes the wine easier to clear and less susceptible to bacterial infection (Akubor *et al.,* 2013). Temperature control during alcoholic fermentation is important to facilitate yeast growth, extract flavors and colors from the skins, permit accumulation of desirable by-products, and prevent undue rise in temperature that might kill the yeast cells. The low temperature and slow fermentation favor the retention of volatile compounds (Fleet, 2013). With reference to other organisms, different bacteria can tolerate different temperature which provides enormous scope for a range of fermentation. Most bacteria have an optimum temperature of between 20 ^{0}C and 30 ^{0}C, but lactic acid bacteria work best at temperatures of 18 ^{0}C to 22 ^{0}C. The *Leuconostoc* sp. which initiates fermentation has an optimum temperature of 18^{0}C to 22^{0}C ^{o}C. The temperature

above 22^0C ^oC favours the *Lactobacillus* spp (Kunkee and Vilas, 2014). Once fermentation is completed, wine is pasteurized at $50\ ^0C$ $-60\ ^0C$ and care should be taken not to heat it to about $70\ ^0C$, as alcohol content would vaporize at a temperature of 75 to78 ^0C (Lamarche *et al*., 2014).

2.4.2   Effect of pH on fermentation

According to Fleet (2013), pH directly affects wine stability. This may be as a result of the fact that at a pH close to neutral (7.0), most microorganisms such as bacterial and molds including some yeasts become more active for fermentation and subsequent spoilage of wine, while pH below 3.5 eliminates most of the microbes and favors only a few of the microorganisms for fermentation. Specifically, the optimum pH for most microorganisms is near the neutral point (pH 7.0). Molds and yeasts are usually low pH tolerant and are therefore associated with the spoilage of foods with low pH. Yeasts can grow in a pH range of 4 to 4.5 and molds can grow from pH 2 to 8.5 but favor low pH. The traditional process of fermentation involves extracting fruits juice and adjusting the pH to 4.0 using sodium bicarbonate and adding yeast nutrient (ammonium phosphate) at 0.14 g/L. During fermentation of fruit juice, reductions of soluble solids are possible from pH between 7.4 and 3.5 to 4.0 in worm fermentation (Mena *et al*., 2012). A pH level of 4.0 may be conducive for the development of unwanted microbes like *Leuconosto coenos,* and this can be prevented by reducing the wine pH to below 3.2 (Meyer *et al.,* 2017). According to Rotter (2008), most fining and clearing agents such as bentonite and Kaolin; Proteins Gelatin, Isinglass, Casein, are more effective in clearing the wine when the pH is below 3. As the strength of the relative charge of suspended particles decreases in the wine, the pH of the

wine increases. At high pH, organic protein fining agents may possess a positive charge insufficient to bind to the negatively charged particulates, thus potentially increasing the turbidity of the wine. This phenomenon is called "over fining" (Nuengchamnong and Ingkaninan, 2017).

2.4.3   Effect of sugar content on fermentation

The main substrate for fermentation of fruits juice into alcohol is sugar. Although other food nutrients such as protein and fats can be broken down by some microorganism in some cases where there is limited sugar. In the presence of sugar, yeast cells have preference for sugar until other factors that affect the growth of yeast become unfavorable. Sugars are the most common substrate of fermentation to produce ethanol, lactic acid, and carbon dioxide. Although sugar is an important substrate of fermentation, higher sugar concentration inhibits the growth of microorganisms (Pino and Queris, 2015).Yeasts are fairly tolerant of high concentrations of sugar and grow well in solutions containing 40 % sugar, and above these concentrations, only a certain group of yeasts such as Osmophilic type can survive. There are only a few yeasts that can tolerate sugar concentrations of 65-70 % and these grow very slowly in these conditions. A winemaker who wishes to make a wine with high levels of residual sugar (like a dessert wine) may stop fermentation early either by decreasing the temperature of the must to shock the yeast or by adding a high level of alcohol (like brandy) to the must to kill off the yeast and create a fortified wine (Dickinson, 2013).

2.4.4   Effect of microorganisms on fermentation

Before the role of yeast in fermentation was understood, fermentation involved wild or airborne yeasts. A few styles such as lambics rely on this method today,

but most modern fermentation adds pure yeast (Kumar *et al.,* 2015). Commercial yeasts of today are quite different from wild strains due to genetic manipulation, allowing them to grow in previously unsuitable conditions (Kumar *et al.,* 2015). The genus *Saccharomyces* which is important in food fermentations has the ability to reproduce much faster. The dominant types of yeast used in fermenting alcoholic beverages are the *Saccharomyces*. Yeasts are of major importance in the food industry as they utilize glucose to produce alcohol and ethanol. For example, to make beer, the ale yeast (*S. cerevisiae*) and lager yeast (*Saccharomyces uvarum*) are used, while in wine, *S. cerevisiae* var *ellipsoideus* and *S. cerevisiae* may be used. The use of different strains of yeasts is a major contributor to the diversity of wine, even among the same grape variety (Kumar *et al*., 2015). According to Saranraj and Stella (2012), the mixture of yeast also known as dual culture (*Torulaspora delbrueckii* and *S. cerevisiae*) can be used to produce a complex fruit wine from pineapple. Some yeast strains are chromogenic and produce a variety of pigments, including green, yellow, and black. Others are capable of synthesizing essential B group vitamins (Kawo and Abdulmumin, 2009). Although there is a large diversity of yeasts and yeast-like fungi (about 500 species), only a few are commonly associated with the production of fermented foods. Bacteria also plays a role in malolactic fermentation; this is because to clarify the wine, the fermented juice maybe transferred into a settling vat, or if made on a smaller scale, into a demijohn, in which suspended yeast cells, cream of tartar and particles of skin and pulp settle to the bottom of the container. As the yeast cells break down within the precipitate, they stimulate the growth of *Lactobacillus* sp. that converts the wine's malic acid into lactic acid. This process is, especially, important in wines made from highly acidic grapes because lactic

acid was a weaker acid than malic acid (bacteria decarboxylate malic acid, thus removing the acidic carboxyl group), and therefore, it mellows the wine's taste (Sahoo *et al.,* 2012).

2.4.5 Effect of acid on fermentation

Acid directly affects wine quality. However, wine owes its acid composition to citric acid, tartaric acid, and some traces of other acids like lactic acid which replaces malic acid during malolactic fermentation. These acids in fruits juice or wine can be determined by titration. Fruit acids are weak acids compared to strong mineral acids such as sulfuric and hydrochloric. In solution, strong acids tend to produce their hydrogen ion (H^+) component nearly completely; weak acids dissociate only about 1 % of their hydrogen ion. Thus, such acid solutions like fruit wine have more hydrogen ions (H+) than hydroxyl ions (OH-). As hydrogen ion concentration increases, the solution becomes more unfavorable for most microorganisms associated with spoilage of wine and acidic foods. However, some molds and yeasts which are needed in the fermentation of fruit juice into wine are usually acid tolerant, and therefore, they are very important in the production of dry wine (wine with a very low or no sugar). According to Selli *et al*. (2013), wine acidity increases as fermentation increases which creates unfavourable environment for spoilages organisms and inhibit their growth.

2.5 Morphology of *Saccharomyces cerevisiae*

Yeasts are usually spherical, oval, or cylindrical in shape and a single cell of *S. cerevisiae* is around 8 μm in diameter. Each cell has a double-layered wall, which is permeable to certain substances. Cell division or reproduction generally takes place by budding in which a new cell forms as a small outgrowth of the old cell,

enlarges and then separates. Although, most of the yeasts reproduce only as single cells, under some conditions, some yeast can form filaments (Sivasakthivelan *et al.,* 2014). Yeasts thrive in habitats where sugars are present, such as fruits, flowers and bark of trees. However, commercial yeasts of today are quite different from wild strains due to genetic manipulation, allowing them to grow in previously unsuitable conditions (Kumar *et al*., 2015).

2.6   Growth Nutrients Requirement of Yeasts

Yeasts are chemoorganotrophic micro fungi that obtain their carbon and energy by metabolism of organic substrates. The more common carbon sources are maltose (as in malt wort for brewing), sucrose (as in molasses for rum production), lactose (as in cheese whey-based beverages) and fructose. Only in fruit juices and wine must is free glucose available together with fructose. *Saccharomyces cerevisiae* cannot fix atmospheric nitrogen, and therefore need a supply of readily assimilable organic nitrogen (amino acids) inorganic nitrogen (ammonium salts) for growth and fermentative metabolism. Nitrogen in yeast fermentation media plays an anabolic role in the biosynthesis of structural and functional proteins (enzymes) and nucleic acids, and a catabolic role in the production of fermentation flavour congeners such as higher alcohols (Sivasakthivelan *et al.,* 2014).

Yeasts need an appropriate supply of inorganic ions in addition to sugar and nitrogen sources in fermentation media. Minerals, especially key metal ions are important determinants of yeast fermentation performance. Phosphorus, Sulphur, potassium and magnesium are key examples of "bulk" minerals required in millimolar concentrations, whilst sodium, calcium, iron, cobalt, zinc,

molybdenum, copper, manganese, nickel and selenium are "trace" elements required in micromolar, or less, concentrations. Complex fermentation media used for fermented beverage production (for example, malt wort, molasses, wine must, cheese whey) normally contains adequate levels of inorganic ions for yeast growth, but supplementation with additional minerals may occasionally be necessary (for example, zinc may be deficient) (Sivasakthivelan *et al.,* 2014). Also, the bioavailability of metal ions in complex industrial fermentation media may be compromised due to precipitation, chelation or absorption. Zinc is an important essential cofactor of the terminal alcohologenic enzyme, ethanol (alcohol) dehydrogenase. Must deficient in zinc may result in slow or stuck fermentations, and this has long been recognized as an occasional problem in the brewing industry. Magnesium is very necessary for yeast in the production of ethanol and it is important to maintain high levels of bio-available magnesium to ensure maximal fermentation performance. Yeasts can synthesize amino acids from sulphate, the most oxidized form of inorganic Sulphur. Phosphorus can be provided to yeasts in the form of phosphate salts and this is essential for the biosynthesis of nucleic acids, phospholipids and ATP. The phosphate content of yeast cells is approximately 3 %–5 % of dry weight and this is mainly in the form of orthophosphate (H_2PO4) which acts as a substrate and enzyme effector.

2.7   Growth Factors Requirement

Growth factors such as vitamins, purines and pyrimidines, nucleotides and nucleosides, amino acids, fatty acids and sterols are required at very low concentrations by *Saccharomyces cerevisiae* for specific catalytic or structural roles. Complex media, such as malt wort or wine must, should contain accessory

growth factors for alcohol fermentations. Nonetheless, commercially available yeast "foods" like ammonium phosphate may also be incorporated to supplement media for optimum yeast growth (Sivasakthivelan *et al.,* 2014).

2.8   Mechanism of Nutrient Utilization by *Saccharomyces cerevisiae*

The yeast plasma membrane is a cellular barrier that controls the entry of nutrients in and out of cell for yeast growth and fermentation. Nutrients transportation into yeast cells across the plasma membrane involves the following mechanisms such as simple net diffusion (a passive or free mechanism), facilitated (catalyzed) diffusion, diffusion channels and active (energy-dependent) transport. The diffusion channels and active transport is aided by the activity of plasma membrane enzyme ATPase, that act as directional proton pumps, creating pH gradients that drive nutrient transport either through proton symporters (in certain sugars and amino acids) or through proton antiporters (with potassium ions). For *Saccharomyces cerevisiae* the mechanism of translocation of sugars is dependent on the sugar being used, yeast species, and fermentation conditions. For example, glucose is transported by facilitated diffusion and maltose by active transport. In fermentation media such as malt wort, glucose exhibits a repressive effect on the assimilation of other sugars such as maltose by *S. cerevisiae*, a phenomenon known as catabolite repression. This may result in slow or incomplete fermentations and the production of off-flavours in beverages.

2.9   Wine Yeast

Yeasts are the most important microorganisms that metabolize grape sugars to ethanol and carbon IV oxide to produce wine. Despite many yeast species associated with grapes and wine, *Saccharomyces cerevisiae* is the driving force of

winemaking. There are numerous yeast genera and species that can be isolated from grape and wine such *as Brettanomyces, Candida, Kloeckera /Hanseniaspora, Kluyveromyces, Metschnikowia, Pichia, Saccharomyces, and Zygosaccharomyces* but most of these genera do not survive long due to their low alcohol tolerance (Kosseva *et al*., 2017). Most yeast die above 5 % ethanol, while *Saccharomyces cerevisiae* strains dominate and complete the alcoholic fermentation. The metabolism of these non-*Saccharomyces* yeasts in the first few days of the fermentation can contribute to wine aroma and flavour. Following great advances and reduced cost of whole genome sequencing, it is now possible to have a much better picture of the dynamics of yeast during fermentation. Metagenomic studies of grapes and wine fermentations have given insight into the number of yeast genera/species present.

2.10   Principle of Wine Making

Since Louis Pasteur demonstrated that wines are produced by alcoholic fermentation of grape juice by yeasts, winemaking process has become a modern industry with scientific research activities in the viticulture and oenology. The main purpose of juice preparation is the prevention of undesired reactions in juices which may lead to defects in wines. This is done through proper sterilization, provision of good physicochemical condition for the growth of desired organism and the use of antimicrobial agents like potassium metabisulphite to prevent growth of spoilage organism.

Fruit wine production techniques are similar to those applied in production of grape wines (Joshi, 2009). On the contrary, extraction of the sugars and other soluble compounds from a pulp of some fruit varieties compared to grapes is often

difficult. Also, must obtained from most fruits has lower sugar content and higher acids content than grapes (Swami *et al.,* 2014). The production of wines from different fruit varieties is the same and includes alcoholic fermentation of the pulp or fruit and only differs in the physicochemical characteristics of fruit varieties and the modifications done prior to alcoholic fermentation (Kosseva *et al*., 2017). The main steps of fruit wine production are the following: fruits (fresh or frozen) reception and preliminary preparation; fruits musts preparation by crushing, squeezing, inoculation (using selected wine yeasts) fermentation of fruit musts clarification aging, and pasteurization.

2.11   Steps in Wine Production

2.11.1 Fruit selection

Fruit quality plays an important role in fruit wine production as it determines the aroma and flavour of wine. The fruit for wine production should have high sugar and low acidity and subject to adjustment when necessary. Sugar should be added to fruit that does not contain sufficient natural to speed up fermentation. The use of fruits that are very acidic in nature like raspberries, strawberries, cherries, and pineapples) for fruit wine production may result in very sour wine taste but can be remedied by the addition of sucrose and water to balance the fruit's acidity (Kosseva *et al*., 2017). Harvesting maturity demands are different for different fruit varieties. Fruit like apples or cherries are preferred slightly over riped while blackberries are better picked before full maturity due to higher pigment concentration at full maturity which leads to deposit pigment in bottle (Kosseva *et al*., 2017). The suitability of fruit for wine production is evaluated based on its

physicochemical parameters, such as dry matter and total soluble solids, colour, pH and reducing sugars.

2.11.2 Must preparation

Must preparation start with maceration, which is the release of fruit pomace (seeds, skins, and pulp) constituent before crushing with the release and activation of hydrolytic enzymes from crushed fruit cells. Maceration improves Must fermentability, thus enhancing the yeast activity. Temperature and contact time are essential factors that determine the course of the maceration process. There are two types of maceration; cold pre-fermentative and low maceration. Cold pre-fermentative maceration is a promising technique that improves the pigment extraction as well as the fruit aroma and flavor transfer to the must (González-Neves *et al*., 2015). During cold pre-fermentative maceration, the must temperature is maintained at 10–15 ºC for several days. At low maceration temperatures the start of fermentation is postponed, and there is contact of fruit parts (pulp, skin, and seeds) takes place without the presence of ethanol (Gómez-Míguez *et al*., 2007). Furthermore, the maceration temperature determines the solubility of water-soluble components, thus increasing the extraction of tannins and anthocyanins (Ortega-Heras *et al*., 2012). The primary barrier that averts the polyphenols release into the most is the skin cell wall of fruit. The increase of porosity of this barrier can be achieved by partial hydrolysis of the polysaccharide compounds (pectins, hemicelluloses, and cellulose) which can be achieved either by mechanical maceration, the use of chemicals such as SO_2 or the use of different maceration enzymes. The use of maceration enzymes has become a widespread practice during winemaking. When commercial maceration enzymes are used at

the early stage of fruit extraction and later on during pressing and clarification, they ensure high quality of fruit wines. The commercial maceration enzyme preparations include pectinases (mainly polygalacturonase, pectin esterase and pectin lyase) as well as small amounts of cellulase and hemicellulase to obtain better cells break down and extraction of cell contents (Ortega-Heras *et al*., 2012; Río Segade *et al*., 2015).

2.11.3 Addition of sulfur dioxide

Sulfur dioxide (SO_2) is still one of the most common preservatives used in wine production owing to its antioxidant and antimicrobial properties. It is indispensable in winemaking because it inhibits the growth of undesirable microorganisms and polyphenol oxidase activity during wine production (Guerrero and Cantos-Villar, 2015). Exposure to SO_2 through consumption of wine can have unfavorable health effects for example diarrhea, urticaria and abdominal pain (Ferrer-Gallego *et al*., 2017). Consumers are demanding for high-quality wines devoid of SO_2 to protect their health. To that effect, scientists are striving with the use of new technologies to replace the use of SO_2 to meet today's consumer demands (Costanigro *et al*., 2014). In recent years, several studies have focused on finding the alternatives to SO_2, thus reducing the use of SO_2 in wines (Nagy *et al*., 2017; Lužar *et al*., 2017). The chemical treatments, such as lysozyme, dimethyl dicarbonate (DMDC) and phenolic compounds are more versatile than the use of physical methods from an economic and technical point of view (Santos *et al*., 2012; Ferrer-Gallego *et al*., 2017). This compound has an essential role in the control of oxidative spoilage of wine, preserving thiols, as

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well as protecting wine against browning and flavour loss (Kritzinger *et al*., 2013).

2.11.4 Alcoholic fermentation of the juice by *Saccharomyces cerevisiae*

Though aroma and flavour are mostly determined by the fruit variety, wine yeasts and alcoholic fermentation (AF) conditions play a major impact on the wine aroma and flavor, as well as the overall wine quality. Wine yeasts produce metabolites known to influence sensory characteristics of wine like higher alcohols, esters, volatile acids, carbonyl compounds, volatile phenols and sulfur compounds (Swiegers and Pretorius, 2005). Due to the differences in fruit composition, yeast strains used for alcoholic fermentation of fruit wines have to adapt to different environments including sugar composition and concentrations and the presence of organic acids. The majority of fruit wine elaboration is based on the use of *S. cerevisiae* strains that allow for rapid and reliable fermentation, reducing the risk of sluggish or stuck fermentation, and microbial contamination (Duarte *et al*., 2010; Berenguer *et al*., 2016). The use of commercial wine yeast can affect the wine composition and sensory attribute and can consequently affect the fruit wine quality (Ginjom *et al*., 2011; Duarte *et al*., 2009). Fast and complete AF of fruit juice sugars to ethanol concentrations above 8 % v/v are essential requirements of selected yeasts strains. Good wine yeasts qualities include sulfur-tolerant, minimal foaming and settling down quickly at the end of fermentation (Fleet, 2008). Knowing the potential differences in volatile biosynthesis between various wine yeast strains is important and should be taken into account during selection of the best yeast that will produce a high-quality wine (Duarte *et al*., 2010).

2.11.5 Malolactic fermentation

Apart from yeast, lactic acid bacteria (LAB) also play a very significant role in carrying out a secondary fermentation, known as malolactic fermentation (MLF) where L-malic acid is converted to L-lactic acid. Malolactic fermentation usually takes place during or end of alcoholic fermentation. Lactic acid bacteria (LAB) simultaneously cause changes in the chemical and physical composition of wines. Different studies have reported changes in the volatile aroma profile of wines after completed MLF (Sumby *et al.*, 2010; Bartowsky and Borneman, 2011). Several oenological parameters namely, alcohol content, temperature, must/wine pH, and sulfur dioxide (SO_2) concentration also affect MLF (Lerm *et al.*, 2010). The major genera of LAB responsible for conducting MLF are *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus* (Bartowsky, 2014; Cappello *et al* 2017). However, *Oenococcusoeni*is most preferred it tolerates harsh environment, fastidious, requires group B vitamins, organic nitrogen and amino acids, most of which can be derived from yeast autolysis. Lack of any of these nutrients will inhibit growth (Fuller *et al.*, 2011). MLF can occur spontaneously due to the indigenous LAB present at the fruit (in musts that have not been subjected to sulfur addition before fermentation) or can be induced by the addition of LAB starter cultures. Some winemakers routinely conduct MLF on apple wine, but red wines, such as blackberry wine are also suitable candidates (Rotter, 2008). MLF performed by *Oenococcusoeni* was successfully used as a technique for deacidification of hard apple cider (Reuss *et al.*, 2010), thus proving the suitability of MLF for apple wines. Sun *et al.* (2016) compared the adequacy of an autochthonous *Lb. plantarum* SGJ-24 strain and *O. oeni*, 31 MBR strains, to conduct MLF in cherry wines. Furthermore, Sun *et al.* (2018) investigated the

interaction between different LAB starter cultures and different pH on the malolactic fermentation of cherry wine. Under mild wine conditions, *Lactobacillus plantarum* promoted malolactic fermentation more efficiently than *Oenococcusoeni*. Furthermore, the increase of biogenic amines content, as well as the reduction of cherry wine color was also noticed.

2.11.6 Clarification processes

Clarification involves the separation of liquid (wine) from any sediment deposits or suspended particles through filtration and adsorption mechanisms. The size and nature of the particles which have to be removed will determine which filtration system and grade are required (Fuller *et al*., 2011). Clarification procedure of fruit wine is similar to that of grape wine. A wine that is not clear after racking and maturation can be clarified by the use of filtering aids (bentonite, tannin/gelatin treatment) and filtration. Bentonite is morillonite clay, widely employed as a fining agent. It exists in a negatively charged state at wine pH, and removes positively charged colloids such as proteins, including enzymes such as oxidases, vitamins, and amino acids, thus increasing microbial and heat stability. Wines are usually cooled and filtered to improve clarification and stability. Proteins and other dissolved materials from the wine are removed before bottling. Otherwise, they may cause haziness, especially when the wine is heated. Vitamin C may be added during or after fermentation to some wines to prevent the oxidation of wine.

2.12 Ageing in Wine Production

The fresh wine usually tastes harsh and has a yeasty flavour. During wine maturation, changes in aroma takes place. Wine ageing process is often divided into two phases: maturation and reductive ageing. Maturation, the first phase,

refers to changes that occur between alcoholic fermentation and bottling and frequently lasts from 6 to 24 months. During maturation, the wine may undergo the above malolactic fermentation, be stored in oak cooperage, racked, and treated by one or more filtration and clarification procedures. The second phase of aging begins with bottling. The second stage is called "the reductive ageing" begins with bottling and mostly occurs in the absence of oxygen. According to some studies, the maturation of fruit wines could be extended from 6 months to 2-3 years, resulting in a clarified wine of mellow taste and fruity flavour (Kosseva *et al.*, 2017).

2.13   Wine Quality

The French oenologist, Emile Peynaud, defined wine quality as the totality of its properties which render it desirable. It is the subjective pleasure provided by drinking which conditions judgment. Therefore, the preference of wine consumption may rely on the personal experience, taste and the perception of quality. However, there are still quantitative components to define wine quality. For instance, the methodology of winemaking, geographical origin and the chemical composition, the taste and mouth-feel sensations, colour, odor and flavor of wine describe its quality and are dependent on chemical composition, winemaking techniques, grape varieties and geographical origin. The quantitative assessment of wine quality can be analyzed or evaluated with trained/untrained panels. The sensory analysis provides detailed characterization of wine's sensory attributes and can give a monetary value to wine or can be a research and development tool (Fanzone *et al.*, 2012). The tasting of wine includes several sensory perceptions such as sweetness, sourness, saltiness and bitterness sensed

by tongue, and astringency, metallic, burning, body or prickling sensations as the mouth-feel sensation sensed by one or more of the trigeminal receptors. These receptors are heat and cold sensors, touch sensors, pain sensors and movement and position sensors. The combination of all these perceptions determines the quality term balance. A balanced wine has a harmony in all dimensions, such as; acidity, sweetness, tannins and alcohol.

2.14   Classification of Wine

Zhao (2005) reported that classification is an intellectual activity, without which there will be problem. Although wine has a wide variety of product range, there is not a generally accepted classification system for it. They can be grouped according to sweetness, color, alcohol content, CO_2 content, fermentation process, geographical region, grape variety or vintage. Initially, according to the alcohol content, wines can be divided into table wines and fortified wines, Table wines usually contain 11–16 % alcohol but can be as low as 7 %. Fortified or dessert wines are generally very sweet and include added brandy, with alcoholic content ranging from 16 % to 23 % (Kosseva *et al*., 2017). The table wines can be subdivided as still and sparkling wines based on their CO_2 contents. Most of the table wines belong to the category of still wines. Still wines can further be classified according to their colours as red, rose and white wines. Most fruit wines belong to still wines group, as they retain no carbon dioxide produced during the fermentation. Sparkling wines, on the other hand, contain a considerable amount of carbon dioxide (Kosseva *et al*., 2017). Apple and pear ciders are the most common examples of sparkling fruit wines.

2.15 Wine Spoilage and Prevention

2.15.1 Sulfur dioxide

Sulfur dioxide (SO_2) is the most commonly used additive to control the growth of microorganisms in wine. However, starting from the late 1970s, the use of sulfite in food has been questioned because of its allergenicity identified by the European Food Safety Authority (EFSA, 2004). EFSA, leading the European Union (EU), the USA, Australia, and other countries to implement labeling frameworks for foods containing more than 10 mg/L of this additive (European Union, EU, 2011). The addition of this compound is legal and effective, and its Preventive treatments to avoid *Brettanomyces* sp. Portugal *et al*. (2014) compared several antimicrobial agents, such as dimethyl dicarbonate (DMDC), chitosan, potassium metabisulphite (PMB), and enological tannins against *Brettanomyces*, and they verified that potassium metabisulfite (PMB) was the most efficient antimicrobial agent in concentrations near the usually applied legal limits for enology. Potassium metabisulfite (PMB) efficiency was also studied in red wines naturally contaminated by *Brettanomyces*. Volatile phenols levels were quantified after long aging of the wines treated with PMB. The results obtained showed that there is a negative correlation between the concentrations of PMB employed and the concentrations of 4-ethylphenol, 4-ethylguaiacol, 4-propylguaiacol, and *Brettanomyces* populations in the studied wine. For wine industry it was important that it was verified that 100 mg/L of total PMB could prevent efficiently *Brettanomyces* growth in the storage red wines and that volatile phenols concentrations were significantly ($P < 0.05$) higher in wines with lower levels of PMB.

2.15.2 Chitosan polysaccharide

Chitosan is a chitin-derived polysaccharide extracted from crustaceous or fungi species. It is an important polyssaccharide because of its high density of amino groups present. In 2011, the use of chitosan in winemaking has been authorized by the EU (Reg. EU 53/2011) for heavy metals, iron and copper cloudiness (100 g/hL) contaminant removal, special ochratoxin (500 g/hL), and for control and reduction of undesirable *Brettanomyces* spp. population (10 g/hL). The antimicrobial activity of chitosan has been mainly based on its positive electrical charge, which is incremented at acid pH and with higher chitosan degrees of deacetylation. Therefore, a higher positive charge density is directly correlated with antimicrobial effectiveness of chitosan (Kong *et al.*, 2010). Portugal *et al.* (2014) showed that chitosan inhibitory effect against *Brucella bruxellensis* was identical in the presence or absence of 12.5 % ethanol in the culture broth, where 62 mg/L of chitosan were enough to reduce 90 % of yeast wine population (MIC50 and MIC90 were defined as the MIC, minimal inhibitory concentration, that inhibited 50 and 90 %, respectively, of the experimental microorganisms, where MBC50 and MBC90 means minimal biocidal concentration).

2.15.3 Oenological tannins

Tannins can react with proteins, causing precipitation of large macromolecular aggregates in wine, mostly established by hydrophobic interactions and hydrogen bonds. The antimicrobial activity of tannins is enhanced by the presence of ethanol (Portugal *et al.*, 2014), Ethanol changes the dielectric constant and modified hydrogen bonds, resulting to cell flocculation and changes in membrane

properties (Soares, 2011). The legal limit for the application of commercial enological tannins against *B. bruxellensis* in winemaking is 0.4 mL/L.

2.15.4 Pulsed electric field (PEF)

Pulsed electric field (PEF) technology constitutes one of the most novel technologies for the pasteurization of foods due to its ability to inactivate pathogenic and spoilage microorganisms at room temperature without modifying the quality of food (Martín-Belloso and Sobrino- López, 2011). Strong electric fields ranging from 26 to 35 kV/cm are produced in 1–4 µs pulses between two electrodes contacting the product (Garde-Cerdán *et al*., 2007). Short-duration high-intensity field strengths cause the electroporation of cell membranes and an increase in their permeability. Cell death is achieved as dielectric breakdown increases permeability of the cytoplasmic and nuclear membranes (Puértolas *et al.,* 2010). Although PEF processing is effective against most microorganisms, yeasts tend to be more sensitive than bacteria (Marselles-Fontanet *et al*., 2009). PEF has been investigated as a means to reduce microbial contamination in wines (Santos *et al*., 2012). Puértolas *et al*. (2009) reported that PEF treatment reduced populations of *B. bruxellensis* by 99.9 % in both grape must and wine. Among yeast, *B. bruxellensis* in must and *Saccharomyces bayanus* in wine were the most sensitive microorganisms to PEF. The wine obtained from PEF-treated grapes was similar to the control wine from a sensory point of view (Puértolas *et al*., 2010).

2.15.5 Phenolic components in wine

Phenolics are organic chemicals in grapes and wines responsible for its taste, mouth feel and colour. They are synthesized in plants through the phenyl propanoid pathway. These compounds are highly important in the growth and

development of plant and are synthesized in response to environmental stress such as pathogen attack, UV irradiation or wounding (Ververidis *et al*., 2007). Phenolic compounds are a large and complex group of compounds affecting the characteristics of red and white wines. They occur at lower concentrations in white wines than in red wines. Their major sources are grape stems, seeds and skins. They can also come from yeast metabolism or from wood cooperage. They contribute to colour, flavor, astringency, and hardness of wine by the esterification reactions with polysaccharides, organic acids, and other phenolic compounds and make insoluble complexes with proteins. Chemically, they are the cyclic benzene groups with one or more hydroxyl groups. They can be divided as flavonoid and non-flavonoid compounds.

2.15.6 Flavonoid groups

The flavonoid group in wine is the major class of phenolic compounds. It includes anthocianin, flavan- 3- ols and flavanols. They are present in the seeds, skins and stems, therefore their concentration in wine is highly affected from winemaking practices such as pressing or maceration in which extraction takes place. They strongly influence wine sensorial characteristics. For instance, monomeric catechins give bitter taste to wine, whereas polymers cause astringent taste. They can be either in the free form or are polymerized to other flavonoids, sugars or non-flavonoids. Those esterified with sugars and non-flavonoids are called glycosides and acyl derivatives, respectively (Oliveira *et al.,* 2011).

2.15.7 Non-flavonoid groups

Group consists of derivatives of hydroxybenzoic and hydroxycinnamic acids, stilbenes (resveratrol) and hydrolysable tannins (Vinyl phenol, guaiacol, and

syringol) which are derived from oak barrels during aging (Oliveira *et al*., 2011). Simple phenols are the phenolic acids including hydroxybenzoic acids (C6C1; vanillic, gallic and syringicacids) and hydroxycinnamic acids (C6C3; coumaric, ferulic and caffeic acids). Although this group is colorless / yellowish, they significantly affect red wine colour through intra- and intermolecular reactions. The most varying composition among cultivars is hydroxycinnamic acids and they commonly esterify with sugars, alcohol or organic acids, mainly tartaric acid (tartaric acid esters of caffeic, p-coumaric and ferulic acids are caftaric, coutaric and fertaric acids, respectively). Hydroxybenzoic acid derivative levels are higher in wines aged in oak barrels. The hydrolysable tannins (ellagitannins: polymer of ellagic acid and glucose) breakdown into ellagic acid (two molecules of gallic acid). The lignins in wood can also degrade into cinnamaldehyde and benzaldehyde derivatives (Jaitz *et al*., 2010; Kelebek *et al*., 2010).

2.15.8 Anthocyanin compounds

Anthocyanins are the main compounds responsible for the colour of young red wines and their synthesis is strictly related to the onset of veraison, unlike flavonols. Veraison is the ripening period during which the grapes undergo several changes such as the change of colour from green to yellow-green for white grapes or red-blue for red grapes, the change of firmness and size of the berry, rising sugar and decreasing organic acid contents (Ivanova *et al.,* 2011). They are predominantly present in the grapes as glycosides, called as anthocyanins. This form increases their chemical stability and water solubility. The five classes of anthocyanins depend on the position and number of hydroxyl groups on the B ring (R1 and R2 positions) and their proportion and amount varies by cultivar and

growing conditions. There are also oligomeric derivatives of anthocyanins that result by the interaction of anthocyanins with other molecules such as, pyruvic acid, vinyl phenol, vinyl catechol, α-ketoglutaric acid, acetone, 4-vinylguaiacol or glyoxylic acid (Pinho *et al*., 2012). For instance, vitisin-A and vitisin-B, the so-called pyran anthocyanins are formed by the condensation of grape anthocyanin, malvidin-3-glucoside with pyruvic acid and acetaldehyde, respectively. During maceration/fermentation, the fermenting yeast releases pyruvic acid while anthocyanins are extracted slowly from the grape skins and diffuses into the wine. The vitisin compounds are of great interest to winemakers as they are more stable and produce deeper colour at pH 4.0 than anthocyanins. It was shown that vitisin-A amount was relatively high short after fermentation (5 mg/L) and its content reduces steadily in the following 6-12 months of aging (Morata *et al*., 2007). Pinotin-A arises from the interaction of malvidin-3-glucoside with caffeic acid through decarboxylation of caffeic acid by the side activities of wine yeast. Due to its slow pathway, this pigment is widely used as an aging indicator in red wines. The concentration of pinotin-A increases enormously by aging depending on the concentration of caffeic acid rather than on malvidin-3-glucoside (Schwarz *et al*., 2004).

2.16   Benefits of Wine to Humans

The benefits of wine abound owing to its bioactive compound composition. Among them is an important compound known as resveratrol which is from the stilbene family of phenolic Compounds. It is synthesized in the leaf tissues of many plants against fungal infection or exposure to ultraviolet light. In grapes, it is present in the skin and seed of grapes.

2.16.1 Cardiac protection

Resveratrol is present in the skin and seed of grapes with higher concentration in red wine following long contact of skin and must during the fermentation process. Its concentration is dependent on grape variety, geographic region, agronomic factors, climatic factors, plant stress conditions and oenological practices. Based on its antioxidant activity, it suppresses peroxidation of lipids which is related to the coronary heart diseases and myocardial infarction (Fernández-Mar *et al.*, 2012). However, there are other studies suggesting that the health benefits of wine consumption could be due to the various phenolic compounds present in wine rather than the effects of dietary resveratrol intake (Xiang *et al*., 2014).

2.16.2 Antioxidation effect

Hydroxytyrosol, which is a phenyl ethyl alcohol, is mostly present in virgin olive oil as well as wine. Most of the studies related to the health benefits of hydroxytyrosol have been developed on the olive oil matrix being the main source of this compound. A daily consumption of up to 2 g/kg body weight is safe despite the health implication (Fernández-Mar *et al*., 2012). Its antioxidant activity lies in scavenging peroxyl, hydroxyl and other free radicals. More so, the antimicrobial activity of hydroxytyrosol has been shown to be higher in gram positive bacteria than gram negative bacteria. Resveratrol, hydroxytyrosol and melatonin present in wine could act synergically for a protection against oxidative stress. However, there are still issues to be addressed about the health benefits of these compounds since the related studies are in the initial stages and still too much variability in the studies (Fernández-Mar *et al*., 2012).

2.16.3 Immune system protection

Chronic inflammation is a critical factor in many human diseases and conditions, such as obesity, cardiovascular diseases, neurodegenerative diseases, diabetes, aging, and cancer, which the ethanol and polyphenol components of wine have the potential to control the immune responses (Magrone *et al*., 2008). Changes in inflammatory biomarkers in human suggest that red wine is capable of protection against various immune-related disorders by both stimulating the innate and adaptive immune responses as well as reducing inflammation. Examples of these changes include suppression of inflammatory cytokine release (such as nuclear factor-kappaB) and induction of anti-inflammatory cytokine release and other protective molecules (interleukins 1α, 6, 10, 12, and interferon-gamma). Inflammatory biomarkers that are reduced by wine drinking include C-reactive protein (CRP), monocyte, and endothelial adhesion molecules which confers protection against infections, has been shown to be released from peripheral blood mononuclear cells in response to red wine polyphenols. Wine is also associated with a reduction in fibrinogen, plasma viscosity, and white blood cell count (Estruch *et al*., 2004; Imhof *et al*., 2004).

2.16.4 Anti-cancerous benefit

Despite the fact that alcohol is a known carcinogen, there is evidence that moderate wine consumption may decrease the risk of several cancers, including colon, basal cell carcinoma, ovarian, and prostate (Bianchini and Vainio, 2003). Consumption of approximately one glass of wine daily was associated with a decreased risk of developing Barrett's esophagus, a precursor to esophageal adenocarcinoma, when compared to heavy drinkers or nondrinkers (Kubo *et al*.,

2009). A meta-analysis found that modest wine consumption had an inverse association for developing lung cancer, for both average wine consumption of less than one drink per day A study of female non-Hodgkin's lymphoma patients found a significantly better five-year overall survival (75 % vs. 69 %) and five-year disease free survival (70 % vs. 67 %) in occasional wine drinkers versus abstainers (Han *et al*. 2010).

In *vitro* and animal studies indicate that red wine polyphenols inhibit angiogenesis by reducing the proliferation and migration of endothelial and vascular smooth muscle cells and the expression of proangiogenic factors vascular endothelial growth factor (VEGF) and matrix metalloproteinase-2) (Oak *et al*., 2005). Evidence that wine polyphenols contribute to the chemo-preventive effects of wine comes from studies performed with grape seed proanthocyanin extract (GSPE). GSPE exhibited toxicity toward human breast, lung, and gastric adenocarcinoma cells, but not normal cells (Bagchi *et al*. 2002; Katiyar, 2008). It protected against tobacco toxicity in oral cells, chemotherapy toxicity in liver cells, and ultraviolet toxicity in skin cells. Modulation of oxidative stress, growth factors, and inflammatory molecules were involved (Katiyar, 2008).

2.16.5 Reduction of cholesterol

Regular moderate wine consumption is associated with beneficial changes in lipid homeostasis. Wine increases high-density lipoprotein (HDL) cholesterol, which helps to remove excess cholesterol from the body. Wine also decreases LDL cholesterol levels, which are associated with a high fat diet. High LDL cholesterol levels are associated with an increased risk of atherosclerosis, obesity, and type 2 diabetes (Njajou *et al*., 2009). Wine consumption with a meal is believed to be

ideal because it allows cholesterol to be cleared before it is deposited in unwanted locations in the body. In a selection of studies measuring lipid effects of wine in healthy humans, a single dose of wine was ineffective at modulating lipid levels. Studies performed for two weeks or more indicated that greater than 200 mL red wine consumption was beneficial. All of the studies with more than one dose indicated that the alcohol component of wine is critical for the beneficial lipid effects, which is supported by the established concept that alcohol raises HDL levels. However, this contradicts observations that grapes, grape juice, and grape extracts positively regulate cholesterol homeostasis (Jiménez *et al*. 2008). Overall, red wine was more effective than white wine, suggesting that wine polyphenols did play a role. There is some evidence that GSE or red wine consumption (150 mL for 3 weeks) may combat obesity by decreasing appetite, especially in females (Djurovic *et al*. 2007).

2.16.6 Gastrointestinal effects of wine

A recent survey of 9,733 older adults found that moderate alcohol consumption is associated with a lower infection rate of *Helicobacter pylori* in wine than beer drinkers (Gao *et al*., 2010). Wine constituents have exhibited antibiotic activity against *H. pylori* isolates and protected against associated gastric damage in mice (Ruggiero *et al*., 2007; Martini *et al*., 2009). On the contrary, white wine has been demonstrated to induce gastroesophageal reflux (GER) in healthy people and GER patients which may be due to an inhibition of postprandial gastric contractions and or disturbed esophageal clearance. Individuals at risk for acid reflux are advised to limit wine consumption to ≤300 mL daily (Pehl *et al.*, 2006).

2.17   Other uses of *Saccharomyces cerevisiae*

2.17.1 Bread making

The use of *Saccharomyces cerevisiae* in bread making is over 10.000 BC. Earliest archaeological evidence for leavened breads was found in the second millennium BC in Egypt and the first millennium BC in North Western China *Saccharomyces cerevisiae* is generally inoculated into bread dough at a concentration of 2 % of the total ingredients. The oxygen from the air entrapped in the dough during mixing is utilized in a couple of minutes by the   yeast cells, and under the anaerobic conditions fermentation reaction takes place.

The optimal conditions for fermentation in the dough are around 34–38 at pH 4.0–5.2 using fresh cells as older cells require longer fermentation time. Addition of fat, salt, or spices can delay yeast multiplication. Three categories of sugar exist on dough namely natural sugars, added or bakers and maltose present in flour.

The yeast cells transform glucose and fructose from the degradation of the more complex carbohydrates such as sucrose, maltose, and starch, to carbon dioxide and ethanol. Maltose, dextrose and sucrose are produced from the starch with the help of enzymes amylases found in the flour or in diastased malt besides the possible addition of fungal amylases added by bakers, while glucose and fructose are transformed into carbon dioxide and ethanol by zymases. As a result of the sugar fermentation, the yeast cells are considered as a leavening agent in baked foods leading to an increase of the bread dough volume from the fermentation gasses, with changes i the structure of the product and also synthesis of organic acids and volatile products that contribute to the taste and flavour of bread.

2.17.2 Production of chocolate

The basic raw material for the production of chocolate is the tropical plant *Theobroma cacao* are (Aprotosoaie *et al*., 2016). However, raw cocoa beans are inedible, being bitter and astringent, thus, are subjected to fermentation to reduce the levels of polyphenols and alkaloids, causing the bitterness and astringency, and to develop flavor that imparts the fine organoleptic of chocolate. After the cocoa rods are opened, cocoa beans with high concentration of citric acid and sugar-rich (10–15 % sugars) cocoa pulp are exposed to the naturally existing wild microflora and left to undergo a spontaneous fermentation (Aprotosoaie *et al.,* 2016).

The succession of microorganism fermentation is complex and dynamic including mainly yeasts followed by lactic acid bacteria (LAB) and acetic acid bacteria (AAB) (Meersman *et al*., 2016). At the beginning of fermentation, yeast under anaerobic and low pH (3–4) conditions ferment the pulp-sugars producing ethanol as well as numerous flavour metabolites that will determine the quality of the final products (Meersman *et al*., 2016; Gutiérrez, 2017). Besides, through the action of pectinolytic enzymes, they degrade gradually the highly viscous cocoa pulp (containing 1.5 % pectin) allowing the air to penetrate into the pulp (Meersman *et al*., 2016) while they also metabolize citric acid causing a pH increase and conditions that favour the growth of LAB and AA. LAB increase pH further as they metabolize citric acid and AAB oxidize ethanol to acetic acid (Meersman *et al*., 2016).

As both ethanol and acetic acid productions are exothermic reactions the temperature grows up to 50. According to experimental results, yeast activity is of paramount importance for the production of high-quality chocolate. Specifically, in pilot-scale cocoa fermentations is carried out in the presence or absence of yeasts (Ho *et al*., 2014). Ho *et al*. (2014) observed that without yeasts there is a reduced production of ethanol, higher alcohols and esters throughout the fermentation while the chocolate produced was of inferior quality compared to the one produced when yeasts were present in fermentation. On the contrary, as the same research team reports LAB and AAB, were not proven necessary for the completion of cocoa fermentation, while their absence did not affect the organoleptic characteristics of the chocolate produced (Ho *et al.,* 2015).

A great variety of yeasts have been isolated and characterized from cocoa beans fermentations, with *S. cerevisiae* being among the most prevalent in several studies (Papalexandratou and De Vuyst, 2011).This fact is attributed to the specific properties of *S*. cerevisiae including its pectinolytic activity, rapid growth at a slightly increased pH and better adaptation to stress conditions of high ethanol concentrations and high temperatures (De Vuyst and Weckx, 2016). As unlike the other industrial fermentation, cocoa fermentation is spontaneous and consequently poorly controlled; inoculation with selected starter cultures could ensure successful fermentations with guaranteed reproducibility (Schwan and Wheals, 2004). To this end, *S. cerevisae* has gained a lot of interest and several studies have exploited strains of the species as starters in mixed or mono-cultures fermentation schemes (Mota-Gutierrez *et al*., 2019). However, the dynamics and contribution of *S. cerevisiae* to cocoa fermentation are clearly depicted in the experimental proof of the studies in which *S. cerevisiae* served as the only starter

culture or in comparative studies in which its presence or absence was the only parameter of differentiation.

2.17.3 Production of bioethanol

The term bioethanol is used to define the amount of ethanol that is produced to be used as a fuel. Bioethanol is produced from the alcoholic fermentation of sugars from sucrose – rich food crops such as sugarcane, sugar beet and a variety of fruits, and starch (corn, rice, wheat) using *Saccharomyces cerevisiae* as the dominant yeast. Under anaerobic conditions, *S. cerevisiae* uses glycolysis to catabolize sugars to pyruvic acid. The pyruvic acid through the enzyme pyruvate decarboxylase is converted to acetaldehyde and carbon dioxide, which in turn is reduced to ethanol by alcohol dehydrogenase with the release of NAD+.

The bioethanol produced from fermentation of food crops is called 'first generation' biofuel. However, since these feed stocks fulfil the needs of animal and human nutrition, there is a controversy concerning their use as fermentation substrates for ethanol production. Therefore, a strategy of 'second-generation' biofuel has been developed, in which non-food substrates belonging to the lignocellulosic biomass (wood, straw, crop and food wastes) are exploited. Subsequently, a 'third-generation' bioethanol has been derived from algal biomass including microalgae and macroalgae (MohdAzhar *et al*., 2017). United State of America use corn as the dominant feedstock for ethanol production, while Canada uses corn and wheat, Brazil sugar cane, China corn, wheat, and cassava, and European countries use primarily wheat and sugar beet to produce bioethanol. United State of America is the largest ethanol producer worldwide today for fuel utilization.

Ethanol production averages over a million barrels (159 million litres) per day with an annualized rate of 16 billion gallons (60 billion litres) in 2017. USA and Brazil are the dominant countries in ethanol production manufacturing over 85 % of the world's fuel alcohol. Bio ethanol can be used alone or mixed with gasoline and exhibits several advantages over petroleum fuel such as higher octane number, broader flammability limits, higher flame speeds and increased heats of vaporization, while on the other hand is less toxic, readily biodegradable and produces lesser air-borne pollutants (John *et al*., 2011).

Although *Saccharomyces cerevisiae* is the dominant sugar fermenter, other yeast species are capable of producing bioethanol from sugar fermentation as well. *Kluyveromyces marxianus* has been investigated (among other applications) for the production of bioethanol from polyfructan substrates (Flores *et al*., 2013). *Dekkera bruxellensis* has been used for bioethanol production from hexoses as products of starch hydrolysis while *Scheffersomyces* (*Pichia*) *stipites* utilizes lignocelluloses substrates (Liang *et al*., 2013).

CHAPTER THREE

3.0   MATERIALS AND METHODS

3.1   Description of the Study Area

The study area was Chanchaga Local Government, Tunga Minna, Niger State, Nigeria. The state lies on latitude 80^0 to 11^0:30' North and longitude 03° 30' to 07° 40' East. The Map of Tunga, Minna showing the area of sample collection is shown in Figure 3.1.

3.2   Collection of Samples

Fresh palm wine, burukutu and pito were purchased from Tunga Minna, Niger-State, Nigeria, while ripped watermelon and carrot fruits were bought from Central Market Minna, Niger State. The packaging materials (sterile bottles and polythene bags) were purchased from Minna. Microbiological evaluation of the samples was carried out under strict and aseptic condition in Microbiology Laboratory, while physicochemical and proximate analysis were carried out in Food Science and Technology laboratory, Federal University of Technology, Minna.
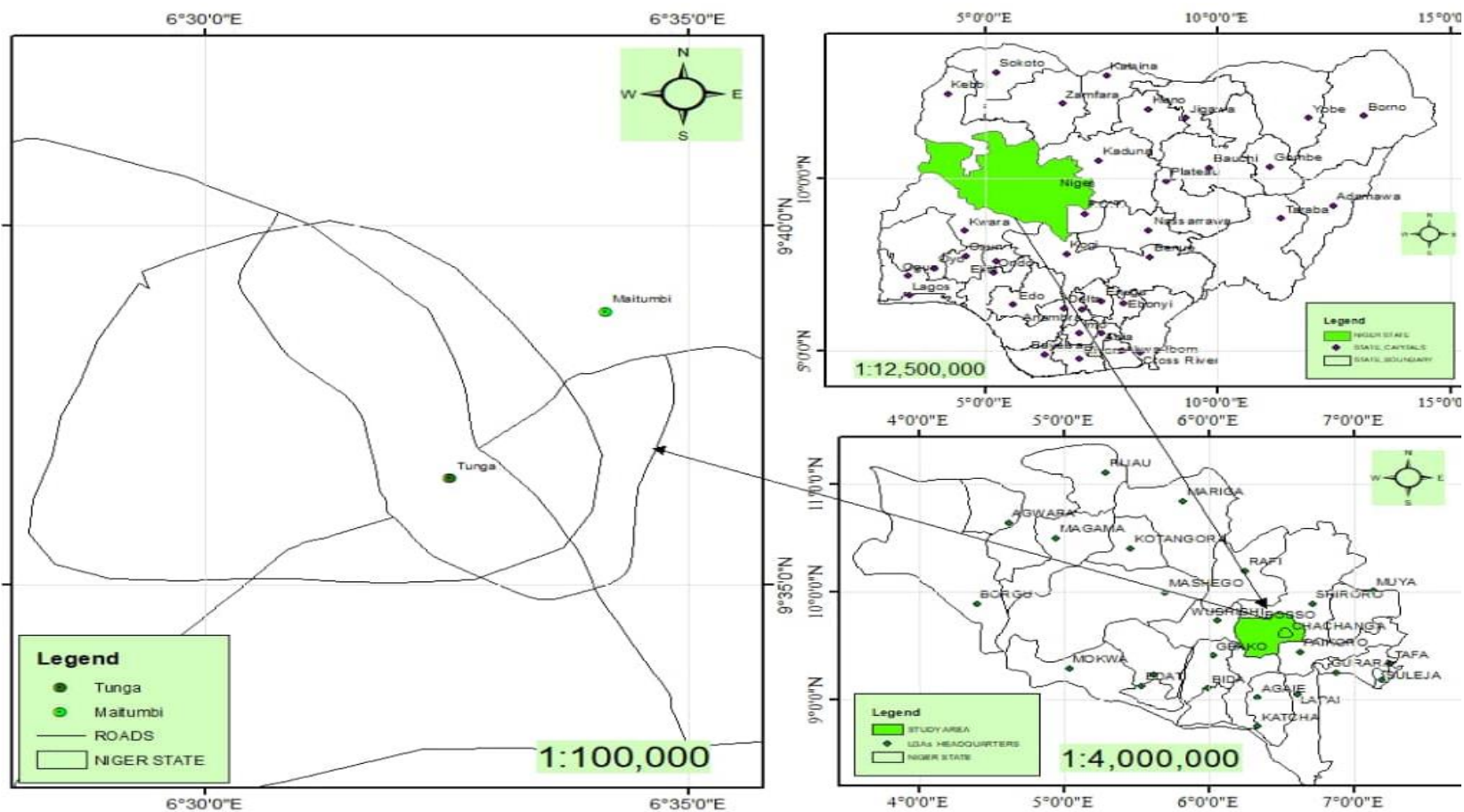
Figure 3.1: Map of Tunga, Minna Niger State Nigeria, showing the collection of sample area (*palm wine*, *burukutu* and *pito*)
Source:federaluniversityofTechnologyminna.(2020).

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3.3 Media Preparation and Isolation of *Saccharomyces cerevisiae*

Sabouraud Dextrose Agar (SDA) was prepared following manufacturers instruction and sterilized at 121°C for 15minutes using autoclave. Dilution of *palm wine*, *burukutu* and *pito* were carried out up to 10^{-1} to 10^{-3} using serial dilution. Sterile water (9 mL) was dispensed into three sets of test tubes and autoclaved at 121°C for 15/minutes. A volume (1 mL) of each sample was pippeted into the first test tube of each set of tubes to make it 10mL and one 1mL taken from the first tube added to the next tube and finally to the third tube. The dilution (1 mL) from the10^{-3} factor was inoculated using pour plate method in SDA agar supplemented with 40 mg/L chloramphenicol to inhibit the growth of bacteria and thereafter incubated at room temperature for 48 h. Visible colonies on the plates were sub-cultured on SDA for another 24 h to obtain pure culture which were stored on SDA slant and preserved in a refrigerator at 4 ^{0}C (Ogodo *et al.,* 2015).

3.4 Morphological Observation of the Yeast Isolates

Distinct colonies on the plates were identified based on their morphological characteristics including colony colour, shape according to the method described by (Ogodo *et al.,* 2015). Microscopic identification was carried out using the method Thais *et al.* (2006) in which 40 % ethanol solution, 0.1 mL potassium hydroxide solution and methylene blue were used. The smear of yeast strain was flooded with 40 % ethanol after heat fixed. The yeast nucleus was hydrolysed with 0.1 mL potassium hydroxide solution for 1 h before staining with methylene blue, observation was made with a light microscope under x40 magnification. The criteria

used in this morphological identification were based on the shape, size, colour using fungal atlas.

3.5   Sugar Utilization and Fermentation Tests

Sugar utilization test was carried out according to the method described by Ogodo *et al.* (2015). Generally, the test is used to determine the ability of certain organisms to utilize and ferment some sugar medium, producing acid and gas or acid only or no fermentation at all. The sugar medium is usually made from peptone water base containing 10 % sugar solution in distilled water and phenol red as indicator. Durham's fermentation tubes were inserted in an inverted position into test tubes containing 10 mL of peptone water with a drop of phenol red indicator and was aseptically plugged with sterile cotton wool. The tubes were sterilized in an autoclave at 121^0C for 15 minutes. Each of the sugar solution containing 1g of glucose, fructose, sucrose and x-xylose was filter- sterilized and aseptically inoculated with 1mL of the standardized inoculum. The tubes were flamed, plunged with a sterile cotton wool and incubated for 72 h at ambient temperature.

3.6   Biochemical Tests

3.6.1   Flocculation test

The Isolates were inoculated in 10 mL of Potato Dextrose broth and incubated at 30 °C for three days. After incubation, tubes were agitated to observe the flocculation rate. The supernatant was carefully decanted and the adhesion of the yeast sediment to the bottles was observed visually (Nwokeke, 2001).

3.6.2 Hydrogen sulfide test

The isolates were inoculated into 10 mL of sterilized triple iron agar and incubated at 37 ^{0}C for 48 h. After the incubation, the tubes were observed for hydrogen sulfide (black colouration) gas production (Okeke *et al*., 2015)

3.6.3 Ethanol tolerance test

The ability of the isolates to grow in higher ethanol concentrations medium was tested by growing them in potato Dextrose broth containing 4 different concentrations of ethanol; 5, 10, 15 and 20 % (v/v) and incubated at 30 ^{o}C for 72 h. The tubes were observed for yeast growth rate at different ethanol concentration after incubation (Okeke *et al*., 2015)

3.7 Molecular Identification of *Saccharomyces cerevisiae*

3.7.1 DNA extraction

Approximately 100 mg of fungi mycelia was grinded with Dellaporta extraction buffer (100 mMTrls pH 8, 51 mL EDTA pH 8, 500 Mm NaCI, 10 mMmcrcapto ethanol). The sample was grinded in 1000 µL of the buffer in a sterilized sample bag, and the mixture collected in a sterile Eppendorf tube with the addition of 40 µL of 20 % SDS. It was followed by brief vortexing and incubated at 65 ^{o}C for 10 minutes. At room temperature, 160 µL of 5 M potassium acetate was then added vortexed and centrifuged at 10000 g for 10 minutes. Supernatant where collected in another Eppendorf tube and 400 µL of cold iso-propanol was added, mixed gently and kept at -20 ^{0}C for 60 minutes. Centrifugation was at 13000 g for 10 minutes to precipitate the

DNA after which supernatant was gently decanted and ensured that the pellet was not disturbed. DNA was then washed with 500 µL of 70 % ethanol by centrifuging at 10000 g for 10 minutes. Ethanol was decanted and DNA air-dried at room temperature until no trace of ethanol was seen in the tube. Pellet was then re-suspended in 50 µL of Tris EDTA buffer to preserve and suspend the DNA.

3.7.2 Polymerase Chain Reaction of the Isolated DNA

ITS gene was used for characterization of fungi. ITS universal primer set which flank the ITS1, 5.8S and ITS2 region was used. PCR sequencing preparation cocktail consisted of 10 µl of 5x GoTaq colourless reaction, 3 µL of 25mM MgCl2, 1 µL of 10 mM of dNTPs mix, 1 µL of 10 pmol each ITS 1: 5' TCC GTA GGT GAA CCT GCG G 3'and - ITS 4:   5' TCC TCC GCT TAT TGA TAT GC 3'primers and 0.3units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water 8 µL DNA template. PCR was carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) with a PCR condition including a cycle of initial denaturation at 94 ^{o}C for 5 min, followed by 35cycles of each cycle comprised of 30secs denaturation at 94 ^{o}C, 30secs annealing of primer at 55 ^{o}C, 1.5 min extension at 72 ^{o}C and a final extension for 7min at 72 ^{o}C.

3.7.3 Agarose gel electrophoresis

The integrity of the amplified DNA of about 1.5Mb gene fragment was checked on a 1 % Agarose gel ran to confirm amplification. The buffer (1XTAE buffer) was prepared and subsequently used to prepare 1.5 % agarose gel. The suspension was boiled in a microwave for 5 minutes. The molten agarose was allowed to cool to 60

ºC and stained with 3µl of 0.5 g/mL ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 minutes to form the wells. The 1XTAE buffer was poured into the gel tank to barely submerge the gel. Two microliter (2 l) of 10X blue gel loading dye (which gives colour and density to the samples to make it easy to load into the wells and monitor the progress of the gel) was added to 4 µL of each PCR product and loaded into the wells after the 100bp DNA ladder was loaded into well 1. The gel was electrophoresed at 120 V for 45 minutes visualized by ultraviolet trans-illumination and photographed. The sizes of the PCR products were estimated by comparison with the mobility of a 100bp molecular weight ladder that was ran alongside experimental samples in the gel.

3.7.4 Purification of Amplified Product

The amplified fragments were ethanol purified in order to remove the PCR reagents after gel integrity. Briefly, 7.6 µL of Na acetate 3M and 240 µL of 95 % ethanol were added to each about 40 µL PCR amplified product in a new sterile 1.5 µL tube Eppendorf, mixed thoroughly by vortexing and kept at -20 ºC for at least 30 min. Centrifugation for 10 min at 13000 g and 4 ºC followed by removal of supernatant (invert tube on trash once) after which the pellet were washed by adding 150 µL of 70% ethanol, mixed and then centrifuged for 15 min at 7500 g and 4 ºC. The supernatant   were again removed and tubes inverted on paper tissue air dried in the fume hood at room temperature for 10-15 min, resuspend with 20 µL of sterile distilled water and kept at -20 ºC prior to sequencing. The purified fragment was

checked on a 1.5 % Agarose gel ran on a voltage of 110 V for about 1hr as previous, to confirm the presence of the purified product and quantified using a nanodrop of model 2000 from thermo scientific.

3.7.5 DNA Sequencing

The amplified fragments were sequenced using a Genetic Analyzer 3130 xl sequencer from applied biosystem using manufacturers' manual, while the sequencing kit used was that of big dye terminator v 3.1 cycle sequencing kit. Bio- Edit software and MEGA 6 were used for all genetic analysis.

3.8 Must Preparation

The fruits (carrot and watermelon) were washed thoroughly with distilled water and then peeled and then weighed. The fruits were then chopped into smaller pieces using a clean knife before transferring them quantitatively into the laboratory blender for crushing. The crushed samples were transferred into a sterile transparent bucket and mixed with distilled water (1:1 w/v). Exactly 2 g of sodium metabisulphate ($Na_2S_2O_5$) was dissolved in 200 mL of water and poured in 1000 mL each of the must and stirred properly. Metabisulphate serves as a sterilizer and also prevent fermentation before the addition of the yeast starter.

3.9 Reactivation of the yeast (*Saccharomyces cerevisiae)*

The method of Amerine *et al*. (2012) was used. Sabouraud dextrose agar (SDA) was prepared according to manufacturer's instructions with the addition of chloramphenicol to eliminate bacterial growth. The stock culture of *Saccharomyces*

cerevisiae was reactivated by subculturing into a freshly prepared solidified SDA and incubated for 24 h at room temperature.

3.10 Preparation of Yeast Starter Culture

The yeast starter culture was prepared from 50ml of each of the must for fermentation, then 5g of sugar and 1mL of 0.5 McFarland standardized inoculum. The mixture of all these were treated with 1g of Potassium phosphate and Ammonium sulphate. Approximately 200 mL of water was boiled and allowed to attain 37 °C and 200 mL of each of the sample (carrot, watermelon and) and carrot and watermelon (mixed) respectively treated with 5g of sugar was added. Exactly 3 g of citric acid was added to each of the preparations and then stirred for proper mixing. Exactly 2 g each of the yeast nutrient namely Potassium phosphate, Ammonium sulphate and Magnesium sulphate were dissolved in 100 mL of distilled water and 400g of sugar poured into the must. Exactly 3.7 mL representing approximately10^8cfu/mL (measured using McFarland standard) of the yeast (*S. cerevisiae*) isolated from palm wine after centrifuging was added to each of the mixture, stirred properly and transferred into fermentation bottles (Ogodo *et al.,* 2015).

3.10.1 Primary fermentation

The method of Okoro (2007) was used for primary fermentation. Yeast was inoculated into the yeas 3 Litres of standardized must using McFarland standard. Primary fermentation of the "must" was carried out in a conical flask which lasted for 4 days with the evolution of carbon (IV) oxide. That was carried out at room

temperature after which the "must" was racked for secondary fermentation. During primary fermentation, aliquots were monitored every 24 hours for physiochemical changes using method of (Amerine *et al*., 2012).

3.10.2 Secondary fermentation

After primary fermentation, the wine was siphoned into a secondary fermenter. Sucrose was dissolved and added to the remaining "must" for secondary fermentation. Secondary fermentation lasted anaerobically for 7 days (Amerine *et al*., 2012).

Bentonite solution (clarifying agent) was prepared and used for clarification. A sachet of bentonite which is in crystal form was dissolved in two litres of boiling water, stirred properly to a gel form and allowed to stand for 24 h. A volume of 150 g of the gel-like bentonite solution was transferred into each of the wine and stirred to mix properly and stored at 2 °C for 30days. A small quantity of the mixture was collected in a clean bottle covered tightly and used to monitor the clarity of the fermented beverage (Ogodo *et al.,* 2015).

Filtration was done after the wine has been clarified using muslin cloth, sieve and siphon tubes sterilized with potassium metabisulfite. The wine was siphoned into the sieve containing four layers of muslin cloth. The filtrates were allowed to mature for a period of three months in a bottle container (Ogodo *et al.,* 2015). The wine was aged at refrigeration condition to mature for three months. The wine was racked by transferring from one bottle container to another to remove sodium pulp and bottled

in sterilized bottles. The matured wine was pasteurized at 68 °C for 15minutes, cooled, stored and ready for consumption.

3.11   Analysis of Physiochemical Parameters of the Wine

pH, titratable acidity, specific gravity (S.G), temperature, total soluble solid (TSS) and percentage alcohol content.

3.11.1 Determination of pH

A digital pH meter was used to determine the pH using the method of McClements *et al.* (2003) in which buffer-4 was used to standardize the pH.

3.11.2 Percentage Alcoholic Content

The alcoholic content was measured using hydrometer. The hydrometer was dropped on the surface of the must, allowed to float and the reading where the surface of the liquid crosses the gravity was recorded. It was done before and after fermentation was completed. The difference in the initial and final value was the alcoholic content.

3.12   Proximate Analysis of the Wine

3.12.1 Moisture content determination

Platinum crucible was washed dried in an oven, cooled in a desccicator and weighed as W1.Two grams each of the samples was weighed into the cooled dried crucibles and the weights recorded as W2. It was transferred into a moisture extraction oven at 105 and heated for 3 h. The dried samples were cooled in a desiccators and reweighed

(w3). The process was repeated until constant weights obtained according to the method described by Association of Official Analytical Chemist, AOAC (2010).

3.12.2 Ash content

Platinum crucible was washed, dried in an oven, cooled in a desiccator and weighed as W1.Two grams each of the sample was weighed into the cooled dried crucibles and the weights recorded as W2 and was evaporated to dryness in a water bath before being charred over a hot plate placed in a fume cupboard until no sooth was given off. They were transferred using a pair of tongs into a muffle furnace set at 55 ^{o}C till fully ashed (colour change to grey) and free of carbon. The samples were removed from the furnace, cooled in a desiccator to a room temperature and reweighed immediately (W3). The procedure was repeated twice more to account for the triplicate readings as described by AOAC (2010).

3.12.3 Fat content determination

The fat content determination was carried out using the Werners Schimidit method of fat acid hydrolysis (AOAC, 2010).The digesting the proteins was done with concentrated hydrolic acid. Liberated fat is extracted with alcohol ethyl ether and petroleum ether, Esters are evaporated and residue left behind is weighed to calculate the fat content.

Five grams of the sample designated as 'W' was heated with 10 mL of concentrated Hydrochloric acid in a boiling water bath to dissolve the fat, the heating continued until the mixture became brown. It was allowed to cool and was transferred into a

separating funnel. Ten (10) millilitres of ethanol was added followed by 25 mL diethyl ether. The mixture was shaken vigorously for one minute and was allowed to stand until upper liquid was practically clear in order to separate the fat content of the sample. Thereafter, a clean dried conical flask was weighed as W1, and the ether layer was transferred into the flask. The extraction was repeated twice with 25 mL of diethyl ether and solvent evaporated in a water bath and subsequently dried at 100 in an oven, cooled and weighed as W2

3.12.4 Crude fibre content determination

Two grams (2 g) of the samples and (1 g) asbestos were added into (200 mL) of 1.25 % of boiled H_2SO_4 for 30 minutes. The solution and content were poured into buncher funnel equipped with muslin cloth and secured with elastic band. This was allowed to filter and the residue was put into 200 mL boiled NaOH and boiling continued for 30 minutes, then transferred to buncher funnel and filtered. It will then be washed twice with alcohol; the material obtained was washed trice with ether. The residue obtained was collected in a clean dried crucible and dried in the moisture extraction oven to a constant weight. The dried crucible was removed, cooled and weighed. Then, the difference of weight (i.e. loss in ignition) was recorded as crucible fibre (F2) and expressed in percentage.

3.12.5 Crude protein determination

The micro Kjeldahl method described by AOAC (2010) was used in the determination of samples crude protein content. It involved three main stages: digestion, distillation and titration. Two (mL) of each of the samples was mixed with

10 millilitres of concentrated H_2SO_4 in eleven heating tubes. One gram of copper sulphate and a speck of selenium catalyst was added into each of the test tubes and mixture heated in a Kjeldahl heating block inside a fume cupboard for 9-10 h till the mixture becomes colourless. The digests were transferred with several washings into a 50- millilitres volumetric flask and was made up to mark with distilled water.

The Micro Kjeldahl distillation apparatus was steamed through for 15 minutes, after which 10 millilitres portion of the digest was poured into the apparatus via the small funnel aperture and was mixed with equal volume (10 mL) of 40 % NaOH solution. The mixtures wee distilled through for about 5-7 minutes to collect enough ammonium sulphate. The distillation were collected with 5 mL volume of 2 % boric acid solution mixed with 3 drops of methyl red indicator contained in a 100 mL conical flask positioned under the condenser tip. The procedure was repeated twice more for each of the eleven samples in triplicate.

3.12.6 Soluble carbohydrate content determination

The soluble carbohydrate content often referred to as Nitrogen Free Extract (NFE) was determined using the methods described by AOAC (2010). The carbohydrate content was calculated as weight by difference between 100 and summation of other proximate parameters as % Ash, % crude protein, %crude fat, % crude fibre and % moisture content as described by AOAC (2010).

3.13   Sensory Evaluation

The wines produced were compared for aroma, colour, clarity, and overall acceptability by a panel of judges on a 5-point hedonic scale, where 5 denotes excellent and one very poor**.**

3.14   Data Analysis

The completely randomized analysis of variance (ANOVA) was used as described by to analyse the data obtained. Mean separation and comparison was done using SPSS version 16.0. Significance was accepted at $P<0.05$ and results were expressed as mean ± standard deviation from the mean.

CHAPTER FOUR

4.0   RESULTS AND DISCUSSION

4.1   Results

4.1.1 Morphological, microscopic and biochemical characteristics of yeast isolates

Morphological, microscopic   and biochemical characteristics of yeast isolates are shown in Table 4.1. A total of seven distinct colonies were isolated from the samples of locally fermented beverages (*palm wine, burukutu* and *pito*). The two distinct colonies isolated from palm wine are denoted as pw1 and pw2 and pw3, while that of *burukutu* and *pito* are denoted as BT1, BT2 and PT1 and PT2. The colours of the Isolates ranges from cream to white, while the shape ranged from circular, long to irregular shape. The isolates viewed under the microscope were oval in shape. All the isolates showed the ability to metabolize glucose, fructose and sucrose with the exception of xylose. They all possess the ability to flocculate and produce hydrogen sulphide with the exception of PW2.

4.1.2 Ethanol tolerance of the isolates

Ethanol tolerance of the isolates are shown in Table 4.2. All the yeast isolates possessed the ability to tolerate 5 % ethanol concentration, moderate growth at 10 % and 15 % concentration and at 20 % concentration, there was a low response for PW2, BK2 and PT 1 as shown in Table 4.2.

Table 4.1: Morphological, Microscopic and Biochemical Characteristics of Yeast Isolates

Sample	Colour	Shape	Glucose	Fructose	D-xylose	Sucrose	Floculation	H_2S
PW1	Cream	Oval	++	+	-	+	+++	+
PW2	Cream	Oval	+	+	-	+	++	-
PW3	Cream	Oval	++	+	-	+	+	+
BT1	White	Oval	+	+	-	+	++	+
BT2	Cream	Oval	+	+	-	+	++	+
PT1	White	Oval	+	+	-	+	++	+
PT2	Cream	Oval	+	+	-	+	+	+

Key: + = Growth - = No growth, Pw=*palm wine,* BT=*burukutu* and PT= *pito*

Table 4.2: Ethanol Tolerance test of the Isolates

Sample	5 %	10 %	15 %	20 %
PW1	+++	++	+	+
PW2	+++	++	+	-
PW3	+++	++	+	+
BT1	+++	++	+	+
BT2	++	++	+	-
PT1	++	++	+	-
PT2	++	++	+	+

Key: PW1: *palm wine* 1, PW2: *palm wine* 2, PW3: *palm wine* 3, BT1: *burukutu* 1,

BT2: *burukutu*2, PT1: *Pito* 1, PT2: *Pito* 2, +: growth, no growth.

4.1.3   PCR amplification of the 16s rRNA region of the yeast isolated from *palm wine*

PCR Amplification of the 16s rRNA region of the yeast isolated from *palm wine* is shown in Plate 4.1 The gel electrophoresis for micrograph of amplified product has 600 bp and sequencing result of the Internal Transcribed Spacer (ITS) region of yeast isolate revealed that pw1 is identical to *Saccharomyces cerevisiae* Chi01 strain in Plate 4.1.

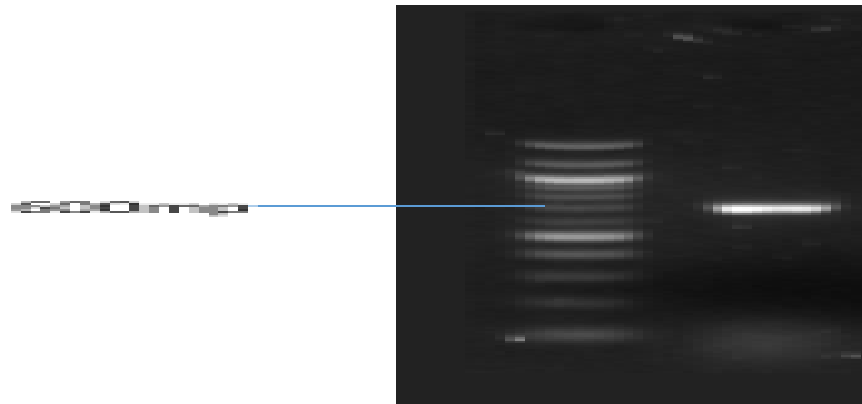**Plate 4.1. Agarose gel Electrophoresis showing the Positive Amplification of the ITS region for the selected Yeast Isolates**.

4.1.4 Basic local alignment search tool (BLAST) analysis

Table 4.3 shows the results of basic local alignment search tool (BLAST) for denaturing gradient gel electrophoresis (DGGE) bands excised of the yeast isolates from *palm wine* identified as *Saccharomyces cerevisiae Chi*01 strain.

Table 4.3: Blast Analysis showing the Relationship between Isolate Sequence

and the most closely related as present in the NCBI data base

Sample ID	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Sample	*Saccharomyces cerevisiae*	1325	1325	98%	0	99.86 %	MT355081 .1

4.1.5   Physicochemical parameters during primary fermentation

Table 4 shows the result of physiochemical parameters of the must during primary fermentation. There was increase in the temperature of the watermelon, carrot and mixed fruit wines (carrot and watermelon) during the period of fermentation. The temperatures were observed to range from $29.00\pm0.33^{o}C$ in control to $32.80\pm0.39^{o}C$ in watermelon and carrot (mixed). The highest temperature was recorded in watermelon and carrot juice (mixed). The pH were all acidic throughout the period of fermentation irrespective of the fruit and ranged from (5.02 ± 0.16) in control, (4.36 ± 027) in watermelon, (4.41 ± 0.28) in carrot and (4.01 ± 0.07) in mixed fruit. The highest soluble solid was recorded in the control $(7.05\pm0.42$ mg/L), while the lowest soluble solid was recorded in watermelon and carrot $(3.21\pm0.13$ mg/L). Specific gravity increased with increase in time. The lowest gravity was recorded in the watermelon and carrot $(1.00\pm0.002$ kg/m^{3}) while the highest was recorded in the control $(1.06\pm0.001$ kg/m^{3}).

Table 4.4: Physico-chemical Parameters during Primary Fermentation

Parameters	Watermelon	Carrot,	Carrot and watermelon (mixed)	Control
Temperature (°C)	31.70 ± 0.49^a	29.10 ± 0.44^a	32.80 ± 0.39^a	29.00 ± 0.33^a
pH	4.36 ± 0.27^b	4.41 ± 0.28^b	4.01 ± 0.07^b	5.02 ± 0.16^b
Titratable Acids (mg/L)	0.041 ± 0.001^b	0.046 ± 0.001^b	0.037 ± 0.000^a	0.032 ± 0.000^c
Total Soluble Solids (mg/L)	5.18 ± 1.21^b	6.20 ± 0.07^b	3.21 ± 0.13^a	7.05 ± 0.42^a
Specific gravity (kg/m³)	1.020 ± 0.002^a	1.03 ± 0.001^a	1.0 ± 0.002^a	1.06 ± 0.001^b

4.1.6 Physico-chemical parameters after secondary fermentation

Physico-chemical of the mixed beverage after secondary fermentation is shown in Table 4.5. There was a decrease in temperature of the beverages after secondary fermentation (Table 4.5). The highest temperature was recorded in the mixed beverage (30.01±0.006 ^{o}C) while the lowest was recorded in carrot (28.04± 0.03 ^{o}C). There was a significant difference between the pH and the total soluble solid (p<0.05). There was a decrease in specific gravity. The highest decrease was recorded in watermelon and mixed (0.98 ± 0.003) while the lowest was1.02 ± 0.003 in carrot.

4.1.7 Physico-chemical parameters of the final fruit beverage

Physico-chemical of the mixed beverage after secondary fermentation is shown in Table 4.6. The temperatures of the final beverages were observed to range from 28.03±0.01 ^{o}C in carrot to 30.00±0.00 ^{o}C in watermelon/ carrot in Table 4.6 The pH of the beverages remained acidic. The highest pH was recorded in carrot (3.75±0.02), while the lowest was recorded in watermelon/ carrot 3.55±0.01 mg/L. The lowest TTS was recorded in watermelon / carrot wine 0.15±0.00 mg/L while the highest TTS was recorded in carrot 0.64±0.071 mg/L. The range of acidity was highest in watermelon and carrot 0.43 ± 0.014 while the lowest acidity was recorded in watermelon /carrot 0.26±0.006 mg/L. The highest S.G was recorded in carrot 1.02±0.00 kg/m^{3}, while the lowest was in watermelon/ carrot 0.97±0.00 kg/m^{3}.

Table 4.5 Physico-chemical Parameters after Secondary Fermentation

Parameters	Watermelon	Carrot	Carrot and watermelon
Temperature (ºC)	29.01 ± 0.003^f	28.04 ± 0.03^f	30.01 ± 0.006^f
pH	3.71 ± 0.003^d	3.82 ± 0.006^d	3.62 ± 0.006^d
Total soluble solids (mg/L)	2.72 ± 0.006^c	3.13 ± 0.03^c	1.92 ± 0.009^c
Specific Gravity (kg/m³)	0.98 ± 0.003^b	1.02 ± 0.003^b	0.98 ± 0.003^b
Titratable Acid (mg/L)	0.37 ± 0.000^c	0.044 ± 0.003^b	0.031 ± 0.003^a
Alcohol	7.31 ± 0.003^e	6.52 ± 0.008^e	8.10 ± 0.06^e

Means with different superscript in the same column are significantly different for the parameter measured ($p < 0.05$)

Table 4.6: Physico-chemical Parameters of the Final Fruit Juice Beverage

Parameters	Watermelon Beverage	Carrot Beverage	Carrot and Watermelon beverage
Temperature (^{0}C)	29.00 ± 0.00	28.03 ± 0.01	30.00 ± 0.00
pH	3.66 ± 0.01	3.75 ± 0.02	3.55 ± 0.01
Total Soluble Solids (mg/L)	0.54 ± 0.026	0.64 ± 0.071	0.15 ± 0.00
Specific Gravity (kg/m³)	0.98 ± 0.03	1.02 ± 0.00	0.97 ± 0.00
Titratable Acid (mg/L)	0.32 ± 0.012	0.43 ± 0.014	0.26 ± 0.006
Alcohol	8.5 ± 0.02	7.3± 0.06	9.05 ± 0.07

4.1.8 Proximate composition of the fruit juice before fermentation

The proximate composition of the fruit juice; moisture content, crude protein, crude fibre, carbohydrate and ash are shown in Table 4.7. Watermelon juice has the highest composition of moisture content with 88.94 ± 0.17 %, crude fibre 1.26 ± 0.01 % and fat 0.62 ± 0.02 % while carrot has the lowest moisture content of 86.73 ± 0.14 and mixed has the lowest fat of 0.46 ± 0.02 %. Carrot has the highest composition of carbohydrate with 9.42 ± 0.32 % and ash 1.30 ± 0.03 %, while watermelon has the lowest carbohydrate content of 7.84 ± 0.21 % and ash 0.66 ± 0.02 %. Carrot has the highest protein content of 1.10 ± 0.01 %. While watermelon has the lowest content of 0.68 ± 0.03 %.

4.1.9 Proximate composition of the wine

The proximate analysis of the fruit wine is shown in Table 4.8. The proximate analysis of the fruit wine showed a significant difference ($p< 0.05$) in moisture content, crude protein and carbohydrate except for percentage fat and ash. The moisture content obtained in the wine as shown in Table 4.8 was 96. 88 % in watermelon, 90.13 % in carrot and 96.69 % in mixed wine. Watermelon has the highest moisture content, while carrot has the least moisture content. The crude protein obtained were 0.54 ± 0.02 in watermelon, 2.95 ± 0.14 % in carrot and 0.66 ± 0.01 % in mixed wine. The highest decrease was recorded in mixed wine, while the lowest decrease was recorded in carrot. The carbohydrate content obtained were 1.35 ± 0.30 % in watermelon, 3.31 ± 0.02 % in carrot, and 1.32 ± 0.21 % in mixed wine

Table 4.7: Proximate Composition of the Fruit Juice before Fermentation

Samples	Watermelon juice	Carrot juice	Carrot and Watermelon juice
Moisture (%)	88.94 ± 0.17^a	86.73 ± 0.14^a	87.80 ± 0.07^a
Crude protein (%)	0.68 ± 0.03^b	0.81 ± 0.12^b	1.10 ± 0.01^b
Crude fibre (%)	1.26 ± 0.01^c	1.24 ± 0.02^c	1.24 ± 0.13^c
Fat (%)	0.62 ± 0.02^{bc}	0.50 ± 0.01^{bc}	0.46 ± 0.02^{bc}
Carbohydrate (%)	7.84 ± 0.21^d	9.42 ± 0.32^d	8.38 ± 0.03^d
Ash (%)	0.66 ± 0.02^e	1.30 ± 0.03^e	1.02 ± 0.01^e

Table 4.8: Proximate Composition of the Fruit Juice after Fermentation

Samples	Watermelon juice	Carrot juice	Carrot and Watermelon juice
Moisture (%)	96. 88± 0.31^a	90.13 ± 0.21^a	96.69 ± 0.05^a
Crude protein (%)	0.54 ± 0.02^b	2.95 ± 0.14^b	0.66± 0.01^b
Crude fibre (%)	0.00 ± 0.00^e	0.00 ± 0.00^e	0.00 ± 0.00^e
Fat (%)	0.32 ± 0.11^{bc}	0.81 ± 0.01^{bc}	0.23 ± 0.13^{bc}
Carbohydrate (%)	1.35 ± 0.30^d	3.31 ± 0.02^d	1.32 ± 0.21^d
Ash (%)	0.91 ± 0.12^c	2.80± 0.03^c	1.10 ± 0.20^c

4.2   Discussion

4.2.1 Morphological, microscopic and biochemical characteristics of yeast isolates

Pure culture of *Saccharomyces cerevisiae* was isolated from palm wine, *burukutu* and *pito*. All the isolates showed the ability to metabolize glucose, fructose and sucrose with the exception of xylose. The isolates ranged from cream to white colour. It suggested that the isolated organism was yeast since the samples were fermented beverages as reported by Ukponobong *et al.* (2017) that isolated a cream and white coloured and oval colonies as viewed under the microscope from locally fermented alcoholic drinks. Xylose was not fermented and could be attributed to lack of enzyme to metabolize the sugar. The yeast isolates flocculated and produced H_2S with the exception of pw2.

The ethanol tolerance test revealed there was intensive growth at 5 % concentration, and moderate at 10 % and 15 %, and at 20 %, there was low growth. The growth could be attributed to toxicity of high alcohol to cell growth. Ethanol tolerance is a unique property of the yeast (*Saccharomyces cerevisiae*) that makes it exploitable for industrial applications as stated (Ukwuru and Awah, 2013).

4.2.2   Physicochemical parameters during the fermentation processes

The physico-chemical parameters of the beverages during fermentation showed that fermentation resulted in increase in temperature. This might be due to the catabolic process of sugar by *saccharomyces cerevisiae* resulting in metabolic heat that ultimately increased the temperature. A similar observation was also reported by (Ukwuru and Awah, 2013). Fluctuations in the temperature of the must were also

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observed during the period of fermentation. The fluctuation could be as a result of biochemical changes occurring during metabolism of substrates by fermenting organism. A similar observation was reported by Ogodo *et al*. (2015) on the production of mixed fruit (pawpaw, banana and watermelon) wine using *saccharomyces cerevisiae* isolated from palm wine.

The pH of the fruit beverages decreased throughout the period of fermentation irrespective of the fruit. Studies have shown that during fermentation of fruits, low pH is inhibitory to spoilage organisms but provides conducive environment for the growth of desirable organisms. Similar observations have been reported for other tropical fruit wines such as banana wine (Obaedo and Ikenebomeh, 2009). Also, low pH is known to give fermenting yeasts a competitive advantage in natural environment (Reddy and Reddy 2005; Chilaka *et al*. 2010). The decrease in pH could be due to accumulation of organic acids during fermentation, and that inhibits the growth of bacteria that can lead to spoilage. Therefore, the wines have a good keeping quality.

Total soluble Solid decreased throughout the period of fermentation. The Total soluble solid obtained were low, ranging from 0.15±0.00 to 0.64±0.07 in carrot and watermelon (mixed) to carrot wine. It implies that consumers are not exposed to taking too much solids into the body. Thus, further reduction in total solids could be achieved by filtration.

The study showed an increase in titrable acidity during fermentation. The increase could be as a result of conversion of organic acid to lactic acid and carbon (1V) oxide. A similar observation was made by Inuwa *et al*. (2011). It was observed that during fermentation of fruits, high acidity gives fermenting yeast a competitive

advantage in a natural environment. Acidity plays a role in determining wine quality by aiding the fermentation process and enhancing the overall acceptability and characteristics of wine as stated by (Awe *et al.,* 2013).

The specific gravity of the fruit wine produced was observed to be decrease during fermentation. This decrease might be due to the type of yeast used in the production *Saccharomyces cerevisiae* isolated from palm wine has been reported by Okeke *et al*. (2015) to reduce specific gravity of fruit wines during fermentation. The observed reduction in specific gravity of the wine and the resultant increase in the alcohol concentration showed the efficiency of the *Saccharomyces cerevisiae* isolated from palm wine and implies that the yeasts is alcohol tolerant. Similar observation was reported by Chilaka *et al.* (2010).

 The alcohol content of the fermenting must increase during fermentation. The increase in the alcohol content can be attributed to yeast metabolism by continuous utilization of the sugar content, ethanol is produced and thus there is an increase in the alcohol content of the fermenting must, this continued until all the available sugar in the fermenting must has been utilized. This result is in consistent with the work of (Awe and Nnadozie, 2015). The final alcohol content of the wine ranged from 7.3 – 9.05%) ranks it among table wines.

There was no microbial contamination of the wine. This revealed the good quality of the wine and implies that the wine was produced under hygienic conditions and is safe for human consumption. A similar observation was reported by (Adedeji and Oluwalana, 2013). Fermentation was carried out under aseptic conditions in order to obtain a good fermentation yield and establish the stability of the whole fermentation process throughout the period. These precautions or measures may have been the

reason why there were no contaminants in the fermentation medium. Pasteurization might also be another reason why there was no contaminant. This means that the heat treatment was sufficient to destroy microbial contaminant in the wine. This is similar to the reports given by (Adedeji and Oluwalana, 2013)

4.2.3 Proximate composition of the wine

The proximate composition is a significant attribute of a beverage. There was an increase in moisture content from 88 – 96 % water melons, 86 – 90 % carrot, and 87-96 % mixed fruit. This could be attributed to differences in the species of the watermelon High moisture content makes beverages suitable as a refreshing and quench-thirsting product which is a characteristic of a good beverage. This is similar to the report given by (Okeke *et al*., 2015). There was an increase in amount of ash obtained. This indicates the presence of mineral component in the fruit and wine. There was a decrease in fat after fermentation of the must. This suggests that the wine could provide protection against excess body lipids (cholesterol) and shows the desirable nutritive quality of the fruit wine produced as reported by (Awe *et al*., 2013). The protein content of the fruit wine decreased after fermentation, which is similar to the report of Awe *et al*. (2013).

4.2.4 Sensory evaluation

The Sensory evaluation of the wine (watermelon, carrot, watermelon and carrot (mixed) was carried out using descriptive analysis. Colour, aroma, clarity and acceptability were evaluated. These characteristics were scored out of 5. The mixed wine sored highest in all the parameters, followed by watermelon and carrot wine. A similar result was obtained by Ogodo *et al*. (2015) in the production of mixed fruit

(pawpaw and watermelon wine). This could be as a result lycopene and carotenoid watermelon and carrot.

CHAPTER FIVE

5.0   CONCLUSION, RECOMMENDATIONS AND CONTRIBUTION OF RESEARCH TO KNOWLEDGE

5.1   Conclusion

The study was based on the isolation of *Saccharomyces cerevisiae* from locally fermented alcoholic beverages (palm wine, *burukutu*, and *pito*) for wine production using carrot and watermelon. *Saccharomyces cerevisiae* Chi01 from *palm wine* has the best potential for wine production as it could metabolize sugar, flocculate, and tolerate alcohol. The physico-chemical parameters and proximate composition of the fermented beverage showed that *Saccharomyces cerevisiae* Chi01 can serve as an alternative to commercial wine yeast. This study showed that watermelon and carrot are good substrates for wine production to preserve the fruits from post-harvest spoilage due to under consumption and inadequate storage facilities.

5.2   Recommendations

i.   Large scale production of *Saccharomyces cerevisiae*Chi01should be promoted to be used as an alternative to commercial yeast.

ii.   Further research on the production of fermented beverages using *Saccharomyces cerevisiae* should be extended to other indigenous fruits and vegetables to test their potential for wine production.

iii.   New studies should be carried out on other fruits and herbs using combination of Saccharomyces *cerevisiae* strains from palm wine. This may result to a product with good taste, flavour and numerous nutrients.

iv.   Genetic modification of *Saccharomyces cerevisiae* Chi01 can be done to enhance yield and reduce cost.

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5.3   Contribution of Research to Knowledge

The study established that *S. cerevisiae* Chio1 was isolated from palm wine and used for the production of carrot-watermelon wine. The pH of the wine averaged 3.55±0.01, acidity 0.26±0.006 mg/L, Total Soluble Solids 0.15±0.00 mg/L. The proximate analyses also showed enhancement in the nutritional value of the wine; Carbohydrate, 1.51±0.01 %, Protein, 0.48±0.14 %, and Fat 0.43±0.02 %. The study suggest that alcoholic fruit beverage can be produced from carrot-watermelon using *S. cerevisiae* Chio1 isolated from palm wine.

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