Bindura University of Science Education

Characterization of yeast isolates from *Ziziphus mauritiana* (Indian Jujube) fruits with potential application in oenology

By

Ethel Joan Swale

B213379B

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Approval form

The undersigned certify that they have read the dissertation titled '**Characterization of yeast isolates from *Ziziphus mauritiana* (Indian Jujube) fruits with potential application in oenology**' and confirm that it is suitable for submission to the Biological Sciences Department, Faculty of Science and Engineering, for assessment.

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I, MR Clayton Siamayuwa, declare that I have supervised this thesis and I am satisfied that it can be submitted to the Biological Sciences Department, Faculty of Science and Engineering, at Bindura University of Science Education.

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Dedication

I dedicate this research to my parents and academic sponsors who made it possible for me to work on this research through financial and moral support.

Acknowledgements

Praise be to God Almighty, who has seen me through to this research project in good health. I'm profoundly grateful to my academic supervisor, Mr C. Siamayuwa, who invested his invaluable guidance, feedback, and encouragement in shaping the outcome of this research. I also extend my gratitude to my colleagues H. Matumbe and I. Dandajena, who helped me with peer-to-peer advice, constructive criticism, and encouragement. The part they played cannot be forgotten. Furthermore, I'm grateful to my institution, led by Professor E. Mwenje, and my faculty department, led by Dr Jinga, for being an environment of growth and a rich niche to carry out the work of this project. Not forgetting all other staff members who played a role in my learning. To my friends and family, I sent a heartfelt gratitude for the unwavering support.

List of abbreviations

TSS – Total Soluble Sugars

TTA – Total Titratable acid

PDA- Potato Dextrose Agar

ANOVA – Analysis of variance

HSD- Honestly Significant Difference

ATP- Adenosine Triphosphate

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Abstract

Ziziphus mauritiana (Indian Jujube) fruits are highly nutritious and abundant, with a potential source of vitamin C and other valuable components. The utelisation of the fruit in the production of various food items, including wine has been explored. Fermentation using only *S. cerevisiae* limits the flavour diversity of the wine, hence the need for exploring wild yeast species with potential application in oenology. . One kilogram of the fruits was sampled from Mashonaland Central fruit and vegetable market and the Bindura University Hub. Yeasts were isolated on Potato Dextrose Agar (PDA) at 30℃ for 72 hours. Morphological and biochemical characterization was done. Fermentative yeasts that fermented glucose, sucrose and fructose were selected for further tests. The twelve isolates purified were further screened down using D-glucose and ethanol tolerance, production of hydrogen sulphide and urease activity. The four isolates selected after screening were used as starter culture for production of *masau* wine. Total Titratable Acidity (TTA) and Total Soluble Sugars (TSS) were analysed on wine at day ten using titration and Brixometer, respectively. Based on the morphological and biochemical characterization, the study identified the genus *Saccharomyces*, *Saccharomycopis*, *Hanseniaspora*, and *Issatchenkia* as potential starter cultures for wine production . The statistical analysis using One-way ANOVA yielded *p-values* 8.56E-08 ≤ 0.05 and 2.59E-12 ≤ 0.05 for TSS and TTA, respectively at $p < 0.05$. The p-values signify a significance difference between the tested groups. Further analysis using Tukey's post-hoc analysis revealed a significant difference between the positive control commercial *S. cerevisiae* and the isolates, except for isolate 11 on both parameters. The results obtained after approaching this research reveal the presence of wild yeasts with potential to be applied as fermentation starter cultures. The study also reveals the weak performance of some isolates hence need to be used in combination with powerful fermentative yeasts e.g. *S. cerevisiae.*

Contents

Chapter 1

Introduction

1.1 Background

Yeasts, which are eukaryotic cells, are adapted to convert sugars into alcohol and carbon dioxide Yeasts are naturally found on the surfaces of fruits, including *Ziziphus mauritiana*, and in fermentable foods, where they present a symbiotic or spoilage risk. The natural microbiota of fruits can serve as a potential source of indigenous yeasts for winemaking and other food fermentations, hence the aim of this research (Nyanga et al., 2007). *Ziziphus mauritiana* fruits, commonly known as *masau*, *Indian jujube,* or *ber* is a fruit traditionally consumed and used in various forms, including making a fruit spirit *(kachasu*), traditional cakes, and porridge (Nyanga et al., 2013, 2007). However, applying the fruit to produce wine in Zimbabwe is still in its elementary stages. *Saccharomyces cerevisiae* has been the yeast species used for wine making. Each yeast species presents a unique pathway for converting sugars into alcohol and other by-products, which could be phenols, esters, or other flavor-enhancing products (Nyanga et al., 2007). The choice of yeast species to use can significantly impact the final wine, resulting in variation in flavor, aroma, mouthfeel, and other wine attributes (Zhang et al., 2024). Therefore, exploring various yeast strains leads to the identification of novel strains capable of producing wine with diverse characteristics and styles (Wang et al., 2023). Through analyzing the performance of yeast isolates in wine production, by considering parameters such as sugar consumption, pH changes, total titratable acidity of the wine, the study aimed to identify the yeast isolates that can be used for wine production.

1.2 Statement of the problem.

Utilization of *S. cerevisiae* as the only yeast for fermentation of *Z. mauritiana* fruit in wine production limits the diversity of the wine flavors and attributes, thereby restraining the utilization of the fruit in wine production (Benito et al., 2019). The need to extract the potentially health benefits from *Z. mauritian*a fruits has driven the need to research various ways the fruit can be utelised. Despite its potential, limited research has been done on the use of natural yeast from the fruit in producing *Z. mauritiana* wine (Nyanga et al., 2013). Characterization of yeast from *Z. mauritiana* fruits is crucial in gaining a deeper understanding of their fermentation capacities,

flavour attributes contribution in wine making, and also spoilage risks. However, there is limited information on the characterization of the yeast on the fruit and its possible application in oenology. This research gap restricts the full utilization of the fruit in wine-making and other fermentation products, and the exploitation of indigenous fruits for biotechnology applications, e.g. food production.

This research aims to cover the research gap by identifying novel yeast strains from the fruit capable of producing wine with good attributes, which ultimately contributes to the diversification of the wine industry in Zimbabwe and the valorization of underutilized fruits.

1.3 Study Aim

To isolate yeasts from *Z. mauritiana* fruits with potential application in the production of wine.

1.4 Research objectives

- To isolate and characterize yeast species with fermentation capacity from the skins of *Z. mauritiana* fruits.
- To assess Total Titratable Acidity (TTA) and the reduction of total soluble sugars. .

1.4.2 Specific objectives

- Isolate yeast from *Z. mauritiana* fruits.
- To compare the performance of indigenous yeasts isolated from *Z. mauritiana* fruits with commercial wine yeasts (S. *cerevisiae)* in terms of sugar consumption and Total Titratable acidity in wine**.**
- To identify the yeast isolates using morphological and biochemical techniques

1.5 Research question

1. What are the morphological, biochemical and physiological characteristics of fermenting yeast species isolated from *Z. mauritiana* fruit?
2. What is the performance of the fermenting yeast isolates from *Z. mauritiana* against the standard *S. cerevisiae* in terms of sugar consumption and total acidity production in fermented wine ?

1.6 Hypothesis

Yeast isolates obtained from *Z. mauritiana* fruits include strains with significant potential applications in winemaking, such as high fermentation efficiency.

1.7 Significance of the study

Characterization of yeast associated with *Z. mauritiana* fruit might lead to the identification of isolates that can serve as alternatives or synergies to commercial yeast strains, aiming for unique flavor profiles and improved wine qualities. As a result, the research aims to expand the oenology industry in Zimbabwe and around the world. The expanded utilization of *the Z. mauritiana* fruit for wine production can generate income worth improving the economic development of the country and the marginalized districts of Muzarabani where the fruit is abundant in Zimbabwe. Most importantly, value addition to indigenous fruits like *Z. mauritiana* aligns with sustainable agricultural practices through food waste reduction and promotion of consumption of healthy foods and beverages by dwellers.

1.8 Assumptions

The research rests on the assumption that yeasts are naturally present on outer parts of *Z. mauritiana* fruits. The second assumption is that yeast from the fruit can be cultured using standard microbiological methods and have unique biochemical and physiological properties due to their adaptation to the fruit environment. Finally, the research assumes that dry fruits will make as good wine as fresh fruits.

1.9 Limitations

By the time of research, fresh *masau* fruits were out of season, and stored samples were used. As a result, the yeast population may vary between seasons affecting the representativeness of the research. Secondly, the alcohometer present at the laboratory was not fully functional and required one-liter volumes, which was a volume too high to use for pilot experiments. Again, only one incubator was functional, hence temperature tolerance was limited to 30℃ and 37℃ only. This prolonged the time frame of experiments as each test had to be done independently.

1.10 Delimitations of the study

Samples were carefully selected to pick unrotten fruits that were visibly not affected by molds. To overcome the absence of a functional alcohometer, a standard assumption was done that each decrease in ^{o}Brix produced 0.5% alcohol. To overcome the limited supply of incubators, tests requiring the same temperature were done the same day before setting the incubator to another temperature.

1.11 Definition of terms

Fermentation is a microbial-mediated process that occurs in the absence of oxygen. The process yields energy, carbon dioxide, ethanol, and lactic acid (Benito et al., 2019).

Oenology is the study of the winemaking process and wine parameters (Wang et al., 2023).

Wine is an alcohol -based product after the fermentation of fruits. It is often characterized by its combination of tartness and sweetness (Kaiser et al., 2014)

Yeasts are eukaryotic single-celled microorganisms under the kingdom fungi. They form colonies similar to bacterial colonies and they are often applied in fermentation and biotechnology (Nyanga et al., 2013).

Chapter 2
Literature Review

2.0 Literature review

This chapter describes the literature relevant to the aim of this research, covering the species description of *Ziziphus mauritiana* tree and fruits, the methodology used, the wine-making procedure, and the wine profile. It also covers literature about the morphological, biochemical, and molecular methodologies used for this research.

2.1 *Ziziphus mauritiana*

2.1.1 Local names.

The species has a number of names in different languages. In Shona, the fruit is known as *masau, Sidr* in Arabic (Chege et al., 2021). In English, the fruits can be identified using either of the names: dunks, jujube, Indian cherry, Indian jujube (Kaarira, 1998), Indian plum, geb, ber or common jujube. In Chinese, the common names for the fruit are date, Chinese apple, and desert apple (Chege et al., 2021). However, the common names of the fruits and tree, which are more universal, are Jujube, Ber, and Indian Jujube (Kaarira, 1998). The diversification of the names shows the diverse distribution of the species in a number of countries or regions.

2.1.2 Description of *Z. mauritiana*

2.1.2.1 Tree

The species is a fruit-forming tree that can grow to a height of 10-15 meters (Mullin, 2003) and 5-8 meters wide on maturity. Mature trees have straight trunks with varying sizes depending on cultivar and the region of growth but on average ranging between 30 and 40 cm length or diameter?. The branches possess thorny, straight protrusions that provide the tree with protection (Mapaure, 2016). The trunk of the tree is coated with dark grey or dull black barks that are irregularly fissured. In the early stages, the species appear to be a scrub with thornbush branches covered with leaves (Orwa, 2009).

2.1.2.2 Flowers

The flowers of *Z. mauritiana* develop on leaf axils and are yellowish-green in colour. They appear in clusters or cymes and have a pleasant faint fragrance (Mullin, 2003). The flowers are 1-2 cm long, 2-3mm wide and exist in clusters of 7-20 flowers (Reference). The flowers have peduncles

2- 3 mm long, pedicels 3-8 mm long and calyx with 5 deltoid lobes (Reference). They are hairy outside while glabrous within. The petals are five for each flower, sub-spathulate, concave and reflexed (Reference).

2.1.2.3 Fruits

Fruits are drupe, globose to ovoid in shape, having size range between 1.5 and 2.5 cm (Mushove, 1995). They have smooth, glossy skins during unripe and early ripening stages (Orwa et.al., 2009). The fruit are green in early stages and change to yellow-reddish then brown at the ripening stage. The pulp is white, juicy, subacid to sweet and crispy in unripe fruits, brown in early ripening stage and caramel brown in dried fruits (Mullin, 2009). The skins of the fruit thicken with maturation. Each fruit carry only one tuberculate and irregularly furrowed stone-shaped seed that has 1-2 elliptic brown kernels. Each kennels is bout 6mm long (Orwa et.al., 2009).

2.1.2.4 Leaves

Z. mauritiana leaves are oval or elliptic in shape (Mullin,2003; Mapaure, 2016). They have sizes ranging between 2-5cm in length and 1-3 cm in breadth (Mushove, 1995; Mullin, 2003). Mapaure, (2016) describes the colour of the leaves of mature plants as dark on the upper surface and pale green/ glabrous on the lower surface. They have entire margins and round, acute tips with a cuneate base (Mushove, 1995).

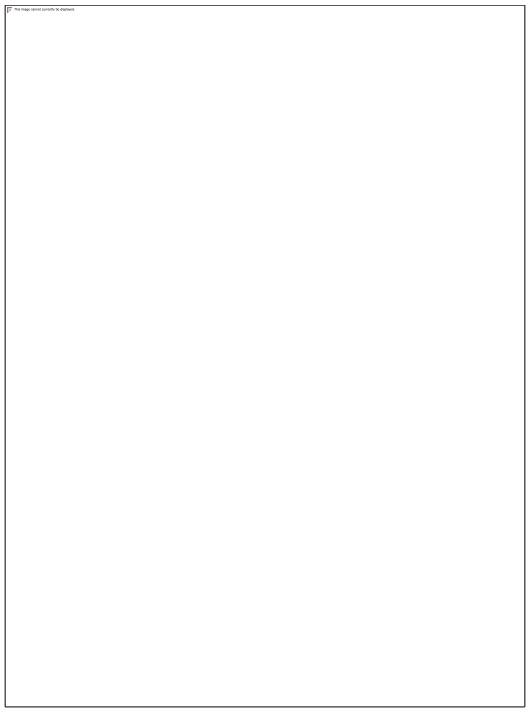


Figure 1: Z. mauritiana tree

Figure 2: Leaves of Z. mauritiana

2.1.3 Taxonomic classification

Ziziphus mauritiana is a dicotylenous herbaceous tree which follows the classification order described in figure 3 below.

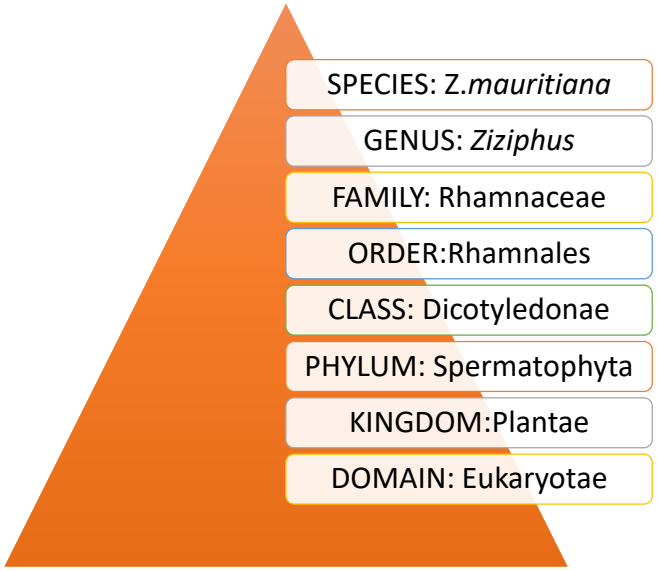


Figure 3 The taxonomic classification of Z. mauritiana

2.1.5 Uses of *Z. mauritiana*

In Zimbabwe, the fruits of *Z. mauritiana* are mostly taken as unprocessed fruits (*masau*) across the country. They are consumed during the early ripening stages and also as dried fruits (Mapaure, 2016; Mushove,1995). In regions of origin, e.g., the Muzarabani area, the fruits are dried and used to make porridge and cakes. The fruits have also been found to be useful in jam and marmalade making (Mushove, 1995). Due to the yeast that is associated with the fruits, they find their application in the making of traditional beverages as supplements e.g. masawu flavoured mahewu (Mapaure, 2016). They are also used to make a distilled alcoholic drink known as *kachasu,* although it is illegal in Zimbabwe. Due to the surplus of the fruits in the country, research institutions are applying these fruits in the production of non- alcoholic beverages, and fruit juices (Mapaure, 2016; Mushove, 1995). The application of the fruits in wine making in Zimbabwe is still in its elementary stages. The fruits hold nutraceutical value in Zimbabwe, as explained by (Mushove, 1995) who described the important role played by the fruit in the treatment of respiratory ailments, e.g. bronchitis, asthma, and normal cough.

Kumar and Kumar, (2017) reported the uses of the leaves of *Z. mauritiana* in tea brewing in India for health benefits. The authors also reported the use of the fruits to make cosmetic products e.g. soaps creams and lotions. In India, the fruits are consumed as nutraceuticals against constipation, diarrheal diseases, and dysentery (Kumar & Kumar, 2017). In Zimbabwe, (Mapaure, 2016) describes the use of the fruits to reduce fever and relieve Malaria symptoms in affected individuals. Hussein and El-Mahdi, (2016) further explained the health benefits of *Z. mauritiana* fruits in India where they are used for wound healing and treatment of other skin conditions.

Furthermore, the fruit is used as animal feed supplements by inhabitants of regions of abundance of the species (Kaarira, 1998; Mapaure, 2016; Mushove, 1995) due to its nutritional contents. Wine making from the fruits has found global attention. The reports from (Bazila Naseer et al., 2021; Karale, 2024; Mapaure, 2016) indicate the vast application of the fruits in wine making from the fruits of *Z. mauritiana* across different cultures.

2.1.7 Fruit microbiota

The fruits of *Z. mauritiana* have been reported to co-exist with yeasts, molds, lactic acid bacteria and even acetic-acid bacteria (Bassey et. al, 2017; Chege, J et al., 2021; Zhang et al., 2024). The

association of yeasts and bacteria species with fruits is not unique to *Z. mauritiana* but a common phenomenon among almost all other fruits. The fruits provide a rich, nutritious niche for the microbiota, thereby sustaining their growth. Understanding the microbiological community of the fruit is important to understand the spoilage risk of the fruits, the fermentation effect of the microbiota, and also the health risks and benefits. Fruits co-existence with lactic acid bacteria, yeasts and molds and other bacteria e.g. *E. coli* and *Bacillus* species has also been reported.

The yeasts found in association have been studied, and studies are still underway to understand their fermenting capacity and spoilage risk to find their biotechnological application. Wild yeast, when fully studied and harnessed, provides potentially valuable biotechnological performance in industries like oenology due to its resilience and adaptation to wild conditions (Zhang et al., 2024). Wild yeasts can diversify the fermentation attributes of beverages and wine thereby creating new wine flavours and profiles different from the common *S. cerevisiae* (Lakew, 2022) .

2.1.7.1 Yeasts

Yeasts are microscopic first-level eukaryotes. Yeast isolates refer to yeast that have been isolated, purified, and characterized. Each yeast isolate represents a unique genetic unit, which can be a species or a strain, and has unique biochemistry, morphology, physiology, and genetics. Studies on *Z. mauritiana* have revealed the association of the fruits with yeast from different genera. The yeast has shown both fermenting capacity and spoilage risk.

Genera such *as Candida, Pichia, Saccharomyces, Hanseniaspora, Saccharomycopsis, Issatchenkia, Auerobasidiuum, Zygoascus, Cryptoccocus, Burella, Fusarium, and Rhodotorula* have been pinpointed as common in the species of concern (Nyanga et al., 2007). Among them all, *S. cerevisiae* is the predominant species (Nyanga et al., 2007). Furthermore, Nyanga et al. (2007) categorized the yeast into fermentative and non-fermentative yeast and grouped the isolates in those two categories. Nyanga et al. (2007) identified *S. cerevisiae, I. orientalis, P. fabianii, S. fibuligera, and H. opuntiae* as the fermentative strains in the ripe and dried fruits. However, the evaluation of yeast isolates in the fermentation of *Z. mauritiana* fruit for wine-making has not been studied in Zimbabwe.

2.1.7.2 Lactic-acid bacteria

Lactic acid bacteria are gram-positive, spherical or rod-shaped prokaryotes that do not form spores and are capable of producing lactic acid as the major fermentation by-product.

Lactobacillus agilis, L. minor, L. confuscus, and *L. fructosus* were isolated from ripe fruits in a research conducted by Nyanga et al. (2007). They isolated *L. minor* and *L. divergens* from the dried fruits (Nyanga et al., 2007). Furthermore, isolation of lactic acid species from the fermented fruit revealed the existence of *Streptococcus* species and all the above strains (Nyanga et al., 2007).

2.2. Oenology

2.2.1 **Use of fruits.**

Over the past decades, the production of wine has diversified from only being dependent on vines to the use of other fruits (Kaiser et al., 2014). The presence of phenols and antioxidants in wine has been reported from both wine and other fruit wines (Tatah & Ayantse, 2023),

2.2.2 Yeast inoculum.

The most commonly used inoculum for wine production comes from the genus *Saccharomyces*, with the predominant species being *S. cerevisiae, S. boyanus,* and *S. paradoxus* (Zhang et al., 2024). However, due to the need to diversify the wine flavors and to produce wine with improved organoleptic properties, non-*Saccharomyces* species are being investigated (Wang et al., 2023). According to (Benito et al., 2019), species that have found their application yet include *Torulaspora delbrueckii, Candida pulcherrima, Lachancea thermotolerans, Metschnikowia pulcherrima, Meyerozyma guilliermondii, and Pichia kluyveri.*

2.2.2.1 ***Saccharomyces cerevisiae***

According to (Wang et al., 2023)*, S. cerevisiae's* wide application comes from its biochemical efficiency and stress tolerance properties. It has a well-defined fermentation kinetics profile. The fermentation is predictable and consistent. The yeast functions well between the pH of 2.8 and 4, which is typical of most wines. It also survives high levels of Sulphur dioxide used for pasteurization of the wine. *S. cerevisiae* also has a tolerance to high sugar and ethanol tolerance of 60% and 15 %, respectively.

S. cerevisiae produces wine with a good aroma and taste. It is responsible for the metabolism of precursors for the primary aroma in fruit wines. It can metabolize several sugars, monosaccharides, disaccharides, and tetrasaccharides. *S. cerevisiae* can ferment D-glucose, D-fructose, D-mannose and D-galactose, which are all monosaccharides. For Disaccharides, it can ferment maltose, sucrose and turanose. *S. cerevisiae* also ferments maltotriose and raffinose, which are both trisaccharide (Wang et al., 2023) .

The ability to ferment so many sugars creates the complex profile of sugars, making it a supreme yeast isolate with wide application.

2.2.2.3 Important parameters in oenology

The most important parameters in oenology include total titratable acidity, total soluble sugars, alcohol content and pH. Further studies may include the phenolic profile of the wine as phenols influence the aroma of the wine and other organoleptic characteristics (Zhang et al., 2024).

Titratable acidity reveals the tartness of wine. It is measured using titration in a neutralization reaction using a strong base, e.g., Potassium hydroxide or Sodium hydroxide 0.1 normality. An endpoint of pH 8.2 is used in relation to tartaric acid as a reference. Phenolphthalein indicator is used as a suitable indicator for the determination of end point as it changes colour from colorless to pink at pH 8.2.

Tartaric acid is used as a reference due to its dominance during secondary fermentation. The reaction follows the chemical equation presented below.

Equation 1

(i) $H_2C_4H_4O_6\ (aq) + 2\ NaOH\ (aq) \rightarrow Na_2C_4H_4O_6\ (aq) + 2\ H_2O(l)$

(ii) $2\ H^+\ (aq) + 2\ OH^-\ (aq) \rightarrow 2\ H_2O\ (l)$

The acceptable total titratable acidity in wine ranges between 5g/L and 7 g/L. The production of tartaric acid is mostly affected by the yeast species used. Each yeast species has a unique set of metabolic reactions that result in the formation of different acids, thereby affecting the total acidity and sensory profile of the wine (Sánchez-Suárez & Peinado, 2024).

Total dissolved solids or sugars determine the sweetness in wine. This parameter is measured using a Brixometer and is expressed in ^{o}Brix. The 1 Brix value equals 1% sugar per 100g of solution (Jaywant et al., 2022). The oBrix value can be used to estimate the final alcohol content of the wine. In cases where the OBrix value of the fruit must is smaller than the expected alcohol content, table sugar (sucrose) can be used to supplement the must. Dissolved reducing sugars are converted to ethanol, Carbon dioxide and water during alcoholic fermentation. The rate of reaction is greatly influenced by enzymes, thereby making the fermentation kinetics of yeast species different within

species. The equation below summaries the chemical reactions that occur during alcoholic fermentation in yeast.

Equation 2 show glucose fermentation reaction

$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$

Based on the equation, a single molecule of sugar gives two moles of ethanol, giving rise to a general formula used to estimate the alcohol content from Brix values. The general rule states that a degree in Brix value by one is equivalent to the production of 0.5 % alcohol by volume, according to a research conducted by (Plugatar et al., 2023) to test the hypothesis.

2.3.0 Morphological characterization

2.3.1.1 Colonies

According to Breakwell etal. (2016) yeast cells form colonies similar to bacterial colonies on agar. Due to the similarity with bacterial colonies, selective media against bacteria e.g. PDA and SDA should be used. Colony morphology is described in regards to color, shape, elevation, edges, opacity, texture and size. Some colony morphologies are distinctive to a specific genus hence may serve as presumptive results for identification.

2.3.1.2 Cells

For the sake of providing more details to morphological characterization beyond using colonies, yeast cells can be analysed using microscopy. Yeast cells can be viewed under a microscope with or without staining, depending on the level of detail required. Often, staining techniques e.g. Gram and Giemsa are used to reveal specific details on the morphology of the cells including budding and cell shape (Breakwell et.al, 2016). Different types of microscopes can be used for viewing the cells but light microscopes are the most commonly used. A magnification from X40 to X100 is mostly used for colony morphologies as it provides clear resolution of the edges and elevation which are difficult to see using a naked eye. Magnification X100 to X400 are used for cell shape, budding style, sporulation and size while X1000 is used for details regarding organelles and membrane packaging (Breakwell et.al, 2016) .

2.3.4 Biochemical characterization

2.3.4.1 Catalase test

Catalase is an enzyme that is responsible for the breakdown of hydrogen peroxide to form water and oxygen (Hadwan et al., 2024). Hydrogen peroxide is a by-product of yeast metabolism or other microbial activities (Taylor & Achanzar, 1972). The presence of this enzyme in yeast cells helps the cells to survive oxidative stress as a result of low oxygen levels thereby remaining viable to continue with alcoholic fermentation (Torrellas et al., 2020) . Increased oxidation in wine is a negative parameter as it can affect wine quality through the production of undesired flavours (Mercanti et al., 2024). Mercanti et al. (2024) explains that high oxidation can accelerate the spoilage of wine.

On the other hand, excessive catalase activity in yeast often interferes with sulfites used as wine preservatives, reducing their efficiency and producing of flavours (Danilewicz & Standing, 2024) . *S. cerevisiae* used in wine making is catalase-positive. It employs mechanisms e.g. using Superoxide Dismutase enzymes (SOD) (Belazz et al., 1991; Gull et al., 2025) followed by using complete!

2.3.4.2 Urease test

The urease test is primarily used to differentiate bacteria and yeast that contain urease enzyme from those that do not. The urease enzyme is responsible for the breakdown of urea to form ammonia and carbon dioxide. This reaction is used as an important highlighter to the pathogenicity of the isolates in yeast. Urease-positive yeasts are likely to be from the genus *Candida*, posing food safety risk to be used in the food industry (Rutherford, 2014).

2.3.4.3 Hydrogen sulfite production

Hydrogen-sulphite production is considered a negative parameter in oenology. This is because of the odd smell that it produces, similar to that of sewage (Bassey et. al, 2017). Hydrogen-sulphite is complex to clean and yeast when left uncleaned, it tints the colour of wine to a darker colour, affecting the organoleptic properties of the wine. Again, the hydrogen-sulphite is reactive to form disulphites and mercaptans which raises risk of allergenic reactions (Waterhouse, Sacks, & Jeffery, 2016) (Bassey et. al, 2017). Production of hydrogen-sulphite by yeast may also indicate growth-related stress, such as limited supply of nitrogen.

2.3.4.6 Glucose tolerance

Yeast like any other cells, suffer osmosis stress when exposed to higher sugar or saline concentrations. Hyperosmosis leaves the yeast cells dehydrated and unable to perform fermentation, which often results in stuck fermentation (Hohmann, 2002). The effect of hyperosmosis is mostly observed by prolonged lag phase as the cells need more time to adapt to the high sugar concentration before carrying out fermentation (Pratt et al., 2003). *S. cerevisiae* has glucose tolerance of up to 60% making it an ideal isolate where the fruit must have higher sugar content or a higher alcohol content. is expected (Bassey et. al, 2017).

By growing the yeast isolates in solid media supplemented with D-glucose, yeast colony morphology and growth can be analysed at once, making it a more convenient method to use for determining sugar tolerance (Bassey et. al, 2017).

2.3.4.7Alcohol tolerance

Ethanol, an alcohol, is one of alcoholic fermentation end products. However, if the yeast used as the inoculum for wine or beer production is not resilient to higher alcohol content, it may suffer end point inhibition, halting the fermentation process (Sánchez-Suárez & Peinado, 2024). The alcohol can also disrupt the cell membrane of the yeast cells, causing cell lysis. Yeast cells that can survive ethanol tolerance of at least 13% are acceptable for fermentation where higher alcohol content is expected (Kaiser et al., 2014). Examples of yeast isolates with higher ethanol tolerance include *S. cerevisiae, Torulaspora delbrueckii* and *Pichia kluyveri* (Nyanga et al., 2007, 2013)

According to Lakew, (2022), solid media supplemented with a corresponding amount of absolute ethanol after autoclaving can be used for determining the ethanol tolerance of yeast isolates at the same time providing the colony morphology which can be used to determine if the ethanol concentration affects the cells. However, broth media used alongside spectrophotometry provide a more accurate result on yeast growth through quantitative analysis (Bassey et. al, 2017) .

Chapter 3

Materials and methods

3.0 Introduction.

This chapter narrates the method that was used to obtain results for this research. It describes the sampling criteria, sample preparation, yeast isolation and characterization, fermentation and DNA extraction**.** Both qualitative and quantitative data collection methods were used on the results.

3.1 Sampling

Dried *Z. mauritiana* fruits (1kg) samples were obtained from Bindura fruit and vegetable market and Bindura University Hub. The origin of the *masau* was confirmed prior to sampling as the research was targeting fruits from the Muzarabani/ Dande area. The samples were transported to the laboratory in a sterile dry sample bag. Whole mature fruits with no blemishes or insect bites were selected from each batch using stratified probability sampling method**.**

3.2 Media Preparation

Yeast isolates were grown in various types of media, a rich source of nutrients that allowed growth. Both solid media and broth were used for the cultivation of yeast isolates. Again, both selective and non-selective media were used**.**

3.2.1 Potato Dextrose Agar

Potato dextrose agar was used for the isolation of the yeast isolates. Dehydrated media weighing 39 g was dissolved in de-ionized water as per manufacturer's guide lines. The media was heated to boiling point before being autoclaved at 121 ℃ for 15 minutes. The media was allowed to cool to 50 ºC and then aseptically dispensed into petri dishes. The plates were allowed to dry before being sealed with parafilm, labelled, and stored.

PDA media slants were prepared dissolving the dehydrated media as per manufacturer's guide line and shaken to ensure homogeneity. One? ml of the media was dispensed into test tubes with caps and heated to boiling in water bath. The tubes were sealed with aluminum foil and autoclaved at 121 ℃ and 15pb for 15 minutes. The tubes were allowed to cool and solidify in a slant position of about 45 degrees to obtain a slant.

3.2.2 Carbohydrate solutions

Carbohydrate solutions containing each of the sugars were prepared using a method described by the (Bassey et. al, 2017). Standard solutions containing protease 10g/L, NaCl 5g/L, phenol red indicator 0.018 g/L, and 1000 ml sterile distilled water were prepared. Each solution was supplemented with 10g of each of the sugars: sucrose, glucose, and fructose. Each test tube was filled with 10 ml of the solutions. Inverted Durham tubes were inserted into the tubes and sealed using a cotton wool plug and aluminum foil. All tubes were autoclaved at 118℃ for 15 minutes except for sucrose, which was sterilized at 121℃ and 15 pb for 3 minutes. The final pH of the tubes was 7.4 +/- 0.2. The tubes were cooled and used immediately afterwards.

3.2.3 Hydrogen peroxide

A hydrogen peroxide solution was prepared by diluting 6% hydrogen peroxide in a ratio of 1: 1 with sterile distilled water to make a 3% solution. The solution was well shaken and used immediately after.

3.2.4 Kliggler Iron agar

Kliggler iron agar from Oxoid was prepared as per the manufacturer's instructions. The media was dispensed into test tubes, making a volume of 10 ml in each tube. All tubes were plugged with cotton wool and sealed with aluminum foil, followed by sterilizing at 121℃ for 15 minutes at 15 pb. The tubes were cooled in a slant position to create media slants.

3.2.5 Urea broth

Urea broth was prepared using the recipe proposed by Christensen (1975). The media was prepared by mixing peptone 1g/L, NaCl 5g/L, monopotassium phosphate 2g/L, phenol red indicator 0.012g/L, glucose 1g/L, urea 20/L and diluting with distilled water to make. The broth was dispensed into test tubes followed by inserting inverted Durham tubes and autoclaving at 121℃ and 15pb for 15 minutes. The media was cooled in a sterile condition and stored.

3.2.6 Carbohydrate strength solutions

Potato dextrose agar (PDA) was supplemented with varying glucose, sucrose, and fructose concentrations to make 5%, 10%, 20%, 30%, and 40% solutions for each carbohydrate. Initially, the PDA used contained 2 % glucose and was subtracted from the final glucose added. Table 1 shows the dilution ratio to formulate 100 ml of the solution.

Table 1 Glucose : water ratio for making glucose concentrations

GLUCOSE CONCENTRATION (%)	MASS OF D-GLUCOSE (G/100ML)	TOTAL GLUCOSE (G) / 100ML	VOLUME OF H_2O ADDED (ML)
5	3.00	5.00	95.00
10	8.00	10.00	90.00
15	13.00	15.00	85.00
20	18.00	18.00	80.00
40	38.00	40.00	60.00

The glucose-supplemented media was sterilized by autoclaving at 121℃ and pressure 15 Ibs for 15 minutes. Empty petri dishes were divided into 4 using straight lines and a marker and labelled with the glucose concentration. The plates were allowed to cool, sealed, and stored.

3.2.7 Infused potato Dextrose broth

Infused potato dextrose broth was prepared by measuring 200g of clean peeled and diced potatoes and boiling in 1 liter water for 30 minutes. The remaining liquid after boiling was served. The solution was supplemented with 20g glucose and stirred to dissolve. The volume was re-adjusted to 1 L by adding sterile water. All tubes were sterilized by autoclaving using 121℃ and 15 Ibp pressure for 15 minutes.

3.3 Sample Preparation.

The fruits were lightly rinsed separately under running water to remove debris, dust, and other physical pollutants. They were then rinsed again using sterile water before being placed in a sterile container and covered with aluminum foil. Samples were prepared using three methods, firstly by crushing the fruits using a pestle and mortar, secondly by soaking the fruits 25g fruits in 50ml water for 2hours with shaking in 10 minutes intervals and lastly by peeling the fruits to remove seed and diluting with sterile distilled water in a 1:2 ratio of fruit as to water with shaking every 10 minutes for 2 hours.

A single ml from each sample was serially diluted up to a 10^{-5} dilution. On Potato Dextrose Agar plates, 0.1 ml of the sample was aseptically inoculated using the spread plate method. The glass rod was dipped into 70% ethanol and flamed between spreads. Samples were incubated at 30 degrees Celsius for 72 hours.

3.4 Morphological characterization.

Colony morphology was analysed using the naked eye and colony counter magnifying glass, paying attention to shape, colour, elevation, edges, texture, and opacity using the method and criteria described by (Kurtzman et al., 2011)

3.4.1 Cell morphology**.**

Yeast cells were analysed using a light microscope, paying attention to shape and budding. Heat fixation method was used, followed by staining the smear with methyl blue for clear view of the cells (Breakwell et.al, 2016).

3.4.2 Biochemical characterization.

3.4.2.1 Carbohydrate fermentation

Cultures, 48 hours old, were used to inoculate each carbohydrate. 1 ml of the yeast culture was added to a test tube containing the carbohydrate e.g. glucose, and an inverted Durham tube (Maragatham & Panneerselvam, 2011) (Lakew, 2022). All tubes were incubated at 35℃ for 48 hours with observation at 24-hour intervals. The colour change of the solution from red-brown to yellow and bubble formation in the Durham tube, or the floating of the Durham tube was recorded as a positive fermentation (Karale, 2024).

3.4.2.2 Catalase activity

A catalase test was done using 3% hydrogen peroxide. Each isolate colony was picked using a loop and placed into a test tube containing 10 ml of the hydrogen peroxide solution (Torrellas et al., 2020). The formation of gas bubbles was recorded as positive (Taylor & Achanzar, 1972), while no bubbling reaction was recorded as a negative result. S. cerevisiae was used as the positive control for the experiment.

3.4.2.3 Urease activity.

Urea broth formulated by (Christensen, 1975) was used. Prior the experiment, each isolate was cultured for 48 hours at 30 ℃ in potato dextrose broth. A milliliter of each isolate was added to 10 ml of the urea broth, vortexed and incubated at 35℃ 24 hours and continued to 48 hours. A colour

change of the solution from yellow to pink was recorded as a positive reaction. No colour change was recorded as a negative reaction.

3.4.2.4 Hydrogen sulphite production test

Hydrogen sulphite production by yeast isolates was tested using Kliggler agar (Oxoid) slants (Lakew, 2022). A colony of each isolate was picked using a sterile inoculation loop. The loop was used to first stub the butt followed by streaking the slant. The slants were incubated at 35℃ for 72 hours with observation at 24 hours interval**.**

3.4.2.5 Sugar tolerance.

To evaluate the sugar concentration tolerance of the isolates, PDA plates with 2%, 5%, 10%, 15%, 20% and 40% glucose were used to determine the growth quality of the cultures. The method used was adapted from (Maragatham & Panneerselvam, 2011) The isolates were streaked on all concentrations and incubated at 30℃ for 48 hours. The growth of the isolates was scored and recorded. Scoring was done on the basis of growth and colony morphology, compared with 2% glucose PDA as a control.

3.4.2.6 Alcohol tolerance

Tolerance of the isolates to different ethanol concentrations was evaluated by growing the isolates in PDA. The media was prepared as per the manufacturer's instructions and allowed to cool. Afterwards, it was supplemented with 5%, 8%, 10%, 13% and 15% v/v ethanol (Maragatham & Panneerselvam, 2011). The concentrations were made from absolute ethanol and distilled water dilutions.

3.5 Wine making

Z. mauritiana fruits were first soaked in 5% sodium bicarbonate for 15 minutes, followed by thorough washing and rinsing. The fruits were dried by blotting on a clean paper towel. The wine-making process was done following the method described by (Malhi, 2018), with adjustments. No enzymes were used for the wine production. Sterilization was done at 80 ℃ for 10 minutes to kill natural microbiota. The Brix value of the wine was adjusted to 20 using table sugar (fructose). Batch fermentation was carried for 10 days under anaerobic conditions. The wine was bottled, sterilized and stored at room temperature in a dark cupboard. All samples were carried out in triplets using the selected isolates.

3.6 Brix measurement

Brix was measured using model 2WAJ Abbe LCD refractometer 0-15% Brix 1.300- 1.700. The value was calculated using the operation procedure from the manufacturer. The machine was calibrated using sterile distilled water and cleaned using distilled water and cotton wool after every sample. Figure 4 below shows the photograph of the exact machine that was used for analyzing the samples

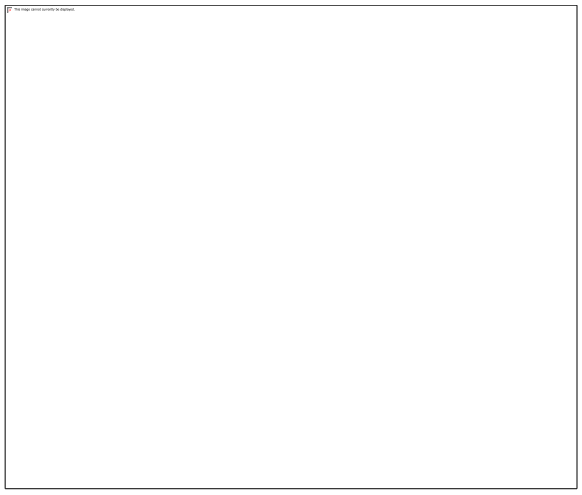


Figure 4 2WAJ Abbe refractometer used for measuring % Brix of wine samples.

The percentage decrease in % Brix was calculated using the formula:

$$decrease\ in\ \%Brix = initial\ \%Brix\ value - final\ \%\ Brix\ value$$

Equation: Calculation for $decrease\ in\ \%Brix\%\ Brix$

3.7 Titration

Wine samples were filtered using a filter paper and 10 mL was measured using a pipette. The wine was diluted using 50 ml of distilled water in a conical flask. To the sample, two drops of phenolphthalein indicator were added and swirled to ensure homogeneity.

For the titration, 0.1 M NaOH (titrant) was filled into the burette. It was dispensed drop after drop into the conical flask containing the analyte (wine). The initial and final burette volumes were calculated and recorded. The total acidity was calculated by applying stoichiometric formula stated below.

Total Acidity (g/L) = [(V_{NaOH} (ml) × M_{NaOH} (ml) × Eq. Wt. of Acid (g/mol)]/
Sample Volume (mL)

where:

V_{NaOH}: Volume of NaOH used

$M_{NaOH:}$ molarity of NaOH used

Eq.wt : equivalent weight of predominant acid e.g. tartaric acid

Sample volume: volume of wine used.

3.8 Statistical analysis

The TTA and TSS data were analysed using one-way ANOVA and Tukey's Honestly Significant Difference method (HSD) using R stats Software, version 4.4.

3.9 Data reliability and study validity

Media preparation was tested for sterility before being used. The sterility test was conducted by incubating the media at 30℃ for 48 hours and observing growth or turbidity. Only sterile media were used for the experiments. All experiments were carried out under sterile aseptic conditions. All experiments were carried out in triplets, and for quantitative data, the mean and standard deviation values were used.

Chapter 4

Results

4.0 Introduction.

This chapter describes the results after critically following the described methodology. The results cover yeast isolation, morphological and biochemical characterization as well as measured wine parameters, total residual dissolved sugar and total titratable acidity. The identified yeasts were *Saccharomycopis, Hanseniaspora, Saccharomyces and Issatchenkia*.

4.1 Yeast isolation and colony morphology

Yeast isolated from *Z. mauritiana* presented different morphological characteristics in colony colour, shape, size, elevation, margins, texture, and opacity on PDA. In total, twenty isolates were purified from the isolation process and preserved on PDA slants for downstream characterization.

Round, irregular and oval-shaped isolates were observed. White and cream were the predominant colors with only one isolate showing yellow colour on PDA. The colony sizes ranged between 0.5mm and 15mm. Colonies also presented different opacity levels with some being fully translucent or opaque, while others were either opaque or translucent on the margins. The colony texture was either creamy, mucoid, brittle or dry.

4.2. Biochemical characterization

4.2.1 Carbohydrate fermentation tests

Of the twenty isolates purified, twelve (60%) showed fermentation characteristics with both colour change from red to yellow and gas production observed as a bubble in Durham tubes. Lactose was only fermented by two (10%) yeast isolates. Isolates fermenting at least three sugars were selected for wine production. Table 2 below shows labelled yeasts isolates classified under fermentive and non-fermentative yeasts.

Table 2: Yeast isolates classified under fermentative and non-fermentative yeasts

FERMENTING YEAST	**2**	**3**	**4**	**5**	**6**	**7**	**9**	**10**	**11**	**12**	**14**	**17**	**S.CEREVISI**
NON-FERMENTING YEAST	1	8	15	16	18	19	20						

4.2.2 Urease test.

All isolates were urease negative except for isolate 20, which showed a positive colour change from yellow to pink without gas production.

Figure 5 illustrates the negative results obtained during the experiment while figure 6 shows the control tubes that were used for the experiment.

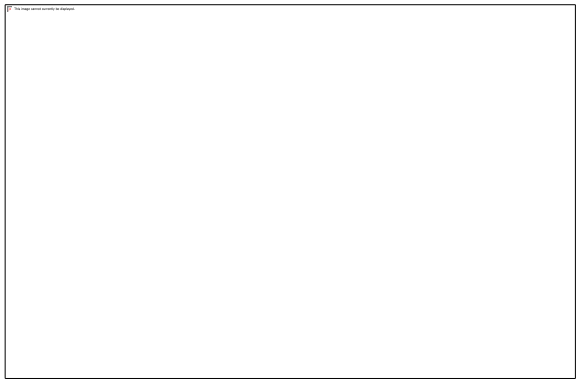


Figure 5: Negative result on all test tubes for urease activity

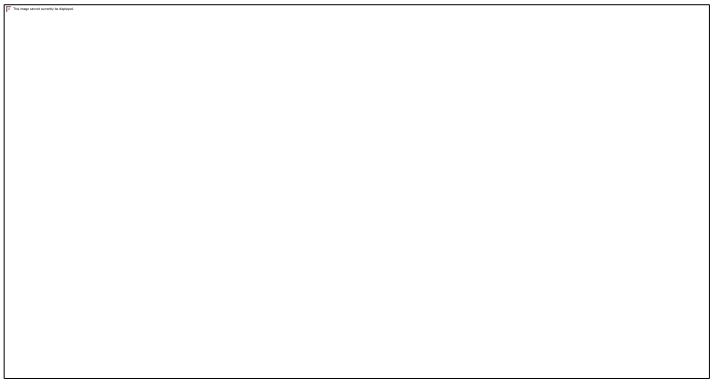


Figure 6: Positive control (left), negative control (center), and positive isolate 20 on Urease activity

4.2.3 Hydrogen sulphide.

All isolates showed negative hydrogen sulphide production results on Kliggler iron agar slants. The butt of the slants was orange and the slant only showed yeast growth. No blackening was observed to represent hydrogen sulphide production. Figure 7 below shows the slants after 48 hours of incubation at 30 ℃.

Figure 7: Negative hydrogen sulphide production on Kliggler iron Agar slants inoculated with yeast isolates.

4.2.4 Catalase test

The graph below shows the catalase results of the yeast isolates presented as either weak positive, moderate positive, strong positive, or negative. Figure 8 and 9 shows catalase vigor of the isolates presented under a bar graph and percentage distribution of the vigor of isolates respectivvely.

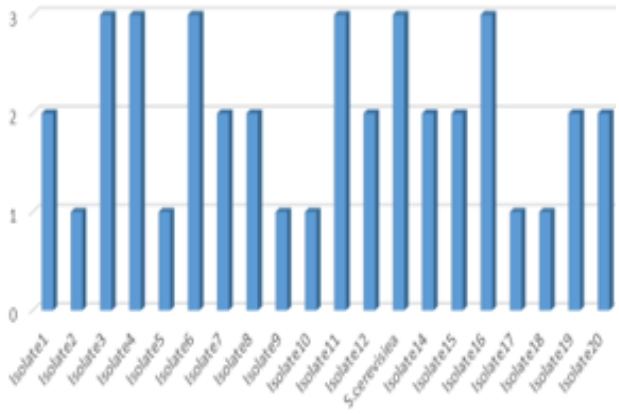


Figure 8: A bar graph representing catalase activity of the yeast isolate

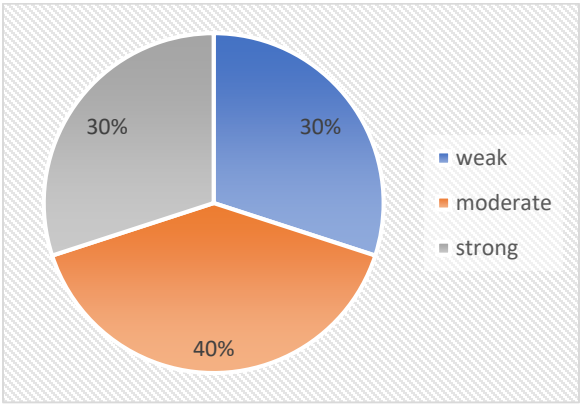


Figure 9: The distribution of catalase reaction vigor of the yeast isolates

KEY: 1. weak positive 2. moderate 3. strong positive

4.2.5 D-glucose tolerance

All twelve isolates identified as good fermenting yeast were used for the experiment. Results on 2% D-glucose (un-supplemented PDA) and 5% showed excellent yeast growth, with full colonization of the inoculated area. All isolates demonstrated growth on 10% glucose, while only the positive control S. cerevisiae showed growth up to 40% glucose concentration**.**

Figure 10: Glucose tolerance of yeasts on different concentrations

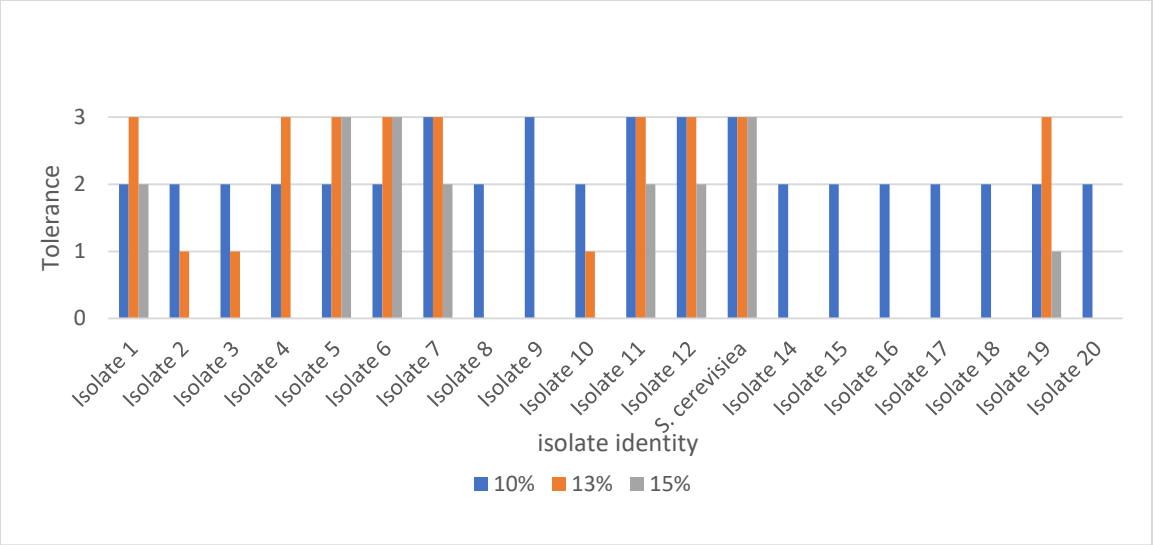
Key : 3 crowded growth, 2 moderate growth, 1 scandy growth, 0 no growth

Figure 11: Yeast isolates growth on 5% glucose PDA

Figure 12:Yeast isolates inhibited growth on 40% PDA except for isolate 13 (S.cerevisiae)

4.2.6 Ethanol tolerance

Isolates demonstrated varying tolerance to ethanol concentrations. Good growth was observed on both 5% and 8%, showing full colonization on the inoculated section. The results of 5% and 8% were not distinct but showed a score of 3 and were hence omitted from Figure 15 below. Tolerance results for 10%, 13%, and 15% are presented on Figure 13 below.



Key : 3 crowded growth, 2 moderate growth, 1 scandy growth, 0 no growth

Figure 13: Ethanol tolerance of yeasts in different concentrations

Figure 14: Yeast growth results in PDA supplemented with 5%, 8%, 10%, 13%, and 15% ethanol obtained after 48 hours of incubation.

4.3 Wine fermentation

Fermenting yeast selected for wine making presented the morphology summarized in the table below. The isolates were selected based on analysis of the results trend of sugar fermentation, ethanol tolerance, and Glucose tolerance.

Table 3: Identity and colony morphology of yeasts used for wine production

COLONY MORPHOLOGY	ISOLATE 5	ISOLATE 6	ISOLATE 11	ISOLATE 7	+VE CONTROL
PRESUMPTIVE GENUS	*Saccharomycopis*	*Hanseniaspora*	*Saccharomyces*	*Issatchenkia*	*S. cerevisiae*
COLOUR	Cream	Cream	cream	White	Cream
ELEVATION	Irregular	Flat	Convex	Convex	Convex
SURFACE	rough	wrinkled	Smooth	Smooth	Smooth
OPAQUCITY	Opaque	Opaque	Opaque	Opaque	Opaque
MARGIN	Entire	entire	Entire	Entire	Entire
SIZE	Large	small	Small	Medium	Small

Table 4: Cell morphology of the isolates selected for wine production

CELL MORPHOLOGY	ISOLATE 5	ISOLATE 6	ISOLATE 11	ISOLATE 7	+VE CONTROL
PRESUMPTIVE GENUS	*Saccharomycopis*	*Hanseniaspora*	*Saccharomyces*	*Issatchenkia*	*S. cerevisiae*
BUDDING POINT	multipolar	bipolar	Round	Unipolar	Round
REPRODUCTION	budding	Budding/pseudo hyphae	budding	budding	Budding
CELL SHAPE	elongated	apiculate	ovoid	Ovoid	Ovoid

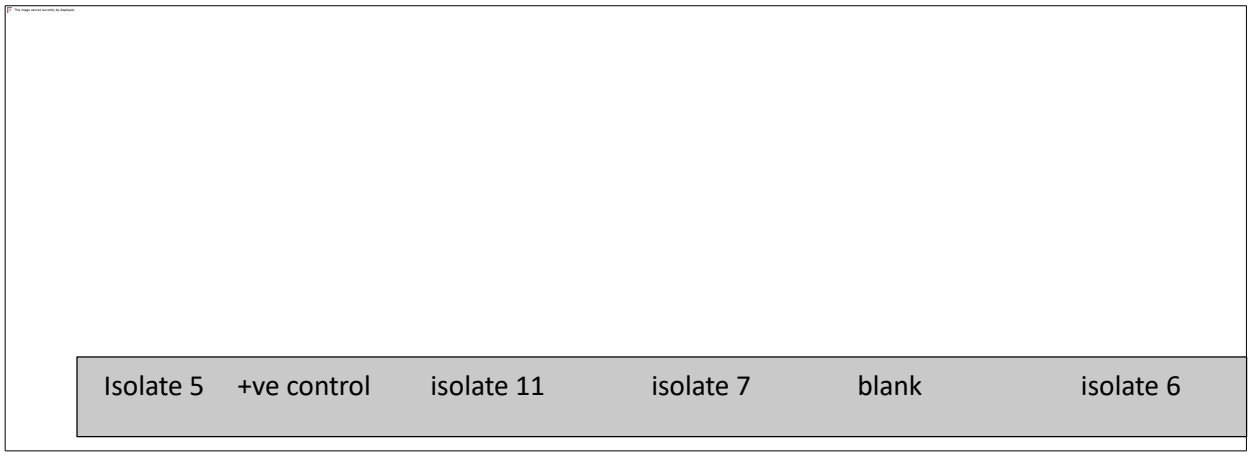


Figure 15: Z. mauritiana wine at day 20

4.10 Brix value at day 10

Table 5: Change in the TSS of wine measured at day 10 and expressed in O Brix

Isolate #	*Initial ^{O}Brix*	*Final ^{O}Brix* *Mean ± S.D*
S. cerevisiae	20.00	4.90 ± 0.02
5	20.00	11.90 ± 0.03
6	20.00	14.30 ± 0.02
7	20.00	9.70 ± 0.02
11	20.00	8.80 ± 0.01

4.11 Titration table

Table 6: Titration values obtained on wine samples using different yeast isolates at day 10

Isolate identity	Positive control	Isolate 5	Isolate6	Isolate7	Isolate 11
Average VNaOH Used (ml)	**6.92 ± 0.02**	**7.63 ± 0.01**	**7.45 ± 0.01**	**7.74 ± 0.01**	**7.14 ± 0.01**
Volume of wine used (ml)	**10.00**	**10.00**	**10.00**	**10.00**	**10.00**
Eq.wt of tartaric acid (g/mol)	**75.00**	**75.00**	**75.00**	**75.00**	**75.00**
TOTAL ACIDITY (g/L)	**5.19**	**5.72**	**5.59**	**5.81**	**5.36**

4.12 Statistical analysis

The data passed the normality test and other requirements of ANOVA. The *p-values* obtained were 8.56E-08 ≤ 0.05 and 2.59E-12 ≤ 0.05 for TSS and TTA, respectively. The Tukey's HSD analysis pack revealed a significant difference between the control and all other isolates except isolate 11 on TTA. The same test revealed a significant difference between the control and all-other isolates on TSS.

Chapter 5

Discussion, summary, recommendations and conclusions

5.0 Introduction

Ziziphus mauritiana fruits present a good niche for the growth of yeasts as observed in most fruits (Lakew, 2022). The wild yeast in the fruits can be used for industrial applications as starter cultures for the manufacturing of alcoholic beverages, fermented dairy products, and non-alcoholic beverages (Zhang et al., 2024). Combining the starter culture species is likely to yield a product with desirable market favoured attributes.

5.1 Discussion

5.1.1 Characterization of yeasts

From the experiment, mixed genera of yeast were isolated from the skin of the fruits. A total of twenty yeast isolates were purified from the sampled fruits. The abundance of yeast isolates on *Z. mauritiana* fruits complies with the results obtained by (Nyanga et al., 2007). The author observed growth of fourteen fermentative yeasts from the analyzed sample. The sampling area was geologically the same, Muzarabani, Mashonaland Central, Zimbabwe. (Lakew, 2022) also supports the notion that fruits provide a good niche for the survival of yeasts, and these yeasts can have industrial applications.

5.1.2 Biochemical characterization.

5.1.2.1 Carbohydrate fermentation

Of the isolates, 60% were fermenting yeasts. These could ferment glucose, fructose, and sucrose to produce carbon dioxide and an acid. Acid production resulted in a decrease in pH, resulting in the colour change of phenol red indicator from red to yellow, as explained by (Hassan and Baydaa ,2018);(Kurtzman et al., 2011). Some tubes showed a colour change with no gas production, indicating sugar assimilation but not fermentation (Aryal, 2019) .

Identification of the isolates as fermentative yeast complies to the work done by (Nyanga et al., 2007), who did the same classification of differentiating fermentative yeasts from non-

fermentative yeasts. The same criteria was used by (Bassey et. al, 2017) in identifying yeast isolates for the production of wine.

5.1.2.2 Catalase activity

The catalase test was conducted on all isolates to ensure the catalase enzyme activity. Although all isolates showed catalase activity, the reaction vigor was different. The vigor was classified as either weak, moderate, or strong following a method used by (Bassey et. al, 2017). Of the twenty isolates, 30% showed strong catalase activity, 40% showed moderate reactivity, while the remaining 30% demonstrated weak catalase activity. The difference in the vigor of catalase activity can be traced back to the genetic build-up and the environmental adaptation of the isolates (Torrellas et al., 2020), (Belazz et al., 1991). The difference in the vigor of the catalase reaction using hydrogen peroxide were also observed by (Torrellas et al., 2020). Moderate activity represented efficient degradation of hydrogen peroxide with no strain on the enzymes. On the other hand, weak catalase activity (30% of isolates) an indication of low oxidative defense, likely as a result of factors such as glucose repression. Finally, strong catalase activity (30% of isolates), on the contrary, is characteristic of yeasts with excellent oxidative stress tolerance, mostly as a result of adaptation mechanisms.

5.1.2.3 Urease activity

A positive urease activity was only recorded for one isolate, Isolate 20. The isolate was discarded from the experiment due to the health risks it poses to the consumers as well as the negative organoleptic properties of the wine that results. Urease activity converts urea into ammonia and carbon dioxide. Urea is naturally present in grapes and also *masau*. The production of ammonia in the wine not only increases the pH (Yu et al., 2023) but also produces an off flavour in the wine, overally decreasing the organoleptic properties of the wine. The increase in the pH also affects the stability of the wine. Wine is more stable under acidic pH range of 3.2- 4.0 (Malhi, 2018). Beyond this pH range, wine is more prone to bacterial and fungal spoilage.

Production of ammonia in wine also raises risk of production of a carcinogenic compound known as ethyl carbamate or urethane. The compound is formulated after the reaction of ammonia and ethanol under warm conditions (Yu et al., 2023). This compound is strongly regulated in alcoholic beverages due to the health risks it provides upon consumption (Program, 2024).

The analysis of the morphological and biochemical characteristics led to the presumptive identification of the yeast isolates as genera *Saccharomyces, Saccharomycopsis, Issatchenkia, and Hanseniaspora as* shown in *Table 3.* The isolates were used for wine production due to their competitive biochemical properties. Presence of the species on the surface *of Z. mauritiana* fruits complies to the results obtained by (Nyanga et al., 2007). It also complies to similar work done by 2025/8/5 and (Bassey et. al, 2017) using street-vended fruits as sources of yeasts.

5.1.2.4 Hydrogen Sulphide production

The experiment on the production of hydrogen sulphide revealed that, from the isolated yeasts on *masau*, none of the isolates was a hydrogen-sulphide producer. For this reason, no isolate was eliminated based on this factor. Hydrogen sulphide production is a negative parameter in wine that can ruin the colour of the wine as well as the taste (Danilewicz & Standing, 2024). The compound, hydrogen sulphide is also identified as a toxin to yeast, hence limiting the proliferation of cells during the log phage of fermentation. Checking for hydrogen sulphide production from yeast intended for wine production and the elimination criteria was used by (Bassey et. al, 2017). From this research, it was observed that only *Candidafamata* and *Candida pelliculosa* survived in the presence of hydrogen sulphide. To the rest of the isolates, hydrogen sulphite was a toxin than inhibited yeast growth. Of the selected isolates for wine production, none of them was a hydrogen producer, which complies to literature complied by (Zai-Bin Xie et al., 2021), (Gautam & Shukla, 2020) and (Allahyari & Gurakan, 2024).

5.1.2.5 D-glucose tolerance

The sugar tolerance test was conducted on all isolates classified under fermentative yeasts. The results of the test were read based on a qualitative scale of 0 to 3, with 0 representing no growth and 3 being crowded growth (Maragatham & Panneerselvam, 2011). The yeast isolates plated on un-supplemented PDA showed similar results to 5% glucose-supplemented PDA, with crowded growth. Yeast cells are eukaryotic and utilize glucose as their primary metabolite for the generation of ATP (Somsen et al., 2000). At low glucose concentrations, the cells receive optimal glucose for their metabolic reactions, e.g. cell replication, thereby proliferating to form lawn growth on the inoculated area (Maicas, 2020). The glucose concentration allows for an osmotic balance between the yeast cells and their environment.

As the concentration increases to 10% and 15%, the osmotic gradient shifts, causing the cells to lose water to their environment due to osmotic pressure. As a result, the cell homeostasis is impaired. Also, water-mediated transport is disrupted, slowing down the transportation of nutrients across the cell. This affects the enzyme activity of the cell as well as the functioning of the cell membrane (Dongdong et al., 2022).

Another phenomenon known as glucose toxicity comes to play. Glucose toxicity results from the increase in concentration of glucose in the environment. In some reactions, glucose crosses membranes via active uptake, which is an energy demanding reaction. As a result, increased concentration of glucose creates an imbalance in the amount of NAD+ and NADH present in a cell at a given time (Somsen et al., 2000). At this state, the metabolic reactions carried out by the cells to fight against the increased sugar concentration lead to the production of genotoxins e.g. methylglyoxal and reactive oxygen species (ROS) (Dongdon et al., 2022). The effects of glucotoxicity can be characterized by reduced cell growth or death.

Further increase of glucose concentration to 20% saw a further decrease in the growth of the yeast. This was observed for isolates 3, 5, 6, 9, 10, 15 and 20. The flaccidity of the cells was phenotypically observed on the colonies, which became pale and dry, and a change in shape (Li et al., 2025). The results suggest a further effect on the osmotic balance of the cells as well as cell damage. However, some isolates, isolates 6, 11, and 12 demonstrated no change from the results recorded on 15%. This suggests the adaptability of the isolates to the environment ensuring no further damage to the cells.

Most interestingly, commercially available *S. cerevisiae* maintained a score of 3 (crowded growth) throughout all concentrations. This suggests the supreme adaptability of the isolate in varying sugar concentrations (Gautam & Shukla, 2020). Prior research by (Liu et al., 2024), reveals that *S. cerevisiae* can withstand sugar concentrations of up to 60%, increasing its industrial application acceptability.

5.1.3 Total acidity of wine

The amount of total acidity in wine reveals the nature of the wine in terms of the balance between tartness and sweetness. Total titratable acidity was determined using the titration method and in reference to tartaric acid. The results obtained reveal that presumptive *S. cerevisiae* had the least TTA value of 5.19 g/L, while Isolate 7 had the highest value of 5.81g/L.

The obtained results are similar to work done by (Nyanga et al., 2007). However, in this research, only *S. cerevisiae* was used as the starter culture, and the results on TA were expressed in relation to citric acid instead of tartaric acid used in this research (Sharma & Mahavidalaya, 2019). *Hanseniaspora* (Allahyari & Gurakan, 2024), *Issatchenkia*, and *Saccharomycopsis* are weak sugar fermenters in comparison with *S. cerevisiae*. For this reason, their ability to degrade organic acids in wine is limited. Moreover, yeasts like *H. opuntiae* have a weak ethanol and sugar tolerance, hence limited in their proliferation as fermentation progresses and ethanol builds up in wine (Fresno et al., 2023).

5.1.4 Reduction in Total Soluble Sugars.

There was an observed decrease in the total soluble sugars in wine from the initial to the final value recorded at day 10 of wine fermentation. The trend behavior was expected as sugars are consumed by fermentation and other metabolic reactions in the cell. However, a notable difference was observed in the percentage decrease with Isolate 11 having the highest decrease and Isolate 7 having the least.

S. cerevisiae is a good sugar fermenter; hence, it has widespread application in the food and biofuel production industries. It is active throughout the whole fermentation process. It is well adapted to survive high sugar concentration up to 60% and against rising alcohol content as a result of fermentation. the decrease in the TSS is inversely proportional to the alcohol content of the sample. The actual ratio used in oenology and viniculture 1: 0.56 ^{o}Brix: alcohol content at 25 $°C$. This ratio is practically used for the estimation of alcohol content. It takes into consideration the use of other sugars in cell respiration as well as other metabolic reactions.

The work by (Benito et al., 2019; Fresno et al., 2023; Sharma & Mahavidalaya, 2019) supports the idea that *S. cerevisiae* has better sugar conversion capacity than the other isolates isolated and used for wine making in this research. However, their work does not despise the important application of non-saccharomyces species in wine making. (Benito et al., 2019) and (Wang et al., 2023) argues the important contribution of non-saccharomyces species in improving the taste, smoothness, and flavour of wine. The author also annotates the need to use these isolates in combination with *S. cerevisiae* in order to obtain both good aroma and flavour as well as alcohol content.

5.1.5 Statistical analysis.

The results of p-value 8.56E-08 ≤ 0.05 and 2.59E-12 ≤ 0.05 for TSS and TTA, respectively, were statistically inferred. This reveals a significant difference between the tested groups. A post-analysis using Tukey's HSD on the TA reveals a HSD value of 0.1094. The data revealed a significance difference between control and isolates 5, 6, and 7. However, there was no significant difference between the control and Isolate 11 (wild *S. cerevisiae).*

The results obtained comply with the literature from (Nyanga et al., 2013, 2007), who reported the presence of wild species of S. cerevisiae with performance comparable to the commercially available species. The results reveal the potential of wild species to be an alternative for wine production.

5.1.6 Significance of results

The results obtained signify the presence of yeast species with fermentative ability on *Z. mauritiana* fruits. The biochemical tests conducted have revealed presence of genus *Saccharomyces, Saccharomycopsis, Issatchenkia, Hanseniaspora* were isolated from the fruits. The statistical analysis reveals the existence of a significant difference between commercially available *S. cerevisiae* and the isolated yeasts. Furthermore, the post-hoc analysis reveals the close relatedness of the commercial yeast and the wild species of S. cerevisiae.

This research adds to the literature already present on the potential application of non-*Saccharomyces* yeasts in the production of wine. It presents clearly the results on critical factors when choosing a starter culture for wine production, including sugar conversion efficiency, TA of the wine, and the residual TSS, which all affect the overall organoleptic properties of the wine.

5.2 Summary

Z. mauritiana fruits co-exist the yeasts that have both fermentation and non-fermentation roles. The fermentative yeast shows variable biochemical properties, including sugar fermentation, ethanol tolerance, and sugar tolerance. Further analysis on the wine reveals the different ability of the isolates in the conversion of sugar to ethanol as well as the build-up of organic acids, measured using titration as total titratable acidity. The existence of genera *Saccharomyces, Saccharomycopsis, Hanseniaspora* and *Issatchenkia* were confirmed by analysis of the morphological and biochemical behavior of the isolates.

The positive control, commercially available *S. cerevisiae,* had the highest percentage decrease in TSS, and the least value of TTA, outperforming all the isolates. There was a significant difference between s. cerevisiae and the isolates using one-way ANOVA @ $p \leq 0.05$. Tukey's post-hoc analysis revealed a significant difference between the control and Isolates 5, 6, and 7, while no significant difference with isolate 11 (presumptive *wild S. cerevisiae*).

5.3 Recommendations

Based on the outcomes of this research, further research is recommended on the method of characterizing yeasts. Use of molecular methods such as Sanger sequencing or Next Generation Sequencing (NGS) and bioinformatics of the data is strongly recommended. Molecular-based methods provide a more accurate way of identifying yeasts at the same time relationships between the wild species and commercially available yeasts can be determined. Alternatively, use of metagenomics on the fruits can reveal other non-yeast species present on the fruits. The use of biochemical testing kits e.g. the API-20 results with better accuracy than traditional methods used in this research and yet they are cost-efficient, requiring basic skills to use them. Furthermore, researching the impact of using starter culture combinations can be investigated, paying attention to alcohol content, TTA, TSS and organoleptic properties of the wine. Alternatively, the use of these combinations can be used to evaluate the shelf-life impact of the wine and the fermentation kinetics during wine processing. Finally, the isolation of yeast from other edible fruits of genera *Ziziphus* can be studied. These species have microflora more adapted to carry than *Z. mauritiana*.

5.4 Conclusion

This research characterized yeast from *Z. mauritiana* with potential application in the oenology of the fruit. The obtained results demonstrate the presence of yeasts with biochemical adaptation suitable for their application in wine-making. The results also review the promising application of wild yeasts in the diverse and variable production of *Z. mauritiana* fruit wine, with favourable organoleptic properties

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APPENDIX

One-way ANOVA on TTA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.789427	4	0.197357	740.0875	2.59E-12	3.47805
Within Groups	0.002667	10	0.000267			
Total	0.792093	14				

Appendix 1 One-way ANOVA on TTA

Appendix 2 Turkey's Post-hoc analysis on TTA

Where :

Control- Commercial *S. cerevisiae*

A – Isolate 5

B- Isolate 6

C- Isolate 7

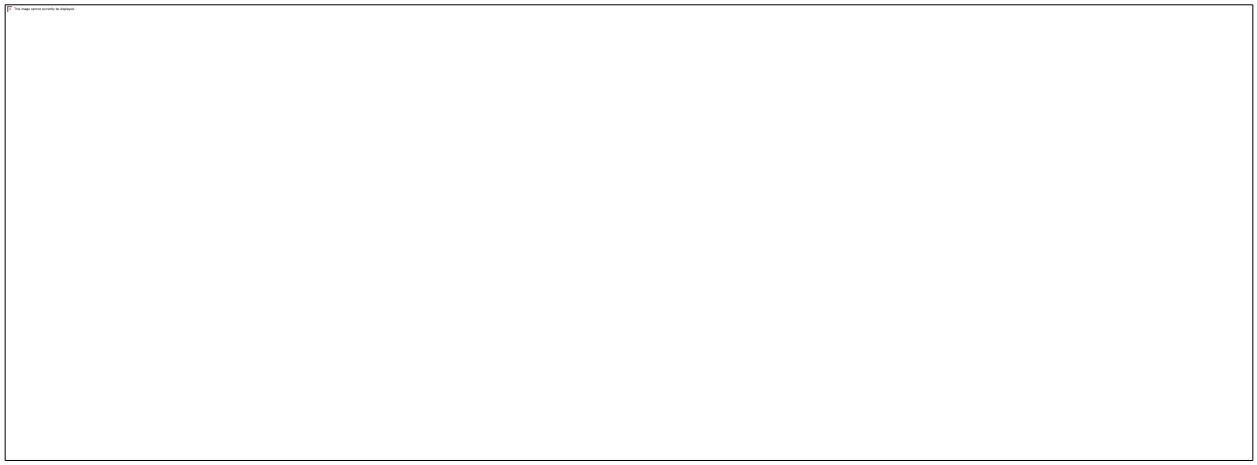
D- Isolate 11

ONE-WAY ANOVA ON TSS

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.763667	4	0.190917	89.77273	8.56E-08	3.47805
Within Groups	0.021267	10	0.002127			
Total	0.784933	14				

Appendix 3 One-way ANOVA on TSS

Tukey's HSD analysis on TSS



Appendix 4 Turkey's Post-hoc analysis on TSS

Where :

Control- Commercial *S. cerevisiae*

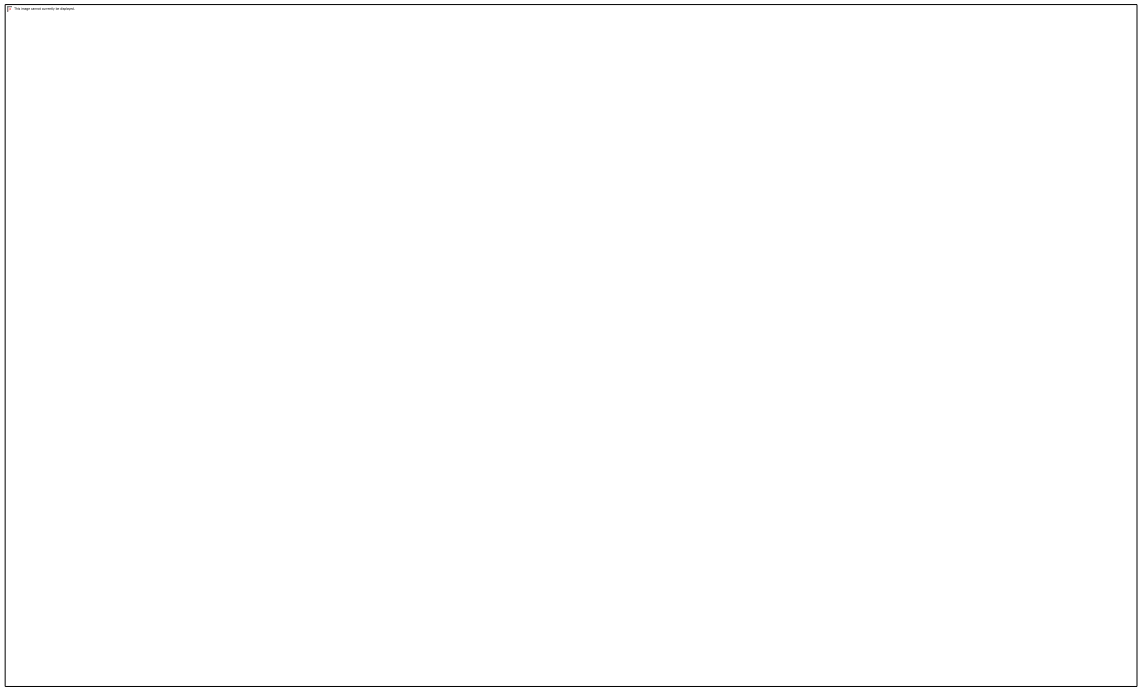
A – Isolate 5

B- Isolate 6

C- Isolate 7

D- Isolate 11

Appendix 5: Titration setup for determining TTA in wine



Appendix 6 : PDA slants cultured with yeast isolates