Alcoholic[1] Fermentation of *Ziziphus Mauritiana* (*Masau*) and *Uapaca Kirkiana* (*Mazhanje*) Fruits in Zimbabwe

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ABSTRACT

In Zimbabwe, most traditional alcoholic beverages are still being produced at household level, owing to limited information on the physio-chemical changes associated with the fermentation process, as well as the micro-organisms involved. The main objective of this study was to carry out traditional fermentation of Ziziphus mauritiana (masau) *and* Uapaca kirkiana (mazhanje), *and isolate and identify yeasts and bacteria associated with the process. Fruit samples were collected, cleaned and blended into pulps for fermentation. Microbiological analyses were carried out on the fruit pulps before and after fermentation. The micro-organisms present were isolated by culturing on selective-media, and their morphologies (colony appearance, cell shape and staining behaviour) were characterised. The confirmation of the presence of yeast was carried out by investigating the ability of micro-organisms to ferment sugars and produce carbon dioxide. After fermentation, the yeast species from* Ziziphus mauritiana *were identified as* Pichia fabianii, Saccharomyces cerevisiae *and* Candida tropicalis, *whilst only* Pichia fabianii *was found in the unfermented fruit pulp. The yeast species isolated from fermented* Uapaca kirkiana *was identified as Candida* krusei, *whilst no yeast species were identified in unfermented pulp. The lactic acid bacteria from* Ziziphus mauritiana *were identified as* Lactobacillus plantarum *and* Streptococcus thermophilus *in both the fermented and unfermented fruit pulps.* Lactobacillus plantarum *was also isolated from both the fermented and unfermented pulps of the* Uapaca kirkiana *fruit. The isolated yeast strains may, therefore, be potential starter cultures for the production of* Ziziphus mauritiana *and* Uapaca kirkiana *alcoholic beverages at industrial scale. The use of these isolates as starter cultures for alcoholic fermentation may lead to the production of commercial beverages of consistent quality.*

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Keywords: *Ziziphus mauritiana*, *Uapaca kirkiana*, lactic acid bacteria, yeasts, fermentation

INTRODUCTION

Household production of several traditional alcoholic beverages using fruits is slowly being replaced by industrially-processed and convenience foods often based on technology developed in industrialised countries (Muhialdin *et al.,* 2022). However, this is a challenge to poor people in Zimbabwe whose livelihoods depend on selling traditional alcoholic beverages (Ngueuleweu *et al.,* 2017). There is therefore a need to develop local fermentation technology suitable for the production of alcoholic beverages from traditional fruits at industrial level in order to maximise profits through the monetisation of knowledge products. Generally, industrial-scale production should be done without losing the unique flavour of the fruits.

Although not much has been done concerning studying the microbiology of the fermentation processes of the local fruits, *Ziziphus mauritiana* and *Uapaca kirkiana*, they are currently being fermented for the production of alcoholic traditional beverages in Zimbabwe (Chawafambira *et al.,* 2020). *Ziziphus mauritiana* is a fruit (Figure 1a) which is locally known as *masau*. The fruit is usually eaten raw or processed into products such as jam which is sold at local markets. It can also be processed into traditional cakes and alcoholic and non-alcoholic beverages. *Ziziphus mauritiana* is naturally fermented under uncontrolled conditions and distilled into a local beverage known as *kachasu*, a process which takes between four to seven days (Nyanga, 2012). In Zimbabwe, the consumption of *kachasu* is illegal because it is alleged to have toxic levels of ethanol that can lead to ill health, and is also associated with cause of sudden death (Nyanga, 2012).

Uapaca kirkiana is an indigenous fruit also known locally as *mazhanje* (Figure 1b). The ripe fruit, which is eaten raw, is mostly sold at local and roadside markets. *Uapaca kirkiana* fruit is fermented into a sweet beverage called *mutandavira* (Oguntoyinbo *et al.,* 2016). The seeds are removed from the pounded fruit, and the pulp is mixed with cold water and then allowed to ferment for three days until the water turns grey or opaque.

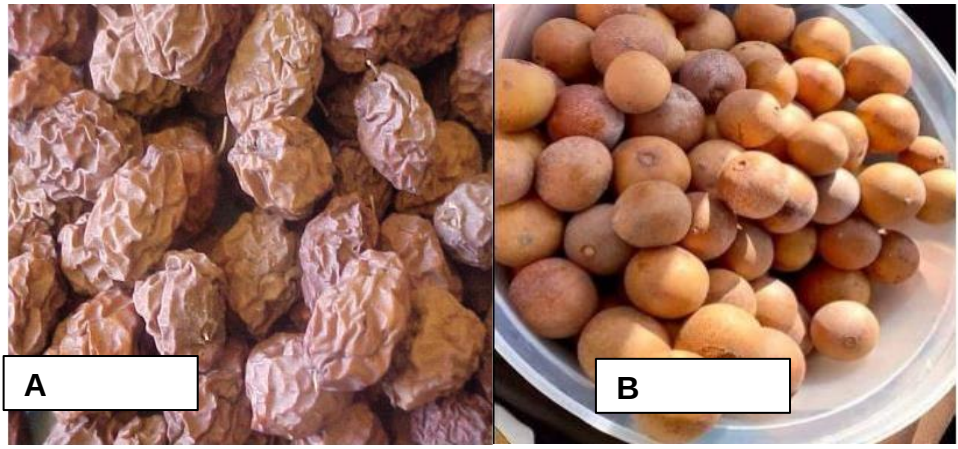


Figure 1: A: *Ziziphus mauritiana,* and B: *Uapaca Kirkiana*

In Zimbabwe, however, the alcoholic beverages from both *Ziziphus mauritiana* and *Uapaca kirkiana* are still being produced at household level owing to limited information on the physio-chemical changes associated with their fermentation processes, as well as the micro-organisms involved (Nemapare *et al.,* 2023). There is, therefore, need to understand the microbiology of alcoholic fermentation of the *Ziziphus mauritiana* and *Uapaca kirkiana* fruits for possible industrial beverage production in Zimbabwe. Thus, this study aimed at identifying the micro-organisms responsible for the spontaneous alcoholic fermentation of Zimbabwe's indigenous fruits, namely, *Ziziphus mauritiana* and *Uapaca kirkiana*.

MATERIALS AND METHODS

SAMPLE COLLECTION AND PREPARATION

Uapaca Kirkiana and *Ziziphus mauritiana* fruits were bought from Mbare Produce Market in Harare, Zimbabwe. Fresh fruit samples were washed using distilled water and cut open using a knife in order to extract the seeds. A total of 100 g of fruit pulp and skin from each fruit sample was homogenised using a blender. A total of 100 ml of distilled water was added to the obtained homogenate, and the pH of the mixture was determined using a pH-25 Benchtop pH Meter with a three-point calibration of pH 4, pH 7 and pH 10. The pH measurements of the samples were made in triplicates.

FERMENTATION

A volume of 100 ml of each fruit mixture was transferred into autoclavable bottles which were tightly sealed to ensure that fermentation occurred under anaerobic conditions. The fruit mixtures were left to ferment on a shaker at 25ºC. After 24 hours of incubation, the lids of the bottles were replaced with a balloon for clear observation of the CO_2 that was going to be trapped and accumulate inside the balloon, slowly inflating it. After 72 hours, the pH of the fermented fruit juices was measured.

ISOLATION OF YEASTS

The fermented and unfermented fruit juices of both the *Uapaca kirkiana* and *Ziziphus mauritiana* fruits were streaked on sabouraud dextrose agar (SDA) plates for isolation of yeasts as explained by Ibrahim and Rahma (2009), with a few modifications. The colonies were observed after plates had been incubated at 28ºC for 48 hours.

ISOLATION OF LACTIC ACID BACTERIA (LAB)

The fermented and unfermented fruit mixtures from both the *Uapaca kirkiana* and *Ziziphus mauritiana* fruits were streaked on the de man rogosa sharpe agar plates for isolation of lactic acid bacteria as explained by Prastujati *et al.* (2022), with a few modifications. The colonies were observed after plates had been incubated at 28ºC for 48 hours.

PURIFICATION OF YEAST AND BACTERIA

Morphologically distinct yeast and LAB colonies were selected and purified by sub-culturing in sabouraud dextrose agar (SDA) and de man rogosa sharpe agar (MRSA), respectively. The cultures were incubated on a rotary shaker at 28ºC and 110 rpm for 48 hours, and the colonies were observed.

MORPHOLOGICAL CHARACTERISATION OF YEASTS

Yeast strains streaked on SDA plates and incubated for 48 hours were examined for their cell morphology (shape, elevation, appearance and colour) using a light microscope at ×100 magnification (Sulmiyati *et al.,* 2019).

LACTO-PHENOL COTTON BLUE STAINING

Cell morphology of the yeast strains was also examined using the lacto-phenol cotton blue staining method. A drop of 70% alcohol was placed on a microscope slide and the specimen was immersed in the drop of alcohol. Two drops of the lacto-phenol cotton blue stain were immediately added before the alcohol dried out. To avoid air bubbles, the cover slip was then gently placed onto the drop of alcohol. The slide was examined using a light microscope at ×100 magnification (Ihuma *et al.,* 2012).

DETERMINATION OF FERMENTATIVE AND NON-FERMENTATIVE YEAST SPECIES

One hundred (100) µl of each pure yeast strain was added to 6% glucose solution in a test tube and then plugged with a parafilm to ensure that sugars would ferment under anaerobic conditions. To determine the production of carbon dioxide gas, a test tube with phenol red indicator was connected to the test tube containing yeast and sugar. The mixtures were incubated at room temperature and observations were made for 30 days (Reiner, 2012).

MORPHOLOGICAL CHARACTERISATION OF LACTIC ACID BACTERIA

Bacterial strains that were streaked on MRSA plates and incubated for 24 hours were then examined to establish their cell morphology (shape, size, elevation, appearance and colour (Nurhikmayani *et al.,* 2019).

GRAM STAIN

Bacterial isolates were etched with two drops of crystal violet on glass slides and left for one minute. Iodine was then dropped onto the slide and left for one minute. Isolates were washed with distilled water, air dried and then slowly mixed with drops of 95% alcohol for 30 seconds. The isolates were washed again with distilled water, air dried and then slowly spilled by safranin for 30 seconds. The isolates were washed with distilled water, air dried and observed using a microscope at ×100 magnification (Gustavo *et al.,* 2021).

CATALASE TEST

To determine the ability of bacteria to degrade hydrogen peroxide, a single colony from each isolate was smeared on a glass object that had been sterilised with alcohol, then dripped with a 3% H_2O_2 solution. The

production of gas bubbles indicates that the bacteria are catalase-positive, while non-production of gas bubbles shows that the bacteria are catalase-negative (Prastujati *et al.,* 2022).

STATISTICS

An unpaired t-test was used in this study to determine the significant differences between the pH means of the two fruit samples.

RESULTS

PH

The pH of *Ziziphus mauritiana* and *Uapaca kirkiana* pulps was measured before and after fermentation (Table 1). Both fruit pulps showed significant differences of pH before and after fermentation, with the two-tailed P values of less than 0.0001. The decrease in the pH after 72 hours confirms occurrence of fermentation, as the pH usually decreases during fermentation due to micro-organisms that consume nutrients and produce organic acids which are released into the medium. There was also significant difference between the pH of *Ziziphus mauritiana* and *Uapaca kirkiana* pulp before fermentation with a two-tailed P value of 0.0034, whilst the difference was 0.0007 after fermentation.

Table 1: pH Values for *Ziziphus mauritiana* and *Uapaca kirkiana* fruit pulps before and after fermentation

pH	Before Fermentation	After Fermentation	P Value
Ziziphus mauritiana	5.33 ± 0.02^{a}	2.21 ± 0.07^{c}	<0.0001
Uapaca kirkiana	6.21 ± 0.14^{b}	3.03 ± 0.05^{d}	<0.0001
P Value	0.0034	0.0007	-

Each value represents mean ± standard deviation of triplicate values. Different superscript letters in the same column or row imply significant difference ($p < 0.05$). Where the same superscript letters appear in the same column or row, there is no significant difference ($p > 0.05$).

FERMENTATION

The (b) bottles from figures 2 and 3 show that carbon dioxide accumulated in the bottles as shown by an increase in the size of the balloons, hence confirming fermentation.

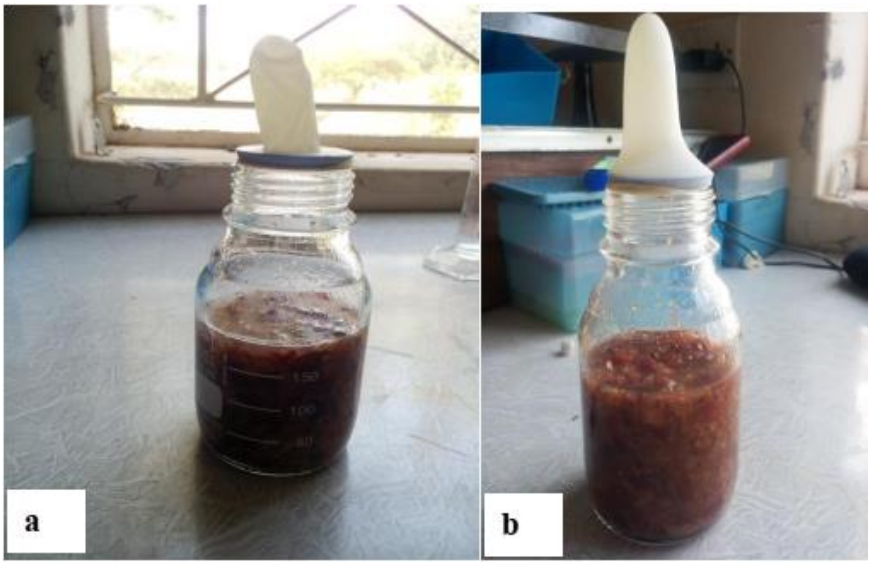


Figure 2: Changes in the size of the balloon as it traps carbon dioxide: (a) 0 minutes prior to fermentation, and (b): 120 minutes prior to fermentation of *Ziziphus mauritiana* fruit pulp

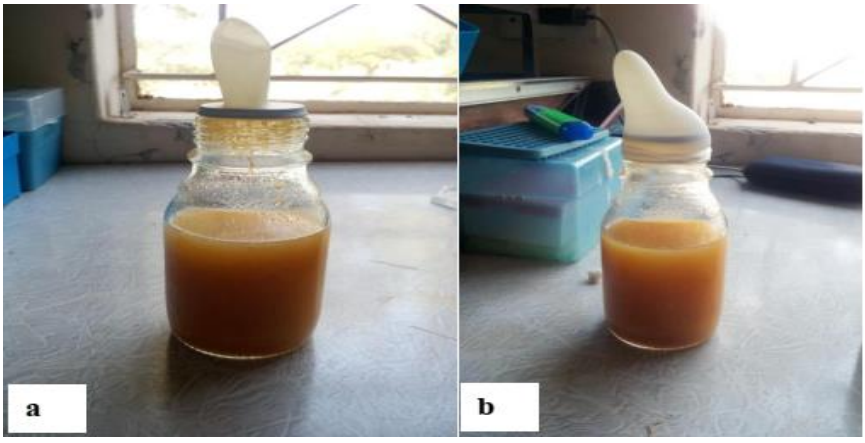


Figure 3: Changes in the size of the balloon as it traps carbon dioxide: (a) 0 minutes prior to fermentation, and (b): 120 minutes prior to fermentation of *Uapaca kirkiana* fruit pulp

YEAST

Table 2 shows the number of yeasts isolated from the fermented and unfermented fruit pulps of both the *Ziziphus mauritiana* and *Uapaca kirkiana* fruits. The yeast isolates obtained in fermented fruit pulp samples were more than those obtained in unfermented samples.

***Table 2**:* Isolated yeasts from the fermented and unfermented fruit pulps of *Ziziphus mauritiana* and *Uapaca kirkiana*

	Number of Isolates	
Fruit Pulp	**Unfermented**	**Fermented**
Ziziphus mauritiana	1	3
Uapaca kirkiana	1	2

LACTIC ACID BACTERIA

Table 3 shows the number of isolates from both the unfermented and fermented fruit pulps. The isolates from *Ziziphus mauritiana* fruit pulps were slightly different, with unfermented samples having more lactic acid bacteria than fermented samples, whilst the isolates from *Uapaca kirkiana* were similar for both the unfermented and fermented samples.

***Table 3**:* Isolated LAB from the fermented and unfermented fruit pulps of *Ziziphus mauritiana* and *Uapaca kirkiana*

	Number of Isolates	
Fruit pulp	**Unfermented**	**Fermented**
Ziziphus mauritiana	3	2
Uapaca kirkiana	1	1

LACTO-PHENOL COTTON BLUE STAINING OF YEAST CELLS

The cells were either spherical or elongated in shape and they all stained pale blue with lacto-phenol cotton blue stain, as recorded in Table 4.

***Table 4**:* Morphological characteristics of yeast cells isolated from unfermented and fermented fruit pulps of *Ziziphus mauritiana* and *Uapaca kirkiana*

Isolate	Colony morphology				
	Shape	Staining behaviour	Colour	Elevation	Appearance
Ziziphus mauritiana					
Unfermented fruit pulp					
1	Spherical	Light blue stained	White tannish	Convex	Glossy
Fermented fruit pulp					
1	Spherical	Light blue stained	White tannish	Convex	Glossy
2	Spherical	Light blue stained	White	Convex	Glossy
3	Elongated	Light blue stained	White	Raised, fringed	Glossy
Uapaca kirkiana					
Unfermented fruit pulp					
1	Spherical	Light blue stained	Cream	Flat wrinkled	Matte, dry
Fermented fruit pulp					
1	Spherical	Light blue stained	Cream	Flat wrinkled	Matte, dry
2	Elongated	Light blue stained	Whitish yellow	Flat wrinkled	Matte, dry

GRAM STAIN

The cells were either rods or spherical in shape, and they all stained purple by gram stain, except for one isolate from the unfermented fruit pulp of *Ziziphus mauritiana* (Table 5).

***Table 5**:* Morphological characteristics of yeast cells isolated from the unfermented and the fermented fruit pulps of *Ziziphus mauritiana* and *Uapaca kirkiana.*

Isolate	Colony morphology				
	Shape	*Staining behaviour*	*Colour*	*Elevation*	*Appearance*
Ziziphus mauritiana					
Unfermented fruit pulp					
1	Rods	Purple stained	Milky white	Convex	Glossy
2	Spherical	Purple stained	Milky white	Convex	Glossy
3	Spherical	Pink stained	Cream	Convex	Glossy
Fermented fruit pulp					
1	Rods	Purple stained	Milky white	Convex	Glossy
2	Spherical	Purple stained	Milky white	Convex	Glossy
Uapaca kirkiana					
Unfermented fruit pulp					
1	Rods	Purple stained	Milky white	Convex	Glossy
Fermented fruit pulp					
1	Rods	Purple stained	Milky white	Convex	Glossy

CATALASE TEST

The presence of catalase activity was only observed in bacterium number 3 from the unfermented fruit pulp of *Ziziphus mauritiana.* This was confirmed by the formation of oxygen bubbles which were observed after bacterial cells were suspended in a droplet of 3% hydrogen peroxide.

FERMENTATIVE AND NON-FERMENTATIVE YEAST SPECIES

Changes in colour from red to yellow indicated the production of carbon dioxide as a by-product of the fermentation process. Colour changes were observed from all the isolated yeasts, thus confirming that all the isolates were fermentative.

DISCUSSION

The first step in the scaling up and technology development for the production of alcoholic beverages from Zimbabwe's traditional fruits is the screening of micro-organisms for use as starter cultures (Akabanda *et al.,* 2014). After 24 hours of spontaneous fermentation of the *Ziziphus mauritiana* and *Uapaca kirkiana* fruit pulps, gas bubbling and frothing were observed, an indication that carbon dioxide was produced as a result of the action of various natural microflora present on the fruit surfaces (Shah & Singhal, 2017). Yeast is usually present on the fruit surface. In this study, yeast on the surface of fruits broke down the sugar to produce carbon dioxide gas that accumulated inside the balloon and slowly inflated it. The decrease in the pH of the *Ziziphus mauritiana* and *Uapaca kirkiana* fruit pulps after fermentation indicates an increase in the acidity of the pulps. The increase in acidity during fermentation was due to the production of carbon dioxide and alcohol by the yeast through the utilisation of natural sugars.

Yeasts isolated from the spontaneous fermentation of *Ziziphus mauritiana* and *Uapaca kirkiana* were identified by comparing them with those of known taxa and standard keys of yeast. Only one species identified as *Pichia fabianii* was isolated from the unfermented pulp of *Ziziphus mauritiana*, and three species identified as *Pichia fabianii, Saccharomyces cerevisae* and *Candida tropicalis* were isolated from the fermented pulp. *Pichia fabianii, Saccharomyces cerevisae* and *Candida tropicali* are wild or natural yeasts that are responsible for the spontaneous fermentation of

fruits (Chu *et al.,* 2023). The nature and number of wild yeasts are determined by a variety of parameters including soil type and the average temperature of the location, as well as rainfall, maturity and the condition of the fruits (Maragatham & Panneerselvam, 2011). The results obtained in this study tally with those previously reported which showed that *non-saccharomyces* are the most common yeast genera identified in the early stages of the fermentation of fruits, whilst *saccharomyces* completes the fermentation process (Garcia *et al.,* 2016).

Two yeast strains, one identified as *Candida krusei* and an unidentified one, were isolated from the fermented fruit pulp of *Uapaca kirkiana. Candida krusei* was also the only isolate from the unfermented pulp of *Uapaca kirkiana* fruit. When viewed under the microscope, the unidentified isolate had flat, wrinkled, whitish yellow colonies that were elongated. This morphology did not match with any yeast of known taxa, hence the possibility that it might be a new strain. In recent studies, *Candida krusei* has been isolated from Japanese sak, ginger beer and baker's yeast (Nakayama *et al.,* 2008), and is widely used in West Africa for chocolate production and cocoa bean fermentation. Heap fermentation in particular is most effective with *Candida krusei* (Jesperson *et al.,* 2005). Candida is an effective fermenter of glucose under aerobic and semi-aerobic conditions. In the current research, the high yielding fermentation by *Candida krusei* is useful, as the build-up of organic molecules during fermentation can cause problems experimentally. For instance, *C. krusei* prevents the build-up of succinic acid via the activity of highly expressed succinate dehydrogenase present in the Krebs cycle. Generally, *Candida krusei* prevents the build-up of succinic acid which is responsible for disrupting the separation of ethanol from other products during ethanol production (Nakayama et al., 2008; Tse *et al.,* 2021). Using this strain for ethanol production is highly advisable since its metabolic activity makes it more suitable in comparison to other similar strains.

Yeast causes alcoholic fermentation, while lactic acid bacteria (LAB) are responsible for lactic acid fermentation in foods. The isolates of lactic acid bacteria (LAB) were identified macroscopically and microscopically. The gram staining method and the catalase test were also used to identify

LAB. The isolates were grown on media that was inoculated with ketoconazole, an antifungal that kills yeast cells (Perez *et al.,* 2006).

Three lactic acid bacterial species — two gram positive and one gram negative — were isolated from the unfermented pulp of *Ziziphus mauritiana* fruit. The two gram positive species were identified as *Lactobacillus plantarum* and *Streptococcus thermophilus*, whilst the gram negative could not be identified. The two species (*Lactobacillus plantarum* and *Streptococcus thermophilus*) were also isolated from the fermented pulp of the *Ziziphus mauritiana* fruit. These species gave a negative test with hydrogen peroxide, and this confirms that they are *Lactobacillus* species as *Lactobacillus* species are typically recognised as catalase-negative (Tajabadi *et al.,* 2013).

Lactobacillus plantarum was also isolated from both the unfermented and fermented pulps of the *Uapaca kirkiana* fruit. *Lactobacillus plantarum* is considered a typical plant bacterium found in most plants — it has been isolated from silage, sauerkraut, pickled vegetables and sourdough, cow dung, dairy products and related environments as well as meat and fish (Corsetti *et al.,* 2016).

CONCLUSION

The yeasts responsible for the fermentation of *Ziziphus mauritiana* were identified as *Pichia fabianii, Sacchromyces cerevisae* and *Candida tropicalis.* The yeast responsible for the fermentation of *Uapaca kirkiana* fruit pulp to produce an alcoholic beverage was identified as *Candida krusei.* Lactic acid bacteria responsible for the fermentation of *Ziziphus mauritiana* were identified as *Lactobacillus plantarum* and *Streptococcus thermophilus*. Lactic acid bacteria from *Uapaca kirkiana* were identified as *Lactobacillus plantarum*.

The isolated yeast strains from both fruits are potential starter cultures for the production of alcoholic beverages from *Ziziphus mauritiana* and *Uapaca kirkiana* at industrial scale. The use of these isolates as starter cultures for alcoholic fermentation can lead to commercial production of beverages of consistent quality.

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