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Selection of Starter from Decaying Oranges and Pineapple for Bioethanol Production

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Authors' contributions

This work was carried out in collaboration among all authors. Author OJB wrote the first draft of the manuscript and managed the literature searches. Author ASM designed the study, performed the statistical analysis and wrote the protocol. Authors OJB and ASM carried out the practical aspect of the research. Author AFT and OSE managed the analyses of the study and were involved in writing the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Screening of yeasts for bioethanol production is an important aspect of industrial microbiology and biotechnology. Optimal yeast nutrition also requires availability of nutrients allowing for growth and ethanol production and ability to withstand the increasing concentration of the medium during fermentation. This study seeks to use appropriate methods to isolate, characterize and identify yeasts isolates with essential attributes for bioethanol production. Microbiological and Physicochemical assessment of the isolated strains was carried out on yeast maintenance media

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for the determination of ethanol, temperature and salt tolerance ability, growth at different pH and temperature, chloramphenicol and nalidixic acid test, determination of killer toxins production capacity of yeast, nitrate and carbon assimilation and sugar fermentation tests were carried out to select the best starter for production.
Fifteen yeasts were isolated belonging to the genera *Saccharomyce*s, *Candid*a, *Rhodotorula*, *Kluvyreomyce*s, *Trichosporon*, *Pichia*. *S. cerevisae* and *K. marxianus* showed efficient physico-chemical attributes. Proximate analysis of fruits juice showed a moisture content between 81.83 – 86.37%, crude protein 1.33 – 2.00%, Ash 0.73 – 0.83%, carbohydrate 11.87 – 15.67% and Ether 0.1 – 0.5%. The total sugar ranged from 1.83 – 13.17, equal mixture of oranges and pineapple juice showed a sugar content of 15 which reduced during fermentation while the alcohol content increased. Organisms were able to tolerate a percentage of 4-30% ethanol content, tolerating 15% sodium chloride which is an index of osmotolerance. At higher concentration, growth reduced, organisms produced catalase, reduced nitrate and showed a variation in utilization of different sugars.
At the end of the screening, *S. cerevisiae and K. marxianus* showed the best attribute essential for bioethanol production and were chosen as starters. The ability of the selected yeast isolates to produce Killer toxins against E. *coli* showed a negative result. Antibiotic sensitivity test was carried out on the selected yeast isolates using chloramphenicol and nalidixic and the two organisms were resistant to the antibiotics tested.
The ability of the organisms to grow in changing environmental conditions and ethanol tolerance are attributes essential for production while *Saccharomyces cerevisiae* showed the highest attribute followed by *Kluveromyce*s *marxinus*. This study concluded that *Saccharomyces cerevisiae* can be employed as starter in the industry for the production of bioethanol and in the conversion of agricultural waste to wealth.

Keywords: S. cerevisiae; starter screening; ethanol-tolerant yeast; nutrient availability; fermentation.

1. INTRODUCTION

The world has been facing a major crisis in recent times due to the problems associated with the production of hydrocarbon products. This is a major concern because of the world economy that is highly dependent on fossil fuel which is exhausted very quickly to meet the continuous energy demand. The excessive consumption of these fossil fuel most especially in urban area have resulted in high level of pollution particularly the emission of greenhouses gases that has adversely affect the environment. The increase in the level of greenhouse gases namely CO_2 in the earth's atmosphere is responsible for global warming [1,2]. The independence in energy source needs measures for creating and utilizing such renewable resources.

Many countries are therefore actively seeking to replace petroleum, coal and gas with renewable energy sources, including the mandatory use of oil fuel-biofuel mixtures, in order to develop a greener global energy market [3].

Most developed and some developing countries in the world are already in the progress of using various renewable materials as energy sources such as trees, crops, agricultural and forestry wastes. One of such renewable biomass that can serve as an alternative to petroleum-based fuel is biofuel. Biofuel are referred to as liquid or gaseous fuels for the transport sector that are predominantly produced from biomass [1]. Biofuel can be bioethanol, biodiesel and biogas. Among biofuels, bioethanol is the largest biotechnological product and the most dominant biofuel globally [4]. Specifically, the United States is the world's largest ethanol producer (60.9 billion liters (bL)), followed by Brazil (30.1 bL), European Union (5.4 bL), China (4.5 bL), Canada (1.8 bL), Thailand (1.5 bL), India (1.2 bL), and Argentina (1.1 bL). The rest of the bioethanol-producing countries account a total production of 2.1 bL (Eliodório et al., 2023).

Traditionally, bioethanol production is usually accomplished by microbial conversion of carbohydrates present in agricultural products [5]. As few yeast strains have been found to possess appreciable characteristics for ethanol production, there is a dire need to explore the potential of indigenous strains of yeasts to meet the national requirements for bio-fuel (Qureshi et al., 2007). Yeasts, being sugar-loving microorganism have been isolated from sugar-rich materials. One of such yeast is *Saccharomyces cerevisiae*.

This is because *S. cerevisiae* exhibits a wide intraspecific variation, only the most adapted strains for the industrial setup should be selected [6,7]. The selection process depends on certain characteristics of the fermentation process; it considers not only ethanol yields displayed by the yeast strains but also characteristics that allow the survival of strains under stress conditions. The dominance (abundance of a strain compared with others) and persistence (presence of the yeast strains throughout the season) of yeast strains depend on a series of features, such as kinetic parameters of yield, productivity, specific growth rate and resistance to different types of stress [8].

Fast and reliable measurements of relevant yeast parameters during fermentation, including concentration, viability, growth rate, cell size distribution, and yeast size with respect to the number of forming cells, would be of practical importance for a better understanding and optimizing fermentative processes [9,10].

Fruits contain high sugar concentration and hence yeast species are naturally present on these and can be easily isolated from fruits. In Nigeria, fruits waste such as decaying oranges and pineapple that have been discarded as a result of their imperfections cause environmental pollution which have affected the health of humans and animals [11]. In Nigeria, decaying fruits always constitute a major environmental pollution before, during and after the harvesting seasons. Post-harvest microorganisms can be divided into those that penetrate the produce on-farm, but develop in their tissues only after harvest, during storage or marketing while there are those that initiate penetration and colonization during or after harvest. Enormous postharvest losses have been attributed to fungal deteriorations [11]. The storage facilities in Nigeria are limited to take care of the large amount of fruits that are harvested yearly because they are highly perishable owing to their high moisture content and environmental conditions that favours microbial growth. Hence the need to turn waste into wealth and reduce spoilage as much as possible. The conversion to bioethanol is a step in the right direction.

Bioethanol, a renewable fuel derived from organic materials such as corn, sugarcane, or other biomass, has a different impact on greenhouse gases compared to the combustion of fossil fuels. Bioethanol is considered a renewable fuel because it is produced from plant materials. While burning bioethanol releases CO_2, the plants used to produce it absorb CO_2 during their growth, creating a carbon cycle that is theoretically carbon-neutral. Bioethanol is renewable as it is derived from crops that can be replanted and grown sustainably. This contrasts with the finite nature of fossil fuels. Bioethanol combustion generally produces fewer pollutants than fossil fuels, contributing to improved air quality. The production of bioethanol involves agricultural practices and land use. Sustainable and responsible land management is crucial to minimize potential negative impacts on ecosystems and food production [12] (EPA Guide, 2023).

Thus, to avoid the environmental pollution due to the decomposition of waste and emission of dangerous gases in the environment, it is necessary to isolate indigenous yeast strains with the required attributes that can be used as a starter in the fermentation industry for bioethanol production. This study aims at achieving this with minimal cost.

1.1 Collection of Samples

Decaying oranges and pineapple waste were collected from different locations and markets in Ile-Ife and its environment. It was collected into sterile Ziplocs material and was transported immediately to the laboratory for microbiological analysis.

1.2 Study Location

The study area were the dumpsites within local markets in Ile-Ife which include Sabo (7º 29′39.33 ″ N 4º 33′ 15. 70 ″ E), Central Market (7º 31′ 02.59 ″ N 4º 30′ 30. 59 ″ E), Mayfair (7º 29′ 27.89 ″ N 4º 32′ 03. 33 ″ E), Akinola (7º 30′ 01.34 ″ N 4º 26′ 17. 27 ″ E),) as well as with decaying fruits dumpsite within Obafemi Awolowo University Staff Quarters.

1.3 Isolation and Screening of Ethanol-tolerant Yeasts

Some pieces of decayed oranges and pineapples were taken and crushed into fine paste. One (1gm) of the sample mixture was serially diluted 10-fold in Maximum Recovery Diluent (MRD) which make up of 0.1 g of peptone and 0.85 g of NaCl in 100 ml of water. Aliquot (100 µl) of appropriately diluted sample was inoculated into Yeast Maintenance Media (YMM) using spread plate method [13]. The

YMM plates were incubated aerobically in an incubator (DSI300D) at 30 °C for 3 days. Single colony formed was picked and the cells were observed under microscope.

1.4 Maintenance of Culture

The culture of yeasts were maintained by sub-culturing on slants using YMM, incubating for 48 hours at 30ºC and thereafter storing in a refrigerator at 4ºC for future use.

1.5 Morphology and Biochemical Characterization of Yeast Isolates

1.5.1 Macroscopic morphology

According to the method of Kreger-van Rij [13] and Kurtzman and Fell [14], the morphology of the vegetative cells of yeast was studied in liquid and on solid media.

1.5.2 Growth on solid medium

In the present study, morphology of cells of the selected isolates and their appearance on YEPD agar media was examined. Fifteen (15 mL) of sterile medium autoclaved at 121 °C and 15 psi was poured into Petri dish and cooled. After cooling, the plates were inoculated by streaking 48 hours old yeast strain and incubated at 30 °C for 48 hours. The different features of the appearance of cultures were recorded; texture, colour and surface of colonies [14].

1.5.3 Growth in liquid medium

Selected isolates were cultured in YPD liquid medium to examine the growth in liquid medium. The medium was fifteen (15 mL) portion of YPD distributed into several McCartney tubes and autoclaved at 121°C and 15 psi and cooled. They were inoculated with half loopful of 48 hours old selected yeast strain and incubated at 30°C for 3 days. After incubation, the cultures were examined for growth visually on the surface of YPD liquid medium and the shape of the cells observed by compound microscope (Olympus, Japan).

1.6 Microscopic Morphology

1.6.1 Direct mount

Direct mount was used to study the yeast morphology microscopically and to determine the purity of the isolates. Wet mount of the yeast isolates was prepared by suspending a portion of 18-24 hours old culture in a drop of distilled water on a microscope slide and covered with cover slip. The preparation was observed with low power objective [15].

1.6.2 Lactophenol mount

This was carried out as described by Fawole and Oso (2007). A thin smear was prepared by taking a speck of the isolate from a 24 hours old culture of the test isolate and emulsified on a clean slide which was mounted in a drop of lactophenol in cotton blue. A cover slip was placed on the slide and observed under the microscope.

1.6.3 Confirmation of the identities of the yeast isolates

The well-established method for manual microorganism identification to the species level, BioMérieux's API identification products test were used to confirm the identities of the different yeast strains. The system offers a large and robust database which is accessible through the Internet-based test. The API test kits for yeasts was API 20C AUX and was used to confirm the identities of the yeast isolates. API strips give accurate identifications based on extensive databases and are standardized, easy-to-use test systems. The kits include strips that contain up to 20 miniature biochemical tests. Set up of the strips is quick, safe and easy to perform. APIWEBTM is a user-friendly website containing all of the API databases for a reliable automated interpretation of API strip results. APIWEB is easy to use key in the biochemical or numerical profile of the strip to obtain the organism identification (https://apiweb.biomerieux.com).

1.7 Physicochemical Characterization of the Isolate

1.7.1 Carbohydrate fermentation test

Tryptone broth was used as a basal medium for the fermentation tests. The ability of the Yeast to use sugar is an important factor for their growth and alcohol production. Yeast fermentation broth media was used for identification and the ability of the yeasts to ferment specific carbohydrates. 0.01% bromocresol purple was used as indicator. Fermentation tubes with 9 mL of basal medium provided with indicator were made as well as 1 mL of 1% sugar was taken in each tube. One Durham tube was introduced in each of the

fermentation tube before sterilization of basal medium. Then the medium was sterilized in autoclave at 121°C and 15 lb/inch^2 for 20 min. The tubes were then inoculated in duplicate with fresh culture of the yeast isolate and incubated at 30 °C for 48 hours. Ability to ferment ten different carbohydrates was examined anaerobically. Capability of fermentation was assessed by checking for gas production (CO_2) in the Durham tubes. The colour change of the fermentation media was a change from deep purple to yellow colour due to the formation of acids and gas [16]. In this study, the fermentation tests of the following carbohydrates and sugar alcohol were observed: glucose, mannose, xylose, sucrose, maltose, lactose, raffinose, mannitol, galactose and meliobiose.

1.7.2 Carbon assimilation

About 5 mL sterile basal medium (yeast nitrogen base) and 1ml of the different carbon source (glucose, maltose, mannitol, sucrose, galactose, melibiose, xylose, lactose, trehalose, at 2% concentration; raffinose at 4% concentration) was inoculated with 0.1 mL of yeast cells suspension made by suspending the growth of 24-48 hours old malt extract agar culture in about 5 mL of sterile distilled water in test tubes. The test tubes were viewed against a black line (approximately 3/4 mm wide) drawn on a white cardboard till it was visible as dark bands. Inoculated basal medium without a carbon source for each isolate served as control. The experimental and control tubes were incubated at 30ºC for four weeks during which period the tubes were observed weekly for amount of growth and pellicle formation [15].

1.7.3 Thermotolerance test

YPD liquid medium was used for detecting thermotolerance and growth in liquid media of selected yeast iisolates. Approximately 10 mL portion of the medium was distributed into McCartney tubes and the medium was autoclaved at 121 °C and 15 psi and cooled. The medium was inoculated with half loopful of 48 hours oldof the selected yeast strains. The initial optical density of each tube was recorded on spectrophotometer at 600 nm against the medium as blank. All cultures were incubated at 25°C, 30°C, 32°C, 37°C, 40°C and 44°C for 3 days and the thermotolerance abilities of the yeast strain determined. The increase in optical density in a tube was recorded as evidence of growth [17].

1.7.4 Ethanol tolerance test

The medium for the detection of ethanol tolerance yeast was modified YPD liquid medium. Each McCartney bottle contained 15 mL of YPD liquid medium with appropriate concentration of ethanol while blank media was used as a control. The medium was sterilized at 121 °C for 15 min in an autoclave and cooled. One mL (1 mL) of various concentrations of absolute ethanol was added from 5 to 30% (v/v), containing 5%, 10%, 15%, 20%, 25%, and 30% of absolute ethanol. Then each was inoculated with half loopful of yeast cell and the initial optical density was measured at 600 nm and incubated at 30 °C for 48 hours. After 48 hours, cell density was further recorded. The increase in optical density in a flask was recorded as evidence of growth. The concentration of alcohol at which the growth of yeasts was just inhibited was assessed as the ethanol tolerance ability of the yeasts.

1.7.5 Growth at different pH in liquid media

YEPD liquid medium was used for detecting the ability to grow at different pH. The medium was autoclaved at 121 °C and 15 psi and cooled. YEPD broth was prepared at pH 2-10. Each McCartney contained 15 mL of YEPD media with different pH and blank media was used as a control. Then each was inoculated by half loopful of yeast cell and measured the initial optical density at 600 nm and incubated at 30°C for 48 hours. After 48 hours, cell density was further recorded at 600 nm for growth.

1.7.6 Osmotolerence test

YEPD broth was prepared containing 6%, 9%, 12%, 15%, 18% and 20% NaCl. Each McCartney bottle contained 15 mL of YEPD liquid media with appropriate concentration of salt and blank media was used as a control. Then each was inoculated by half loopful of Yeast cell and measured the initial optical density at 600 nm and incubated at 30°C for 48 hours. After 48 hours cell density was further recorded at 600 nm [17].

1.7.7 Chloramphenicol and nalidixic acid resistance test

Sensitivity to Chloramphenicol and nalidixic acid were evaluated by growing isolates in MEA in the presence of 30 µg/ml discs. In this study YPD agar medium was used for detecting yeasts for

chloramphenicol and nalidixic acid resistance. Chloramphenicol and nalidixic acid disc (30 µ/L) were placed into the center of the already inoculated Petri dish. Then the plate kept at 30°C for growing. The zone of inhibition by the disc was recorded as an evidence of chloramphenicol and nalidixic acid sensitivity.

1.7.8 Determination of killer toxin production capacity of yeasts

First the target bacteria (*Escherichia coli*) were inoculated into Nutrient broth for 24 hours. Ten millilitre (10 mL) Molten Agar (3%) were added to already inoculated Nutrient broth. Media was poured on plate and left to solidify. The selected Yeast isolate were streaked on plate in 2 to 3 rows and it was incubated at 25 °C for 24 hours. Thereafter, clear zone of Inhibition was observed.

1.7.9 Nitrate reduction test

Sterile nitrate peptone water medium in test tubes containing inverted Durham tubes was inoculated with a loopful of 18-24 hours old broth culture of isolates. Inoculated tubes were incubated at 35±2 ºC for 5 days with sterile uninoculated control. Reduction of nitrate by the organisms in the medium was detected by adding to each tube after incubation Griess-Ilosvay's reagents (0.5 mL of 1% Sulphanilic acid in 5 N acetic acid followed by 0.5 mL of 0.6% of dimethyl-α- Naphthylamine in 5 mL acetic acid). The development of a red colouration within few minutes indicated the presence of nitrite produced from the reduction of nitrate. Negative results in tubes showing no colouration were confirmed by the addition of zinc dust. The development of red colouration on the addition of zinc dust indicates the presence of nitrate, thus no reduction had taken place. The presence of gas in Durham tubes indicated the production of Nitrogen.

1.7.10 Growth in 0.1% actinidine

YPD liquid medium was used for detecting yeasts ability to grow in 0.1% actinidine. Each test tubes contained 5 mL of YPD liquid media with 0.1% actinidine and blank media was used as a control. The medium was sterilized at 121°C for 15 min in an autoclave and cooled. Actinidine (0.1%) was aseptically added to the test tubes containing different organisms. Then each test tubes incubated at 30 °C for 48 hours. The presence of turbidity of medium indicated a positive test.

1.7.11 Determination of proximate composition of the fruits juice

The proximate composition and chemical characteristics of wholesome and decaying oranges and pineapple was measured for moisture content, protein (N × 6.25), crude fiber, fat, ash and carbohydrate. Total crude fiber was determined using the methodology described by Kirk and Sawyerr (1990). The moisture content, ash, fat protein, and crude fiber content of the wholesome and decaying oranges and pineapple was determined according to standard methods [18]. Total carbohydrate was determined by the difference according to Kirk and Sawyerr (1990).

2. RESULTS

In this study, a total number of fifteen (15) yeast isolates were isolated from the decaying oranges and pineapple. The culture was identified as yeast based on colony morphology (Table 1), microscopic examination and budding formation. Yeast isolates formed butyrous and smooth white raised colonies on YEPDA medium. The budding stage of the yeast isolates was observed under (40X) microscope and confirmed to be yeast with API kit for yeast identification. After 3 days of incubation at 30°C, heavy, dry climbing pellicles were formed on the surface of YEPD broth medium.

In this study, yeast isolates showed variation in terms of utilization of ten different sugars (Table 2). The selected strains for production namely *S. cerevisiae* was able to utilized Glucose, sucrose, maltose, meliobiose, galactose, mannose and fructose, xylose and trehalose but failed to grow on lactose, mannitol and raffinose, maltose and meliobiose.

K. marxianus on the other hand was able to utilize seven sugars but was unable to grow on raffinose, Changes in physicochemical assessment of the isolates is described in Table 3 Some organisms can successfully tolerate up to 15% sodium chloride salt in the media and this is an index of osmotolerance. However, at higher concentration, growth reduced. Some organisms can tolerate up to 20% absolute ethanol in the media and this is an index of ethanol tolerance. However, at higher concentration growth reduced (Table 4). Five of the organisms that showed highest ethanol tolerance value were then selected to test their ability to grow at different temperature and pH.

Table 1. Morphological characterization of yeast associated with decaying oranges and pineapple

Isolate code	Colour	Shape	Size	Type of Edge	Elevation	Surface	Opacity	Gram's Reaction
1	White	Circular	Small	Tentate	Raised	Rough	Opaque	+
2	Yellow	Circular	Big	Lobate	Flat	Smooth and Shiny	Transparent	+
3	Pink	Circular	Big	Entire	Raised	Smooth	Opaque	+
4	Cream	Circular	Small	Lobate	Raised	Smooth and Shiny	Transparent	+
5	White	Circular	Small	Entire	Flat	Smooth	Translucent	+
6	White	Circular	Small	Entire	Raised	Smooth	Opaque	+
7	Cream	Spherical	Big	Tentate	Flat	Dull	Opaque	+
8	Cream	Circular	Big	Tentate	Flat	Dull	Translucent	+
9	Cream	Circular	Big	Lobate	Flat	Smooth	Transparent	+
10	White	Circular	Small	Lobate	Flat	Smooth	Opaque	+
11	Cream	Circular	Medium	Entire	Raised	Smooth	Translucent	+
12	Off-white	Oval	Small	Entire	Slightly Raised	Smooth	Opaque	+
13	White	Circular	Small	Entire	Flat	Smooth and shiny	Transparent	+
14	Cream	Circular	Medium	Tentate	Flat	Smooth	Opaque	+
15	Yellow	Circular	Big	Lobate	Raised	Rough and dull	Opaque	+

Table 2. Biochemical characteristic of yeast associated with decaying oranges and pineapple

Isolate code	Glucose	Sucrose	Xylose	Lactose	Mannitol	Raffinose	Maltose	Meliobiose	Mannose	Galactose	Growth in 0.1% Actinidine	Nitrate Reduction Test	API Confirmatory	
													Test	Probable identity of isolate
1	++	++	++	++	++	-	++	+	+	++	+	+	+	*Trichosporon asahii*
2	++	++	++	++	++	+	+	+	+	+	+	+	+	*Trichosporon aesteroides*
3	++	++	++	++	++	-	-	-	+	+	+	+	+	*Rhodotorula mucilaginosa*
4	+	+	+	-	-	-	-	+	+	+	+	+	+	*Pichia meri*
5	++	+	++	++	+	+	-	+	+	+	+	+	+	*Trichosporon mucoides*
6	++	++	+	-	++	-	-	-	++	++	+	+	+	*Candida fructus*
7	+	+	+	++	-	-	++	+	+	++	+	+	+	*Trichosporon cutaneum*
8	+	++	++	++	++	+	+	+	+	-	+	+	+	*Candida albican*
9	-	-	++	-	-	-	-	-	+	-	+	+	+	*Candida catemulata*
10	+	+	+	-	-	-	-	-	+	+	+	+	+	*Candida parapsilosi*
11	++	++	++	++	++	-	-	-	+	+	+	+	+	*Kluyveromyces marxianus*
12	++	+	++	-	-	-	+	+	++	+	+	+	+	*Saccharomyces cerevisiae*
13	++	++	++	-	++	-	-	-	-	-	+	+	+	*Candida albican*
14	+	-	+	-	-	-	-	-	-	-	+	+	+	*Kluyveromyces fragilis*
15	-	-	-	-	-	-	-	-	-	-	+	+	+	*Candida valida*

KEY: ++ Positive and can produce gas, + positive and cannot produce gas, - Negative

Table 3. Osmotolerance test salt concentration (g)/ growth (optical density)

Isolate code	6	9	12	15	18	20
1	0.647±0.67 [a]	0.941±0.91[a]	0.628±0.93 [a]	0.628±0.61 [a]	0.789±0.85 [a]	0.644±0.79 [a]
3	0.245±0.67 [a]	0.351±0.91[a]	0.689±0.93 [a]	0.689±0.61 [a]	0.869±0.85 [a]	0.006±0.79 [a]
4	0.729±0.67 [a]	0.342±0.91[a]	0.427±0.93 [a]	0.427±0.61[a]	0.819±0.85 [a]	0.045±0.79 [a]
7	0.890±0.67 [a]	0.864±0.91 [a]	0.636±0.93 [a]	0.636±0.61 [a]	0.689±0.85 [a]	0.125±0.79 [a]
10	0.237±0.67 [a]	0.277±0.91 [a]	0.253±0.93 [a]	0.253±0.61 [a]	0.023±0.85 [a]	0.486±0.79 [a]
11	0.118±0.67 [a]	0.283±0.91 [a]	0.141±0.93 [a]	0.141±0.61 [a]	0.203±0.85 [a]	0.146±0.79 [a]
13	0.167±0.67 [a]	0.169±0.91 [a]	0.100±0.93 [a]	0.10±0.61 [a]	0.142±0.85 [a]	0.849±0.79 [a]
15	0.417±0.67 [a]	0.439±0.91 [a]	0.338±0.93 [a]	0.338±0.61 [a]	0.440±0.85 [a]	0.012±0.79 [a]
17	0.623±0.67 [a]	0.790±0.91 [a]	0.450±0.93 [a]	0.450±0.61 [a]	0.470±0.85 [a]	0.758±0.79 [a]
19	0.502±0.67 [a]	0.520±0.91 [a]	0.250±0.93 [a]	0.250±0.61 [a]	0.825±0.85 [a]	0.237±0.79 [a]
24	0.388±0.67 [a]	0.381±0.91 [a]	0.692±0.93 [a]	0.692±0.61 [a]	0.468±0.85 [a]	0.398±0.79 [a]
25	0.219±0.67 [a]	1.278±0.91 [a]	0.108±0.93 [a]	0.108±0.61 [a]	0.808±0.85 [a]	0.664±0.79 [a]
26	0.597±0.67 [a]	0.851±0.91 [a]	0.137±0.93 [a]	0.137±0.61 [a]	0.808±0.85 [a]	0.527±0.79 [a]
34	0.082±0.67 [a]	0.073±0.91 [a]	0.006±0.93 [a]	0.006±0.61 [a]	0.053±0.85 [a]	0.013±0.79 [a]
35	0.077±0.67 [a]	0.097±0.91 [a]	0.169±0.93 [a]	0.169±0.61 [a]	0.033±0.85 [a]	0.001±0.79 [a]

Values are the means ± standard deviation of determinations on three replicate growths. Means with different superscript within Rows are not significantly different at p<0.05.

Table 4. Ethanol tolerance test

ETHANOL CONCENTRATION	Ethanol (v/v)/ growth (O.D)					
ISOLATE CODE	**5**	**10**	**15**	**20**	**25**	**30**
1	0.919±0.8^{c}	1.075±2.7$^{b, c}$	0.960±0.7$^{b, c}$	1.018±0.8^{b}	0.373±0.4^{a}	0.265±0.3^{a}
3	0.938±0.8^{c}	0.774±2.7$^{b, c}$	0.663±0.7$^{b, c}$	0.608±0.8^{b}	0.480±0.4^{a}	0.499±0.3^{a}
4	1.068±0.8^{c}	0.909±2.7$^{b, c}$	0.645±0.7$^{b, c}$	0.836±0.8^{b}	0.176±0.4^{a}	0.242±0.3^{a}
7	0.89±0.8^{c}	0.853±2.7$^{b, c}$	1.283±0.7$^{b, c}$	0.241±0.8^{b}	0.379±0.4^{a}	0.266±0.3^{a}
10	0.895±0.8^{c}	0.452±2.7$^{b, c}$	0.729±0.7$^{b, c}$	0.791±0.8^{b}	0.045±0.4^{a}	0.179±0.3^{a}
11	1.314±0.8^{c}	0.346±2.7$^{b, c}$	0.462±0.7$^{b, c}$	0.389±0.8^{b}	0.387±0.4^{a}	0.038±0.3^{a}
13	1.747±0.8^{c}	0.837±2.7$^{b, c}$	0.974±0.7$^{b, c}$	0.619±0.8^{b}	0.253±0.4^{a}	0.204±0.3^{a}
15	1.482±0.8^{c}	0.826±2.7$^{b, c}$	0.977±0.7$^{b, c}$	0.699±0.8^{b}	0.129±0.4^{a}	0.281±0.3^{a}
17	0.727±0.8^{c}	1.302±2.7$^{b, c}$	0.99±0.7$^{b, c}$	1.32±0.8^{b}	0.237±0.4^{a}	0.207±0.3^{a}
19	1.183±0.8^{c}	0.964±2.7$^{b, c}$	0.962±0.7$^{b, c}$	1.063±0.8^{b}	0.069±0.4^{a}	0.119±0.3^{a}
24	1.293±0.8^{c}	0.530±2.7$^{b, c}$	1.06±0.7$^{b, c}$	0.552±0.8^{b}	0.452±0.4^{a}	0.045±0.3^{a}
25	1.009±0.8^{c}	0.633±2.7$^{b, c}$	0.305±0.7$^{b, c}$	0.368±0.8^{b}	0.139±0.4^{a}	0.302±0.3^{a}
26	1.141±0.8^{c}	0.583±2.7$^{b, c}$	0.35±0.7$^{b, c}$	0.356±0.8^{b}	0.178±0.4^{a}	0.023±0.3^{a}
34	1.447±0.8^{c}	0.308±2.7$^{b, c}$	0.777±0.7$^{b, c}$	0.746±0.8^{b}	0.192±0.4^{a}	0.058±0.3^{a}
35	1.717±0.8^{c}	1.093±2.7$^{b, c}$	1.211±0.7$^{b, c}$	0.184±0.8^{b}	0.141±0.4^{a}	0.057±0.3^{a}

Values are the means ± standard deviation of determinations on three replicate growths. Means with different superscript within Rows are significantly different at p<0.05

Table 5. Growth at different pH

pH/Growth	1	4	7	17	19
2	0.207±16[a]	0.361±16 [a]	0.392±16 [a]	0.132±16 [a]	1.02±16 [a]
3	0.397±17 [a]	0.262±17 [a]	0.024±17 [a]	0.488±17 [a]	1.022±17 [a]
4	1.001±03[b]	0.821±03[b]	0.843±03[b]	0.84±03[b]	0.932±03[b]
5	1.104±11[a, b]	0.497±11[a, b]	0.937±11[a, b]	0.688±11[a, b]	0.901±11[a, b]
6	0.825±20[a, b]	0.133±20[a, b]	0.708±20[a, b]	1.168±20[a, b]	1.258±20[a, b]
7	0.825±17[a, b]	0.288±17[a, b]	0.672±17[a, b]	1.169±17[a, b]	1.236±17[a, b]
8	0.623±09[a, b]	0.612±09[a, b]	0.916±09[a, b]	1.04±09[a, b]	0.965±09[a, b]
9	0.605±07[a, b]	0.622±07[a, b]	0.666±07[a, b]	0.997±07[a, b]	0.796±07[a, b]
10	0.466±11[a, b]	0.962±11[a, b]	0.957±11[a, b]	0.929±11[a, b]	0.576±11[a, b]

Values are the means ± standard deviation of determinations on three replicate growths. Means with different superscript within Rows are significantly different at p<0.05.

Table 6. Growth at Different Temperature
Temperature (ºC)/ growth (O.D)

ISOLATE CODE	25	30	32	37	40	44
1	1.043±11[b]	1.136±15[b]	1.198±11[b]	0.474±12[a, b]	0.277±19[a, b]	0.278±12[a]
4	1.031±11[b]	0.360±15[b]	0.598±11[b]	0.487±12[a, b]	0.786±19[a, b]	0.375±12[a]
7	1.487±11[b]	1.006±15[b]	1.175±11[b]	0.701±12[a, b]	0.431±19[a, b]	0.206±12[a]
17	0.953±11 [b]	1.129±15[b]	1.147±11[b]	0.947±12[a, b]	0.858±19[a, b]	0.380±12[a]
19	0.843±11[b]	1.143±15[b]	1.123±11[b]	1.049±12[a, b]	1.368±19[a, b]	0.885±12[a]

Values are the means ± standard deviation of determinations on three replicate growths. Means with different superscript within Rows are significantly different at p<0.05

Table 7. Proximate analysis of oranges and pineapple

SC	MC %	Protein%	EE%	Ash%	CF%	Carbohydrates%	pH	TTA (T/10)	TS %	TRS %
1	86.37±0.21^{a}	0.80±0.1^{f}	0.10±1.67^{f}	0.77±0.06^{b}	0.10±1.7^{a}	11.87±0.06	3.6±0.00	0.83±0.0^{b}	1.83±0.15^{a}	0.13±0.06^{a}
2	83.83±0.15^{c}	1.53±0.15^{c}	0.27±0.05^{d}	0.80±0.1^{a}	0.17±0.06^{b}	13.40±0.26^{b}	4.7±0.00	0.33±0.0^{b}	9.80±0.10^{b}	4.53±0.15^{a}
3	85.03±0.15^{b}	1.33±0.05^{d}	0.40±0.1^{b}	0.83±0.1^{a}	0.13±0.06^{b}	12.27±0.20^{d}	4.5±0.00	0.27±0.0^{b}	10.12±0.15^{a}	5.23±0.15^{a}
4	82.33±0.15^{e}	1.70±0.2^{b}	0.50±0.1^{a}	0.83±0.06^{b}	0.27±0.06^{b}	14.34±0.23^{c}	4.9±0.00	0.50±0.1^{a}	12.12±0.15^{a}	5.90±0.15^{a}
5	81.83±0.21^{f}	1.30±0.1^{e}	0.33±0.15^{d}	0.73±0.06^{b}	0.13±0.06^{b}	15.67±0.45^{a}	3.9±0.06^{b}	0.23±0.0^{b}	2.57±0.15^{a}	0.30±0.10^{b}
6	83.23±0.15^{d}	2.00±0.1^{a}	0.37±0.06^{c}	0.77±0.06^{b}	0.23±0.06^{b}	13.4±0.26^{b}	5.1±0.00	0.60±0.1^{a}	13.17±0.15^{a}	7.07±0.15^{a}

Values are the means ± standard deviation of determinations on three replicate fermentations. Means with different superscript within Rows are significantly different at p<0.05.

***PARAMETERS**: Moisture Content %; Protein%; Ether Extract (Fat) %; Ash %; Crude Fibre %; Carbohydrates (By Difference) %; pH; Total Titratable Acidity (T/10); Total Sugars %; Total Reducing Sugars %.* ***KEYS:** 1 = Decaying Oranges, 2 = Medium Oranges, 3 = Wholesome Oranges 4 = Pineapple and Oranges, 5 = Decaying Pineapple, 6 = Wholesome pineapple*

Table 8. Production of killer toxins by yeast isolates

Isolate's name	Killer Toxin Test
Trichosporon asahii	-
Trichosporon aesteroides	-
Rhodotorula mucilaginosa	-
Pichia meri	-
Trichosporon mucoides	-
Candida fructus	-
Trichosporon cutaneum	-
Candida albica	-
Candida catemulata	-
Candida parapsilosi	-
Kluyveromyces marxianus	-
Saccharomyces cerevisiae	-
Candida albica	-
Kluyveromyces fragilis	-
Candida valida	-

Table 9. Antibiotics sensitivity test

Isolate Name	Chloramphenicol
Trichosporon asahii	-
Trichosporon aesteroides	-
Rhodotorula mucilaginosa	-
Pichia meri	-
Trichosporon mucoides	-
Candida fructus	-
Trichosporon cutaneum	-
Candida albica	-
Candida catemulata	-
Candida parapsilosi	-
Kluyveromyces marxianus	-
Saccharomyces cerevisiae	-
Candida albica	-
Kluyveromyces fragilis	
Candida valida	-

The Yeast isolates were able to grow at 25-40°C. Little growth was observed at 44°C (Table 5). The selected yeast isolates grew at lower pH. The isolate were able to grow at pH 10. Maximum growth was seen at pH 6 (Table 6).

At the end of the screening, *S. cerevisiae and K. marxianus* showed a better attribute essential for bioethanol production and could be employed in the fermentation industry as a starter for bioethanol production.

The proximate analysis of the decaying orange and pineapple juice singly and in combination is shown in Table 7.

The ability of the selected yeast isolates to produce Killer toxins was carried out against *E. coli*, showed negative result i.e., (no clear zone of inhibition by the Yeast was observed). This is shown in Table 8.

Antibiotic resistance test was carried on the selected yeast isolates using chloramphenicol and nalidixic and the two organisms were resistant to the antibiotic mentioned above because there were no clear zones around the yeasts isolates and the result was recorded. This is shown in Table 9.

The ability of the selected yeast isolates to produce Killer toxins was carried out against *E. coli*, showed negative result i.e. (no clear zone of inhibition by the Yeast was observed).

This is shown in Table 8. Antibiotic sensitivity test was carried on the selected yeast isolates using chloramphenicol and nalidixic acid and the two organisms were resistant to the antibiotics.

3. DISCUSSION

In this study, a total number of fifteen (15) yeast isolates were isolated from the decaying oranges and pineapple. The organisms were identified as yeast based on colony morphology, microscopic examination and budding formation. Yeast isolates formed butyrous and smooth white raised colonies on YEPDA medium. The budding stage of the yeast isolates was observed under (40X) microscope and they were confirmed to be yeast. After 3 days of incubation at 30°C, heavy, dry climbing pellicles were formed on the surface of YEPD broth medium. This agrees with an earlier report by Meghana 2014). This also agrees with the work of Maria et al. (2022) which showed that the knowledge generated on the indigenous yeast populations in industrial fermentation processes of bioethanol producing distilleries allowed the selection of well adapted bioethanol- producing strains.

Isolates however showed variation in terms of utilization of ten different sugars. The selected strains for production namely *S. cerevisiae* was able to utilize Glucose, sucrose, maltose, meliobiose, galactose, mannose and fructose, xylose and trehalose but failed to grow on lactose, mannitol and raffinose. *K. marxianus* on the other hand was able to utilize seven sugars but was unable to grow on raffinose, maltose and meliobiose. This is contrary to the report of Meghana) (2012) who reported earlier that some species of microorganism select their carbon sources in which they grow. Thermotolerant strains reduce the costs involved in cooling the fermentation vessel, and the role of trehalose in thermotolerance has already been established [19].

Temperature is important to the growth of microorganisms. However, temperature below the optimum or above it may be detrimental to the organisms especially when it comes to production. Industrial microorganisms however act best at optimum temperature and pH which was observed in this study and corroborated by the work Mir naiman and Mohammed [20] (2014) and Patil et al. (2016).

Moreover, Anderson et al. (1986) and Ueno et al. (2003) reported that thermotolerant yeast can produce > 6% ethanol within 24 hours at 40 °C. The result of this study does agree with Armament et al. (2014) who reported some yeast isolate that can tolerate up 44 °C temperature. It is important to note that the results of the thermotolerance analysis for this study is very significant. These thermotolerant yeast could promote high yield of ethanol at high temperature. The use of high thermotolerant yeast in fermentation industry is highly important as its exhibit rapid metabolic activity and a high fermentation rate with high product output and minimized contamination [21,22]. However, the use of thermotolerant fermenting yeasts can improve its efficiency by allowing fermentation to occur at temperatures above 40°C using different technology. The screening of thermotolerant yeast strains capable of fermentation at high temperatures, which are deemed suitable for bioethanol production have been reported by authors like Choudhary et al. [21] and Li et al. 22].

Furthermore, the ethanol concentrations are the major influencing factors during the fermentation process. Ethanol generally inhibits growth and its toxic to cell. Increase in ethanol concentration during fermentation leads to a reduction in the fermentation process [23]. As there is a constant decrease in growth, ethanol tolerance of a strain is taken at a concentration of ethanol after which there is a sharp decline in growth. The ability of yeast to tolerate high concentration of ethanol is important from commercial point of view, because of the fact that production of higher levels of ethanol requires the strain to be able to tolerate higher concentration of ethanol in the medium. Due to the fact that the plasma membrane of unicellular organisms is in direct contact with the surrounding medium, it is likely that its characteristic will influence tolerance of cells to all kinds of changes occurring during fermentation. However, the physiological basis for ethanol tolerance in yeast remains obscure (Meghana et al., 2012).

In this study, all selected yeast strains showed maximum growth at 5% ethanol concentration. Some strains showed good growth at 10 and 15% concentration. Few are able to tolerate up to 20% absolute ethanol concentration. Sener et al. [24] reported that ethanol accumulation in fermenter inhibits specific growth rate, specific ethanol production rate, cell viability and substrate consumption. Tikka et al. (2013) also reported the tolerance of seven yeast strains isolated from fruits. In his study, maximum of 12% ethanol tolerance by one of the strain was reported. Mir naiman and Mohamme (2014) also reported two yeast strain of *S. cerevisiae* that can tolerate 14% ethanol. Nwachukwu et al. [20] reported the level of ethanol tolerance of 16%

(v/v) by *S. cerevisiae* isolated from raffia palm wine. It is important to note that the results obtained in the analysis of ethanol tolerance are highly significant as it forms the basis for the selection of effective strains for bioethanol production. In this study, two of the yeast strains namely *S. cerevisiae* and *K. marxianus* can tolerate 20% ethanol. This was supported by a recent study by Li et al. [22] who reported that a higher fermentation broth and ethanol concentration can greatly reduce the distillation cost and promote economic cost savings, especially the use of plant biomass would substantially produce potentially huge scale of bioethanol.

The utilization of sugar during fermentation is one of the important physiological features of yeast strains used for ethanol production in the industry as it influences the rate of production and the yield in addition to the physiological growth of yeast (Sathees et al., 2011, Meghana et al., 2012.). At high sugar concentration, osmotic pressure increases in the fermenting medium which can be inhibitory to many yeasts. In this study, yeasts were able to grow at 20% sodium chloride concentration. This agrees with Osho [25] who reported maximum of 20% sugar tolerance for *S. cerevisiae* BSOSU 0269. Mir naiman and Mohammed [20] and Kusumawadee [26] also reported similar result on yeast isolated from rotten fruits waste and soil sample for bioethanol production. The results of the analysis of salt concentration obtained in this study has no effect on the selection on the starters for bioethanol production.

In addition, it was reported by Mongi et al. [27] that the initial pH affected the levels of the alcohols production. It is worthy to note that in this study, the selected yeast isolate was able to grow in a wide pH range from 2 to 10, but *S. cerevisiae* and *K. marxianus* showed remarkable growth at pH 6. The results of the pH analysis for this study is significant.

Proximate analysis of fruits juice was carried out to determine the physical and chemical properties of the fruits juice. The wholesome and decaying oranges, wholesome and decaying pineapple and a mixture of the two were analyzed. The physical properties studied included pH, titratable acidity, total sugar and total reducing sugar with value ranges of 3.60 – 5.10, 0.23– 0.83%, 1.83 – 13.17% and 0.13 – 7.07%, respectively. Chemical composition of the juice blends and the reference sample showed moisture content with a range of 81.83 – 86.37%, crude protein 1.33 – 2.00%, Ash 0.73 – 0.83%, carbohydrate 11.87 – 15.67% and Ether 0.1 – 0.5% respectively. The total sugar ranged from 1.83 – 13.17 in which the total sugar for mixed oranges and pineapple juice is 12.12 which agree with the reports of El-Sheikha et al. [28] who recommended equal mixture of oranges and pineapple juice to be 12.15% in bioethanol production. The result of the proximate analysis showed a decrease in the sugar content of the decaying oranges and pineapple. This may be due to the fact that some organisms were already growing on them which resulted in spoilage and the depletion of nutrients; hence the need for addition of sugars. Sucrose was added (7.5g) to the mixed orange and pineapple juice to give a mixture with higher sugar concentration and this gave a level that will help to sustain the microorganisms throughout the period of fermentation so that the nutrient will not be used up completely before the termination of the experiment for a better and desirable alcoholic content.

S. cerevisiae produced better yield when used singly (92%) and also *K. marxianus* (40%). The work of Gabriela et al. (2023) showed that for a good yield in fuel ethanol production, genetically modified strains of the yeast is needed which produced a 11% higher ethanol production from sucrose by the modified industrial yeast compared to its parental strain.

There was however a reduction when they were used in combination (20%). This agrees with the work of Artur et al. [29] that shows that large scale and non-aseptic fermentation of sugarcane feedstocks into fuel ethanol in biorefineries represents a unique ecological niche, in which the yeast *Saccharomyces cerevisiae* is the dominant organism. They recorded a decrease in ethanol concentration which was referred to as enzyme “jamming” effects, similar to what was observed when using the combination of the exogenous enzymes and endoglucanase enzymes secreted by the yeast strain most likely exceeded a critical enzyme concentration, resulting in poor hydrolysis [30,29]. But this is contrary to an earlier report by workers such as Brooks [5]; Querol et al. [31]; Kusumawadee [29]; Sathees et al. (2017) and Sathees et al. [32,33-40].

This may be due to different enzymes produced by the yeasts and their enzymatic activities, as we have reported that the two yeasts strains did

not produce killer toxins against themselves during growth and production of bioethanol. The reason for this in this work cannot be ascertained as this is not the focus of this work (Adeyemo et al., 2023b In press) [41-50].

4. CONCLUSION

It was concluded from this study that yeast are endogenous organisms isolated from decaying fruit juice as the organisms were not from any other source outside. Growth and maintenance of cultures and even selection is usually a great task in industries. Industrial microorganisms must not be toxic or pathogenic, their genes must be easily manipulated which agree with the work of Ana et al. (2021) and must be able to undergo simple microbial processes in production of important end products. This we have been able to achieve in this research using simple, cheap and readily available raw materials for the isolation and maintenance of such industrially important microorganisms.

This study concluded that *Saccharomyces cerevisiae* can be employed as starter in the industry for the production of bioethanol and in the conversion of agricultural waste to wealth. Turning waste to wealth is an area that can be explored by providing solution to the problem of waste disposal management through prevention, recycling, minimization, reuse and conversion in our societies and globally. Efforts should be directed more into ways of waste minimization and turning waste to energy in the form of bio-ethanol and bio-fuel production. These have less environmental pollution and release of carbon dioxide gases which is responsible for the greenhouse effect and the retention of heat in the atmosphere is minimized. The use of biofuels should be encouraged.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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