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ISOLATION, CHARACTERIZATION OF ETHANOL-TOLERANT YEAST FROM DECAYING ORANGES AND PINEAPPLES FOR THE PRODUCTION OF BIOETHANOL: A WASTE MANAGEMENT OPTION

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ABSTRACT

The increase in population has led to a high demand for petroleum, a nonrenewable fossil fuel; excessive use of which results in global warming causing health and environmental problems. Therefore, there is a need for urgent replacement with renewable energy such as Bioethanol. The study focuses on isolating, identifying, and confirming ethanol-tolerant yeasts from decaying oranges and pineapples obtained from local markets within the Ibadan Metropolis. Standard morphological, biochemical, analytical profile index (API), and physicochemical tests were carried out on the yeasts and ethanol produced respectively. The fruits were crushed, juice extracted and yeasts isolated with Yeast Extract Agar (YEA) and screened for ethanol, NaCl, and antibiotics tolerance. The brix level, titratable acidity, pH, and specific gravity of the ethanol produced were determined during fermentation from day zero to fourteen. The yeasts isolated were *Candida valida*, *Pichia meri*, *Saccharomyces cerevisiae*, *Kluveromyces fragilis,* and *K. marxianus*. The pH and TTA were monitored from day 0 to day 14, a decrease in pH from 4.57 to 3.07 and an increase in TTA from 0.03 to 0.51 was observed as fermentation progressed. There was a significant reduction in reducing sugar level (brix) from 1.10 to 0.20; and specific gravity from 1.036 on day 0 to 1.005 on day 14. The lower the specific gravity, the higher the alcohol content. This means that the alcohol content increases as the specific gravity reduces. Moreover, the pH and the specific gravity reduced, and the ethanol content increased with fermentation time. This research concluded that decaying oranges and pineapples can serve as substrates for isolating of ethanol-tolerant yeasts and producing bioethanol, thus presenting a viable waste management solution.

Keywords: Decaying fruits, yeast, fermentation, bioethanol, renewable energy.

INTRODUCTION

Excessive use of fossil fuels has resulted in global warming and climate change. Therefore, there is a thrust towards replacing fossil fuels with cleaner and renewable fuels such as bioethanol and biodiesel. Moreover, due to the rapid consumption of conventional fossil fuels and their unpredictable changes in prices, there is an urgent need to develop an alternative renewable source of energy e.g., bioethanol for the national energy securities (Sharma, 2006).

A biofuel is a fuel generated from biomass rather than from the formation of the geological process of oil and fossil fuel. Biofuel is also called non-conventional fuel and fossil fuel, petroleum, coal, and natural gases are called conventional fuel. Bioethanol is the most common and widely used liquid biofuel in the world (Farrell *et al.,* 2006). It is particularly used as an alternative fuel to petrol. There is a lot of energy stored in lignocellulose (Mosier *et al.,* 2005) and it is important to find ways of making it easier to get the energy and extract it in the form of sugars that can be fermented to produce bioethanol and other products.

Zainab and Fakhra (2014) stated that biofuels are a superior alternative fuel solution that can address the energy crisis and reduce pollution issues. Since the need for bioethanol as a universal energy source has been increasing, bioethanol production must be increased using cheaper and eco-friendly raw materials. The process of creating bioethanol from renewable sources of lignocellulosic materials has the potential to decrease the world's increasing dependency on petroleum, mainly due to its ability to lower the net emissions of carbon dioxide, a significant greenhouse gas (Bušić *et al.*,

2018).

Lignocellulosic raw materials which include fruit and vegetable wastes, forestry waste, agro-residues, etc. can be used to produce bioethanol. As bioethanol is preferable to gasoline fuel in many ways, it is being researched extensively as a renewable fuel source. Compared to oil, ethanol produces energy that is less carbon-intensive and renewable. It is a biofuel made by biochemical processes from biomass (Babu *et al*., 2014).

Fruit wastes are rich in cellulose and hemicelluloses and have low lignin contents, mostly loosely placed between cellulose and hemicelluloses (Zheng *et al.,* 2009). This makes these wastes interesting raw materials for the production of bioethanol. The utilization of lignocellulosic raw materials, which are viewed as a renewable energy source, for the production of bioethanol could also contribute to the reduction of carbon dioxide emissions.

Fruit wastes are rich in sugars and carbohydrates which can be recovered and utilized for bioethanol production through fermentation by microorganisms such as fungi (yeasts and molds) and bacteria (Jahid *et al.,* 2018).

Yeasts have been isolated from a variety of natural sources like leaves, flowers, fruits, etc. (Tournas, 2005). It is typically isolated from sugar-rich materials because it is a sugar-loving microbe. Because fruits have significant sugar content, yeast species are naturally found there and are easily separated from them. The population of microflora on the substrates always depends on the pH of the substrate. Since fruits are acidic, they are predominantly inhabited by yeasts (Temudo *et al*., 2008).

This research work seeks to isolate and identify ethanol-tolerant yeasts associated with decaying oranges and pineapple for the production of bioethanol through the fermentation process using ethanol-tolerant yeasts isolated from fruit wastes.

MATERIALS AND METHODS

Collection of Samples

Decaying fruit (orange and pineapple) wastes were collected at various markets in Ibadan. It was collected into sterile Ziplocs material and was transported aseptically to the microbiology laboratory at First Technical University, Ibadan for microbiological analysis.

Sterilization of Materials

Unless otherwise stated, all glassware used for this study was sterilized in a hot air oven at 160° C for 2 h. The inoculating wire loop and needle were sterilized by flaming to red hot in a Bunsen burner flame. Materials such as microscope slides were sterilized with 70% v/v ethanol. Media were sterilized in an autoclave at 121° C for 15 min and prepared according to manufacturers' instructions (Mohapatra and Prabhakar, 2017).

Preparation of Sample for Inoculation

The decaying oranges and pineapples were dipped into 200 mL of distilled water for 10 min and rinsed to remove secondary contaminants. Some pieces of freshly infected area were taken using a sterile knife and crushed. One (1 gram) of the crushed samples was added to 9.0 mL of sterile maximum recovery diluent into test-tube and serial dilution of each was carried out (Ben-David and Davidson, 2014).

Preparation of Media

Yeast Extract Agar (YEA) was used as the sole source of nutrients for cell culturing. According to the manufacturer's directive, eight grams of the yeast extract powder and fifteen grams of agar were dissolved in 1 L of distilled water, it was homogenized and autoclaved at 121° C for 15 min, followed by dispersion of medium in sterile Petri dishes and were allowed to solidify before inoculation (Zarei *et al*., 2016).

Isolation of Yeasts

Using the spread plate method (Kreger-Van-Rij, 1984), appropriate dilution (0.1 mL) was plated separately on the solidified YEA plates. The plates were incubated aerobically in an incubator at 25° C for 2 days. Single and distinct colony was isolated in a pure form by sub-culturing on freshly

prepared culture agar plates incubated at 25° C for 48 h. Distinct colonies were picked for identification and further studies. Distinctive morphological properties of each pure culture on the agar plates were observed and counted (SFU/ml).

Preservation and Identification of Yeast Isolates

A single colony of representative isolates was purified following the dilution plating technique in yeast extract agar. Pure colonies were inoculated into agar slants in a sterile environment and stored in a refrigerator at 4° C to preserve and maintain the original morphology of the microorganisms for further studies (Bergey *et al*., 2005). Purified yeast isolates were identified through colonial, morphological, physiological, and biochemical tests following the methods described by Oyeleke (2009) and Tille and Forbes (2014).

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 was 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 kit 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. The setup 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 makes it easy to use the key in the biochemical or numerical profile of the strip to obtain the organism identification (https://apiweb.biomerieux.com).

Screening of Isolates for Ethanol Production

The yeast isolates were screened through growth at different concentrations of ethanol, NaCl concentration, pH range, temperature, and antibiotic susceptibility using tetracycline (Desai *et al*., 2012).

Extraction of Juice from Decaying Oranges and Pineapples

The oranges were peeled after washing in clean water to achieve surface sterilization. They were cut into half. Each half was squeezed by hand to extract the juice. The juice was poured through a strainer to remove any seeds or large pulp and it was pressed by a big spatula to release more juice. The pineapple was peeled, and the brown spots were removed. It was cut into smaller pieces and the hard core removed. It was blended in a laboratory blender until smooth, the blended pineapple was strained through a mesh strainer into a flask while pressing with a big spatula to squeeze out all the juice (Sharma *et al*., 2017).

Production of Bioethanol from Decaying Fruit Juice

Bioethanol production includes media preparation, fermentation of fruit juice for bioethanol production, and physicochemical analysis such as pH, Brix, and specific gravity (Amerine and Kunkee, 2005).

Fermentation media preparation

The fruit juice obtained from decaying oranges and pineapples was used as a fermentation media for the study. Sucrose was added (1gram) to 100 mL of the decaying fruit juice and mixed thoroughly in sterile bottles before the fermentation media was sterilized in an autoclave at 121° C for 15 min and cooled before inoculation (Dowe and McMillan, 2001).

Standardization of Yeast Cells

The McFarland Standard method was used to standardize the number of cells that were inoculated into the media. Yeast cell density of 0.5 McFarland standard which provided an optical density comparable to the density of yeast suspension with approximately 1.5 $x10^8$ (CFU/mL) was used for the fermentation of the juice obtained from decaying oranges and pineapples (Beal *et al*., 2020).

Fermentation of fruit juice for bioethanol production

A 24-h old culture of yeast cells was added aseptically to sterile fermentation broth media (100 mL) singly (yeast only) and in combination,

the bottles were shaken gently to form a homogeneous suspension in an aseptic condition. The bottles were tightly covered and incubated at room temperature for 14 days. The sample was taken at an interval of days 0, 7, and 14 for bioethanol production. Some physiochemical analyses were monitored along this interval (Zabed *et al*., 2014).

Physicochemical Analysis

The physicochemical parameters that were used to monitor the progress of the fermentation of decaying orange and pineapple juice during fermentation include pH, titratable acidity, brix level (total sugar), and specific gravity.

Determination of pH

Using a pH meter (Hanna Instruments 8021), the pH of the fruit juice samples was determined. The pH meter was cleaned using cotton wool soaked in ethanol and calibrated with standard buffers before it was used to take the readings. It was later dipped into the fermenting medium and was left to read for two minutes (Laopaiboon *et al*., 2007).

Determination of titratable acidity % of ethanol

The titratable acidity was expressed as a percentage of acidity and analyzed using the method of Tyl and Sadler (2017). A known amount of the sample (10 mL) was titrated against standardized 0.1M NaOH, and a few drops of phenolphthalein solution were used as an indicator to generate a pink color endpoint that should last for 15 seconds. This process was used to determine TTA.

% ethanol = (mL of 0.1M NaOH (titre) x normality)/ (ml of sample x 1000) …eqn 1

Determination of ethanol brix level (total soluble sugar)

A refractometer was used to calculate the sugar content or Brix. Two drops of the material were placed on the refractometer's prism using a clean, dry applicator, and the value was read (Wilson *et al*., 2012).

Determination of specific gravity of ethanol

The hydrometer was used to estimate the specific gravity (Babu *et al*., 2014). It was carefully placed into a test jar containing the orange and pineapple must, and it was dipped in the liquid. The specific gravity values on the glass stem were measured at eye level at the point where the liquid's 20° C surface crossed it.

RESULTS

In this study, a total of fourteen (14) yeast isolates were isolated from the decaying oranges and pineapples. Table 1 shows the mean microbial load associated with decaying pineapple and oranges from different locations which ranged from 3.6 x 10^4 to 4.3 x 10^6 SFU/g. The organisms were identified as yeast based on morphological characteristics (Table 2), microscopic examination, and biochemical tests (Table 3). The yeast isolates were observed to form smooth, curved, white, cream, and raised colonies on the Yeast Extract Agar (YEA).

Table 1a: Mean Microbial Load of Yeasts Associated with Decaying Pineapples From Three Different Locations.

Location	Microbial count (SFU/g)
Challenge	8.1×10^4
	5.0×10^5
	4.3×10^6
Oje	9.0×10^4
	7.0×10^5
	4.0×10^6
Bodija	3.6×10^4
	9.0×10^5
	2.0×10^6

The mean microbial load of yeast isolates from decaying oranges is shown in Table 1b in (SFU/g). The highest mean microbial load of yeast isolates observed from oranges collected from Bodija was 3.0×10^6 SFU/g.

Table 1b: Mean Microbial Load of Yeasts Associated with Decaying Oranges from Three Different Locations

Location	Colony count (SFU/g)
Challenge	9.7×10^4
	8.8×10^5
	2.6×10^6
Oje	3.6×10^4
	9.0×10^5
	2.0×10^6
Bodija	1.3×10^4
	8.0×10^5
	3.0×10^6

The colony morphology of the organisms associated with decaying pineapple and orange on yeast extract media (YEA) are respectively shown in Table 2a and Table 2b, some of the features such as; cream coloration, circular shape, raised elevation, smooth edges, etc. were recorded.

Table 2a: Morphological Characteristics of Yeast Isolates from Decaying Pineapple

Isolates code	Color	Shape	Size	Margin	Elevation	Surface
PC1	Creamy	Circular	Small	Smooth	Raised	Wet
PC2	Creamy	Circular	Big	Smooth	Flat	Dry
PC3	White	Circular	Small	Undulate	Flat	Dry
PO1	Creamy	Circular	Small	Smooth	Raised	Wet
PO2	White	Irregular	Big	Undulate	Flat	Dry
PO3	White	Irregular	Small	Curled	Raised	Dry
PB1	Creamy	Circular	Medium	Smooth	Flat	Wet
PB2	White	Irregular	Small	Undulate	Raised	Dry
PB3	Creamy	Oval	Small	Entire	Raised	Wet

KEY: PO-Pineapple Oje; PC- Pineapple Challenge; PB-Pineapple Bodija; 1- Isolate one; 2- Isolate two; 3- Isolate three

Table 2b: Morphological Characteristics of Yeast Isolates from Decaying Oranges

Isolates code	Shape	Size	Elevation	Color	Margin/Edges	Surfaces
OO.1	Irregular	Small	Flat	White	Undulate	Dry
OO.2	Circular	Big	Flat	Cream	Entire	Wet
OO.3	Circular	Big	Flat	White	Curled	Dry
OC.1	Irregular	Big	Flat	Cream	Undulate	Wet
OC.2	Irregular	Big	Flat	Cream	Undulate	Wet

KEYNOTE: OO-Orange 'Oje Market', OC- Orange 'Challenge Market', 1- Isolate one, 2- Isolate two, 3- Isolate three

The isolates were Gram stained and all were Gram-positive when observed under the microscope. Biochemical tests were carried out to characterize the yeast isolates, all isolates were catalase positive, some isolates were able to liquefy gelatin and reduce nitrate to nitrite, and some were able to ferment sugars and produce gas. The biochemical characteristics of yeast isolates from decaying pineapple and orange are respectively shown in Table 3a and Table 3b.

The API test kit for yeasts, API 20C AUX and was used to confirm the identities of the isolates.

Table 3a: Biochemical Characteristics and Sugar Fermentation Test of Yeast Associated with Decaying Pineapples

Isolates code	Glucose	Fructose	Lactose	Xylose	Galactose	Sucrose	Mannitol	Raffisnose	Inositol	Trehalose	Arabinose	Catalase	Nitrate Reduction	Gelatin	Gram Rnx	3ºC	20ºC	70ºC	6.5% NaCl	3% NaCl	3.9 pH	9.4 pH
PO1	++	++	+-	++	+-	++	++	++	+-	++	-	+	+	+	+	-	+	-	-	+	+	-
PO2	++	++	++	++	++	++	++	++	+-	+-	-	+	+	+	+	-	+	-	-	+	+	-
PO3	++	++	++	++	++	-+	++	++	+-	++	-	+	+	+	+	-	+	-	-	+	+	-
PC1	++	++	+-	++	+-	++	++	++	+-	++	-	+	+	-	+	-	+	-	-	+	+	-
PC2	++	++	++	++	++	++	++	++	+-	++	-	+	+	+	+	-	+	-	-	+	+	-
PC3	++	++	+-	++	++	-+	++	++	+-	++	-	+	+	-	+	-	+	-	-	+	+	-
PB1	++	++	+-	++	+-	++	++	++	+-	++	-	+	+	+	+	-	+	-	-	+	+	-
PB2	++	++	--	++	-+	--	+-	++	--	-+	-	+	+	-	+	-	+	-	-	+	+	-
PB3	++	++	+-	++	+-	+-	++	++	--	+-	-	+	+	+	+	-	+	+	-	+	+	-

KEY: + = positive reaction, - = negative reaction, ++ = positive reaction & can produce gas, + - = positive reaction & cannot produce gas

Table 3b: Biochemical Characteristics and Sugar Fermentation Test of Yeast Associated with Decaying Oranges

Isolate code	Glucose	Sucrose	Xylose	Lactose	Mannitol	Raffinose	Trehalose	Fructose	Inositol	Galactose	Gram staining	Catalase	Arabinose	Gelatin liquefaction	Nitrate Reduction	3 °C	20 °C	70 °C	3.9 pH	9.4 pH	3% NaCl	6.5 % NaCl
OO.1	+G	+G	+G	+G	+G	+G	+G	+G	+	+G	+	+	-	+	+	-	+	-	+	-	+	-
OO.2	+G	+G	NG	+G	+G	+G	+G	+G	+	+G	+	+	-	+	+	-	+	-	+	-	+	-
OO.3	+G	NG	+G	NG	+G	NG	+G	+G	-	+G	+	+	-	-	+	-	-	-	+	-	+	-
OC.1	+G	+G	+G	+G	+G	+G	+G	+G	+	+G	+	+	-	+	+	-	-	-	+	-	+	-
OC.2	+G	+G	+G	+G	+G	+G	+G	+G	+	+G	+	+	-	+	+	-	-	-	+	-	+	-

KEY: + = positive reaction, NG= NO gas, - = negative reaction, +G = positive reaction & gas production

The yeast isolates were screened for ethanol tolerance in 2%, 4%, 6%, 8%, and 10% ethanol concentrations, a large number of the isolates were able to tolerate 2%, 4%, 6%, and 8% ethanol while few were able to tolerate 10% ethanol. Further screening in 1g and 0.1g of tetracycline was carried out, many of the isolates did not grow in 0.1g of tetracycline. The screening of ethanol-tolerant yeasts in decaying pineapple and orange are shown in Table 4a and Table 4b respectively.

Table 4a: Screening for Ethanol-Tolerant Yeast Associated with Decaying Pineapples

Isolate code	2% Ethanol	4% Ethanol	6% Ethanol	8% Ethanol	10% Ethanol	0.1g Tetracycline	1g Tetracycline
PO1	+	+	+	+	+	+	-
PO2	+	+	+	+	+	-	-
PO3	+	-	-	-	-	+	+
PC1	+	+	+	+	+	+	-
PC2	+	+	+	+	+	-	-
PC3	-	-	-	-	-	+	+
PB1	+	+	+	+	+	+	_
PB2	+	-	-	-	-	+	+
PB3	+	+	+	+	+	-	-

Table 4b: Screening for Ethanol-Tolerant Yeast Associated with Decaying Oranges

Isolate code	2% ethanol	4% ethanol	6% ethanol	8% ethanol	10% ethanol	0.1g Tetracycline	1g of Tetracycline
OO.1	+	+	+	+	-	+	-
OO.2	+	+	+	+	+	+	-
OO.3	+	+	-	-	-	+	-
OC.1	+	+	+	+	+	-	-
OC.2	-	-	+	+	+	-	-

KEY: +ve shows growth, -ve shows no growth

The probable organisms identified as *Pichia meri*, *Saccharomyces cerevisiae,* and *Kluyveromyces marxianus* for decaying oranges and *Candida valida, Pichia meri, Kluyveromyces fragilis, and Saccharomyces cerevisiae* for decaying pineapples are respectively shown in Table 5a and Table 5b.

Table 5a: Probable Identity of Yeast Isolated from Decaying Pineapples

Isolate code	API CONF.	Probable organism
PO1	+	*Candida valida*
PO2	+	*Kluyveromyces fragilis*
PO3	-	-
PC1	+	*Candida valida*
PC2	+	*Pichia meri*
PC3	-	-
PB1	+	*Candida valida*
PB2	+	*Saccharomyces cerevisiae*

Table 5b: Probable Identity of Yeast Isolated from Decaying Oranges

Isolate code	Probable organism
OO.1	-
OO.2	*Kluyveromyces marxianus*
OO.3	-
OC.1	*Pichia meri*
OC.2	*Saccharomyces cerevisiae*

The percentage occurrence of yeast isolates from decaying pineapple and orange from different locations are represented in a pie chart and respectively shown in Figure 1a and Figure 1b.

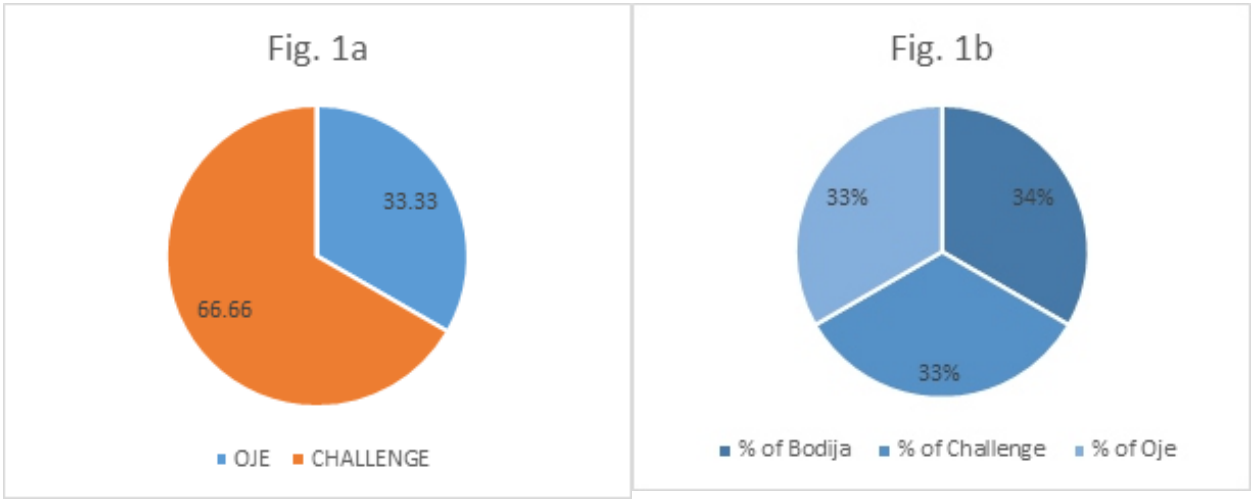

Figure 1a: Percentage of occurrence of organisms isolated from decaying pineapples from different locations; **Figure 1b**: Percentage occurrence of isolates isolated from decaying oranges from different locations.

During the fermentation process, the pH level was monitored from day 0 to day 14, decrease in pH was observed as fermentation progressed (Figure 2a and Figure 2b).

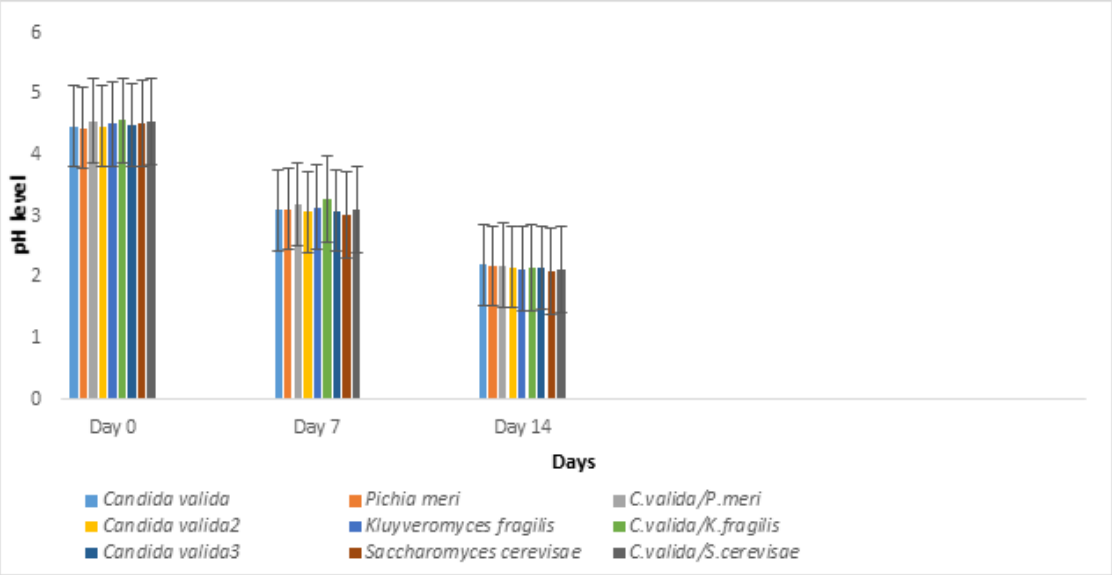

Figure 2a: Changes in pH of Fermenting Pineapple Juice

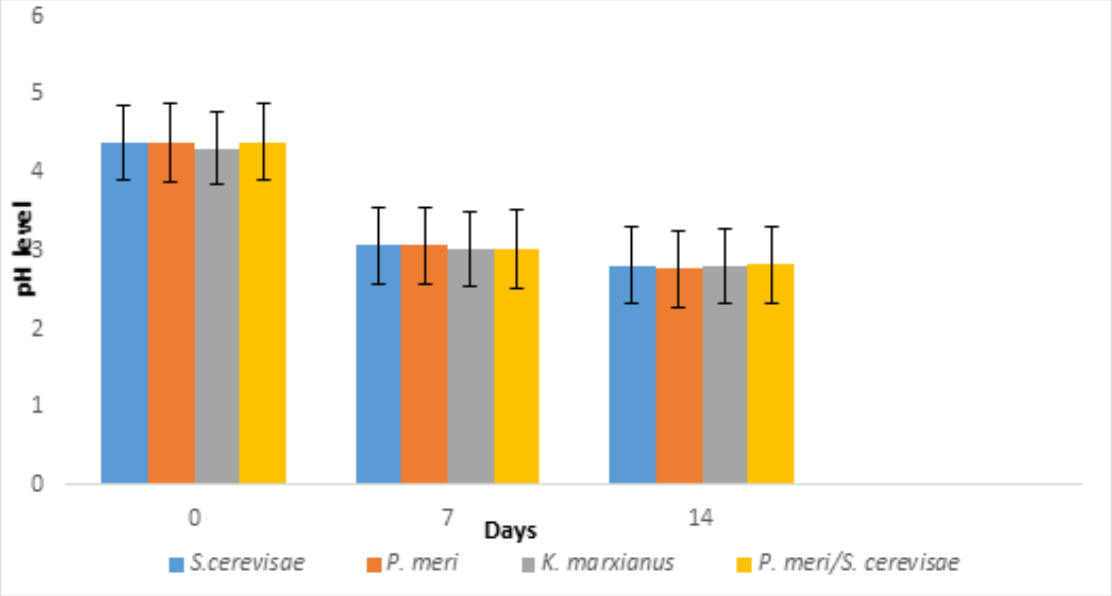

Figure 2b: Changes in pH of Fermenting Orange Juice

The titratable acidity was also monitored and an increase in the titratable acidity was observed as fermentation progressed from day 0 to day 14. Changes in pH and titratable acidity for both fruits are shown in Figure 3a and Figure 3b respectively.

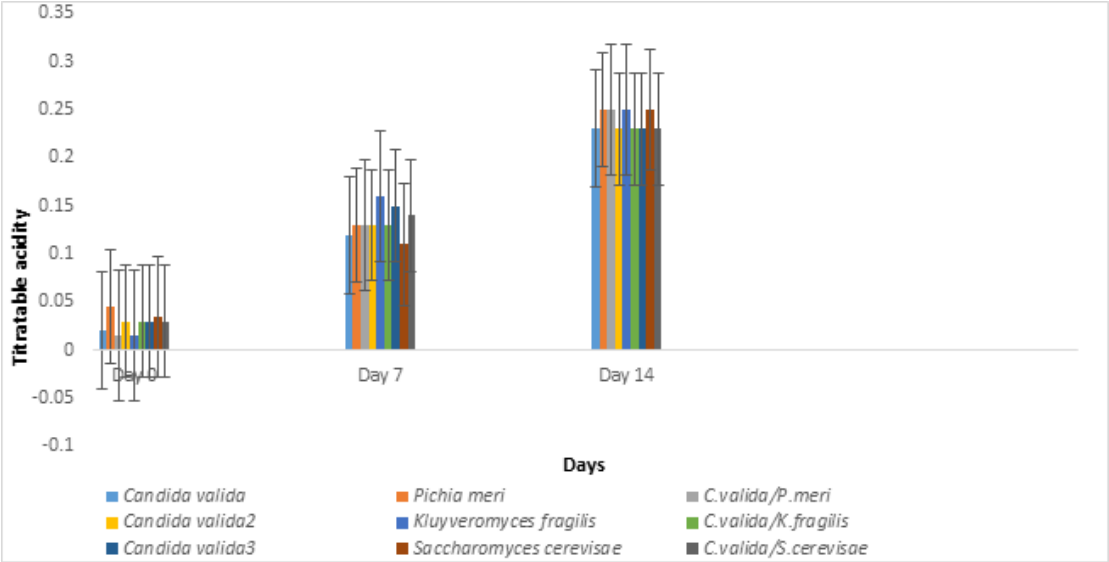

Figure 3a: Changes in Titratable Acidity (% Ethanol) of Fermenting Pineapple Juice

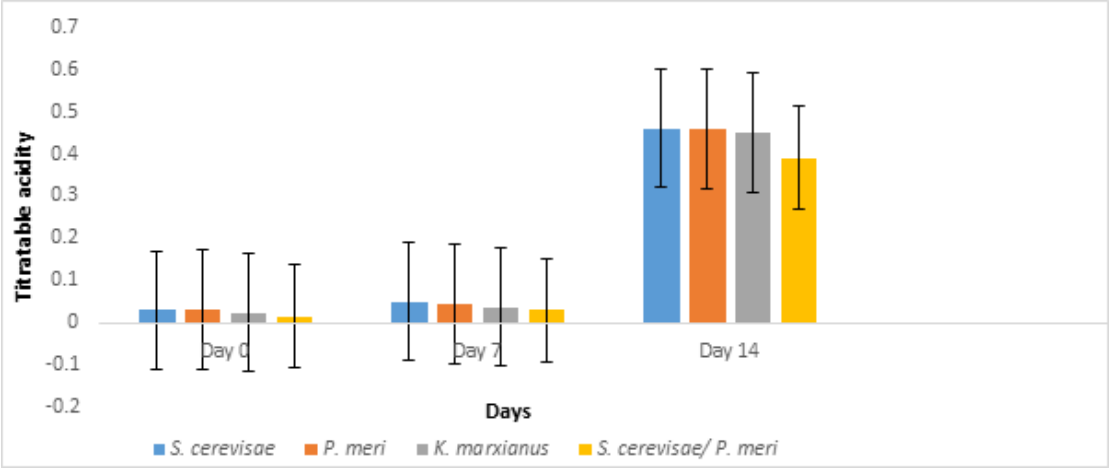

Figure 3b: Changes in Titratable Acidity (% Ethanol) of Fermenting Juice

The reducing sugar level (brix) and specific gravity were monitored from day 0 to day 14, decrease in brix level and specific gravity was observed as the fermentation progressed with the lowest value of 0.20 on day 14 and the highest value of 1.10 on day 0 for brix level, the specific gravity reduced as the fermentation progressed from 1.036 on day 0 to 1.005 on day 14. Changes in the brix level of fermenting pineapple and orange juice are shown in Figure 4a and Figure 4b respectively.

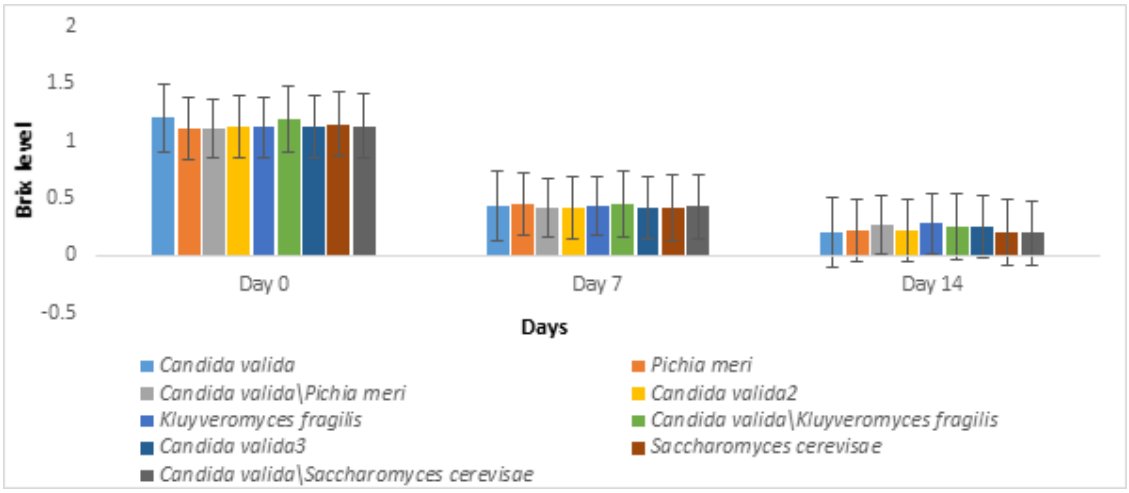

Fig. 4a: Changes in Brix Level (Reducing Sugar) of Fermenting Pineapple Juice

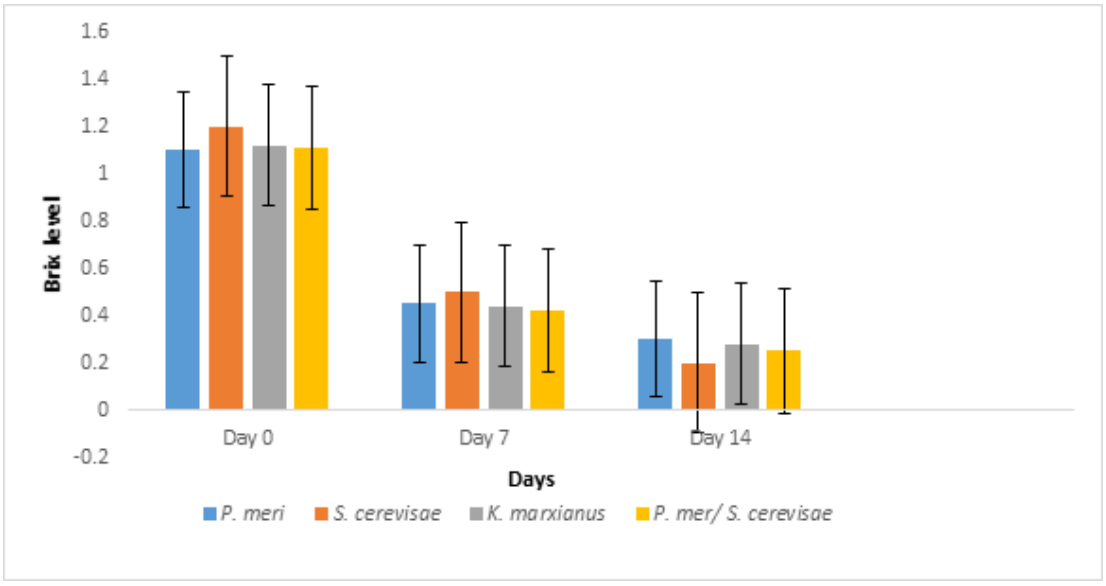

Figure 4b: Changes in Brix Level (Reducing Sugar) of Fermenting Orange Juice

Changes in the specific gravity of fermenting pineapple and orange juice are shown in Figure 5a and Figure 5b respectively.

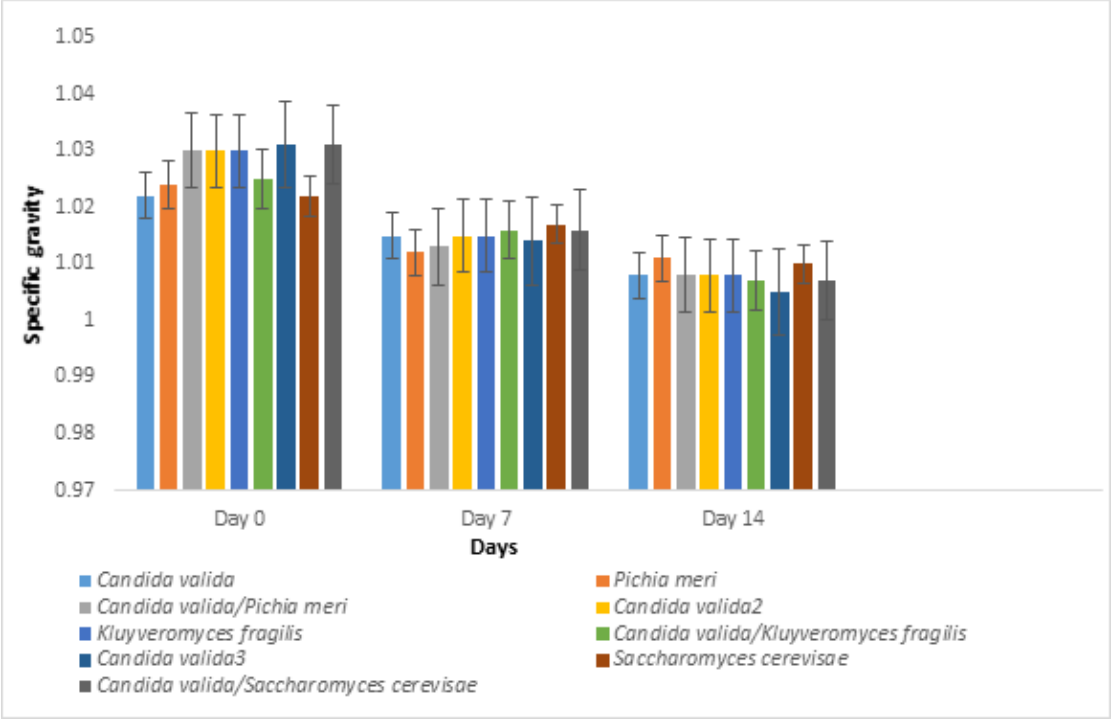

Figure 5a: Changes in Specific Gravity of Fermenting Pineapple Juice

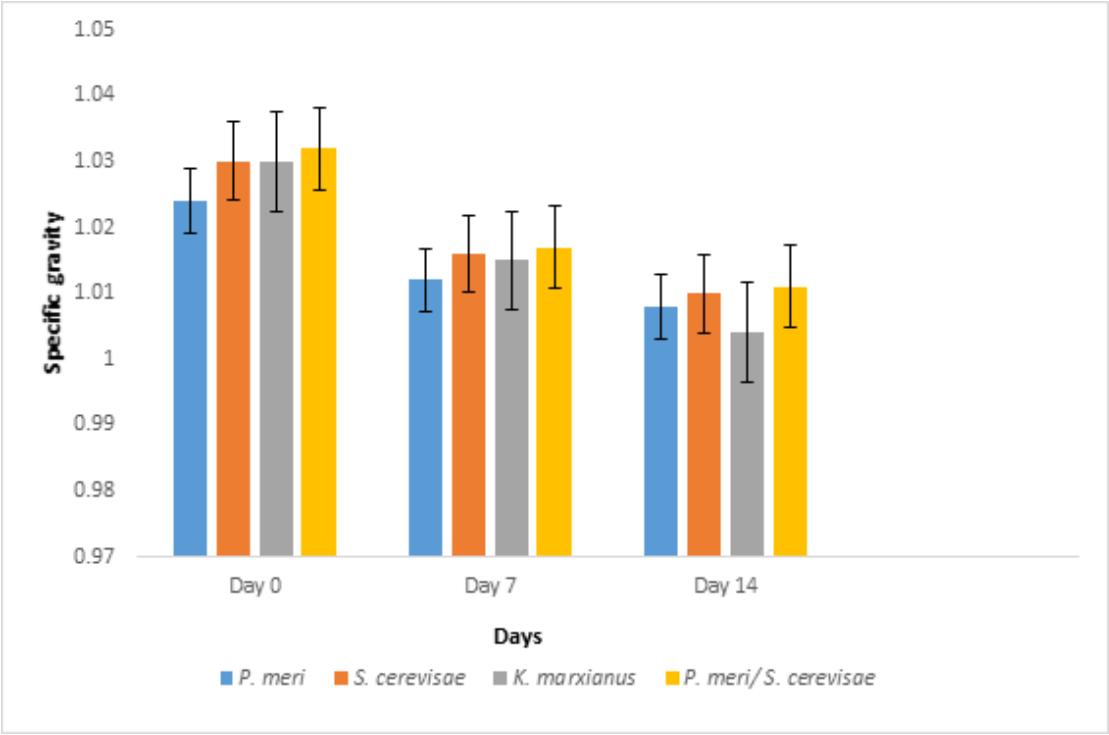

Figure 5b: Changes in Specific Gravity of Fermenting Orange Juice

DISCUSSION

Bioethanol is a volatile renewable energy source that can be produced from biomass such as fruit wastes. The use of decaying fruit wastes as biomass helps to reduce environmental pollution, and mitigate carbon-dioxide generation at the same time. This study focuses on the production of bioethanol from discarded orange and pineapple fruits, typically wasted in local markets in Ibadan. These fruits were selected for bioethanol production as they are good sources of carbohydrate and lignocellulosic material

naturally. Following a colonial visual examination of cultures, five yeast strains were isolated from the decaying oranges (*Citrus sinensis*), and nine yeast strains from the decaying pineapples (*Ananas comosus*) were collected from local markets in Ibadan. Three distinct yeast species from decaying oranges and five from decaying pineapple were identified and used for bioethanol production. Based on their colony, biochemical, and physiological characteristics, the yeast species were identified as *Saccharomyces cerevisiae, Klyuveromyces marxianus, Pichia meri, Kluyveromyces fragilis,* and *Candida valida*.

In addition, the yeast strains were further subjected to growth at different temperature ranges (3°C, 20°C, and 70°C), pH ranges (3.9 and 9.4) and NaCl concentrations (3% and 6.5%) to test for their thermotolerance, osmotic tolerance, and pH tolerance which are important factors affecting the growth of yeasts. Further screening was carried out to select ethanol-tolerant yeast strains by growing the strains in 2%, 4%, 6%, 8%, and 10% ethanol concentrations. They were also screened with different grams of tetracycline (1g and 0.1g). The present study revealed that all isolates were ethanol-tolerant. The range of ethanol tolerance obtained in the present study was 2-10% which correlates with the previous reports of Belitz *et al.* (2008). Isolates that can tolerate high ethanol concentrations will be suitable for the production of bioethanol as they can tolerate the ethanol content during production.

Alcoholic fermentation is significantly impacted by pH levels. The ethanol that is created during fermentation has a pH between 4 and 6. In this investigation, it was shown that as fermentation continued until day 14, the pH of the fermenting media decreased. According to Hashem *et al.* (2021), the optimum pH range for ethanol production is 5-6 due to the good yeast growth over the pH range of 3.5–6.5, which results in a decrease in pH as fermentation progresses.

Titratable acidity is a crucial quality that the fermentation process requires (Chohan *et al.*, 2020) and it depends on the biochemical composition of fruit juice used in the alcoholic fermentation and the process parameters of fermentation. Following the production process, the titratable acidity in this study increased from day 0 to day 7, this is in alignment with Oiwoh *et al.* (2018). Hashem *et al.* (2021) also reported an increase in titratable acidity (% ethanol) as the pH decreases.

The lower the specific gravity, the higher the alcohol content. This means that the alcohol content increases as the specific gravity is reduced. This is being corroborated by an earlier report by Oiwoh *et al.* (2018). Moreover, pH reduced as the specific gravity reduced and the ethanol content increased with fermentation time. Hashem *et al.* (2021) reported a decrease in pH and specific gravity as the ethanol content increased and fermentation time progressed. Marcos *et al.* (2020) reported that when yeast converts sugars in the wort to alcohol and carbon dioxide, the wort becomes less dense and the specific gravity drops. By comparing the specific gravity before fermentation (known as original gravity, or OG) to that after fermentation (final gravity, or FG) and applying a formula it is possible to calculate the alcohol content. A specific gravity bottle is usually used to measure the specific gravity of a liquids.

The ability to produce ethanol from these fruit juices (orange and pineapple) was tested for all isolates by inoculating single strains and mixed cultures into pretreated fruit juice. Sucrose was added to the fermenting medium to provide enough nutrients for the yeasts to utilize throughout the fermentation process for 14 days. These findings stated that the isolates could be used at the industrial level for fermentation of various raw materials to obtain an increased production of bioethanol.

CONCLUSION

This study concluded that the importance of alternative energy sources has become even more necessary not only due to the continuous depletion of limited fossil fuel stock but also for a safe and better environment. Decaying orange and pineapple fruit juice may serve as a good substrate as they contain sufficient amounts of carbohydrates naturally which can be used for the production of bioethanol. The results obtained from this study reveal a strong indication of yeast's great potential in the production of ethanol.

Although the determination of the bioethanol yield was not observed, the result for the brix level and the titratable acidity are pointers to the fact that bioethanol was generated as the results agree with previous research. Therefore, bioethanol is considered a fuel of the future and can solve the problem of pollution and energy crisis.

PROSPECT

Evidence from existing new studies suggests that the distillation of the produced bioethanol, and the percentage yield of the bioethanol produced can be determined.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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