SELECTION OF STARTER FROM DECAYING ORANGES AND PINEAPPLE FOR BIOETHANOL PRODUCTION

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ABSTRACT :

The global energy crises and cost of production, transportation and distribution of the various products is a pointer to the fact that scientist should across the globe ought to harness ways of getting alternative sources of energy which will be renewable, sustainable, efficient and cost effective. In Nigeria, decaying fruits always constitute a major environmental pollution during the harvesting season. There is need to convert these wastes to wealth, hence this study. This study seeks to isolate, screen and characterize ethanol-tolerant yeast from decaying oranges and pineapple and select the best starter for bioethanol production. Samples were collected from different locations within Ile Ife and transported aseptically to the Laboratory. Microbiological and Physicochemical assessment of the isolated strains were on yeast maintenance media. Ethanol, temperature and salt tolerance ability, growth at different pH and temperature, chloramphenicol and nalidixic acid resistance, determination of killer toxins production capacity of yeast, nitrate and carbon assimilation tests were carried out to select the best starter for the production of bioethanol.

Fifteen yeasts were isolated belonging to the genera *Saccharomyce*s, *Candid*a, *Rhodotorula*, *Kluvyreomyce*s, *Trichosporon* and *Pichia*. *S.cerevisae*and *K. marxianus*and showed efficient physico-chemical attributes. A temperature of 30°C and pH 6 was the optimum for the growth of isolates tolerating 15% w/v of Nacl and 20% v/v absolute ethanol; produced catalase, reduced nitrate and fermented different sugars. They were resistant to chloramphenicol and nalidixic acid and did not produce any toxin against tested organisms of the same and different species, hence they were chosen as starters for bio-ethanol production. Ability of the organisms to grow at different temperature and the ethanol tolerance are the attribute essential for selecting the best starter. *Saccharomyces cerevisiae* gave the highest attribute essential for bioethanol production followed by *Kluveromyces 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, Waste utilization, Decaying oranges and pineapple, Ethanol-tolerant yeast

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1. INTRODUCTION

"The world has been facing a major 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 green houses 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" (Ballesteros *et al.*, 2006; Naik *et al.,* 2010).ce. "The independence in energy source needs measures for creating and utilizing such renewable resources. 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"(Ballesteros *et al.*, 2006). "Biofuel can be bioethanol, biodiesel and biogas. Among liquid biofuel, bioethanol is an attractive alternative fuel because it is a renewable bio-based resource and it is oxygenated thereby provides the potential to reduce particulate emissions in compression–ignition engines"(Hansen *et al.*, 2005).

"Traditionally, bioethanol production is usually accomplished by microbial conversion of carbohydrates present in agricultural products" (Brooks, 2008). "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 is fruits. 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 decayed oranges and pineapple that have been discarded as a result of their imperfections cause environmental pollution which have affected the health of humans and

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animals. Thus, to avoid the environmental pollution due to the decomposition of waste, it is necessary to isolate an indigenous yeast strains with the required characteristic that be used as a starter in the fermentation industry for bioethanol production. This study aims at achieving this with minimal cost as much as possible.

2.1 Collection of Samples

Decaying orange 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.

2.2 Study Location

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

2.3 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 (Kreger-van Rij, 1984). 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.

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2.4 Maintenance of culture

The culture of yeast was maintained by sub-culturing on slants using YMM, incubating for 48 hours at 30 ^{0}C and thereafter storing in a refrigerator at 4 ^{0}C for future use.

2.5 Morphology and Biochemical Characterization of Yeast Isolates

2.5.1 Macroscopic morphology

According to the method of Kreger-van Rij (1984) and Kurtzman and Fell (1997), the morphology of the vegetative cells of yeast was studied in liquid and on solid media.

2.5.2 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 petridish and cooled. After cooling, the plates were inoculated by streaking 48 hours old yeast strain and incubated at 30 °C for 48 hours. The following features of the appearance of cultures were recorded; texture, colour and surface of colonies". (Kurtzman and Fell, 1997)

2.5.3 Growth in liquid medium

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

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2.6 Microscopic morphology

2.6.1 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 (Kurtzman *et al.*, 2011).

2.6.2 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.

2.7 **Physicochemical Characterization of the Isolate**

2.7.1 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 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 allowed to incubate at 30 °C for 48 hours. Ability to ferment ten different carbohydrates was examined anaerobically. Capability of fermentation was assessed by looking for the formation of gas (CO_2) in Durham tube and

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colourchange of the fermentation media Deep purple to yellow due to the formation of acids and gas (Warren and shadomy, 1991). In this study, the fermentation tests of the following carbohydrates and sugar alcohol were made: glucose, mannose, xylose, sucrose, maltose, lactose, raffinose, mannitol, galactose and meliobiose.

2.7.2 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 tube. The test tube was 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 carbon source for each isolate served as control. The experimental and control tubes were incubated at 30 ^{o}C for four weeks during which period the tubes were observed weekly for amount of growth and pellicle formation (Kurtzman *et al.*, 2011).

2.7.3 Thermotolerance Test

YPD liquid medium was used for detecting thermotolerance and growth in liquid media of selected yeast isolates. 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 old selected yeast isolates. The initial optical density of each tubes 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 for observing thermotolerance of yeast strain. The increase in optical density in a tube was recorded as evidence of growth (Armanult, 2014).

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2.7.4 Ethanol tolerance Test

The medium for the detection of ethanol tolerance yeast was modified. YPD liquid medium was used for detecting yeasts for ethanol tolerance. Each McCartney 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 varied 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 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. 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 of yeasts.

2.7.5 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.

2.7.6 Osmotolerence Test

YEPD broth was prepared containing 6%, 9%, 12%, 15%, 18% and 20% of NaCl. Each McCartney 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

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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 (Armanult, 2014)

2.7.7 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, Sample collection using the method of Kirby *et al*. (1966). 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) was placed into the center of the already inoculated Petridish. 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.

2.7.8 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 inoculate Nutrient broth. Media was poured on plate and let 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.

2.7.9 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 ^{0}C for 5 days with sterile uninoculated control. Reduction of nitrate by the

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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.

2.8Growth in 0.1% actidine

YPD liquid medium was used for detecting yeasts ability to grow in 0.1% actidine. Each test tubes contained 5 mL of YPD liquid media with 0.1% actidine and blank media was used as a control. The medium was sterilized at 121 °C for 15 min in an autoclave and cooled. 0.1% of actidine 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.

2.9 Determination of proximate composition of the fruits juice

The proximate composition and chemical characteristics of good and bad 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 good and bad oranges and pineapple was determined according to standard methods (AOAC, 2000). Total carbohydrate was determined by the difference according to Kirk and Sawyerr, 1990.

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3.1 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 them to be yeast. After 3 days of incubation at 30°C, heavy, dry climbing pellicles were formed on the surface of YEPD broth medium.

3.2 Biochemical Characteristic of Yeast Associated with Decaying Oranges and Pineapple

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. *K. marxianus* on the other hand was able to utilized seven but was unable to grow on raffinose, maltose and meliobiose.

Table 1: Morphological Characterization of Yeast Associated with Decaying Oranges and Pineapple

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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	+

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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% Actinide	Nitrate Reduction Test	Probable identity of isolate
1	++	++	++	++	++	-	++	+	+	++	+	+	*Trichosporonasahii*
2	++	++	++	++	++	+	+	+	+	+	+	+	*Trichosporonaesteroides*
3	++	++	++	++	++	-	-	-	+	+	+	+	*Rhodotorulamucilaginosa*
4	+	+	+	-	-	-	-	+	+	+	+	+	*Pichia meri*
5	++	+	++	++	+	+	-	+	+	+	+	+	*Trichosporonmucoides*
6	++	++	+	-	++	-	-	-	++	++	+	+	*Candida fructus*
7	+	+	+	++	-	-	++	+	+	++	+	+	*Trichosporoncutaneum*
8	+	++	++	++	++	+	+	+	+	-	+	+	*Candida albica*
9	-	-	++	-	-	-	-	-	+	-	+	+	*Candida catemulata*
10	+	+	+	-	-	-	-	-	+	+	+	+	*Candida parapsilosi*
11	++	++	++	++	++	-	-	-	+	+	+	+	*Kluyveromycesmarxianus*
12	++	+	++	-	-	-	+	+	++	+	+	+	*Saccharomyces cerevisiae*
13	++	++	++	-	++	-	-	-	-	-	+	+	*Candida albica*
14	+	-	+	-	-	-	-	-	-	-	+	+	*Kluyveromycesfragilis*
15	-	-	-	-	-	-	-	-	-	-	+	+	*Candida valida*

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

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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 (Table 3). 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 was reduced (Table 4). Five of the organisms that shows highest ethanol tolerance value were then selected to test their ability to grow at different temperature and pH.

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* show 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 decayingorange 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).

Antibiotic resistance test was carried on the selected yeast isolates using chloramphenicol and nalidixic and the two organisms were sensitive to the antibiotics mentioned above as zones of inhibition were observed round the organisms.

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Table 3.:Osmotolerance Test

Isolate code	Salt concentration (g)/ growth (Optical Density) 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 $p<0.05$.

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Table 4: ETHANOL CONCENTRATION **Ethanol (v/v)/ growth (O.D)**

ISOLATE CODE	5	10	15	20	25	30
1	0.919 ± 0.8^{c}	$1.075 \pm 2.7^{b, c}$	$0.960 \pm 0.7^{b, c}$	1.018 ± 0.8^{b}	0.373 ± 0.4^{a}	0.265 ± 0.3^{a}
3	0.938 ± 0.8^{c}	$0.774 \pm 2.7^{b, c}$	$0.663 \pm 0.7^{b, c}$	0.608 ± 0.8^{b}	0.480 ± 0.4^{a}	0.499 ± 0.3^{a}
4	1.068 ± 0.8^{c}	$0.909 \pm 2.7^{b, c}$	$0.645 \pm 0.7^{b, c}$	0.836 ± 0.8^{b}	0.176 ± 0.4^{a}	0.242 ± 0.3^{a}
7	0.89 ± 0.8^{c}	$0.853 \pm 2.7^{b, c}$	$1.283 \pm 0.7^{b, c}$	0.241 ± 0.8^{b}	0.379 ± 0.4^{a}	0.266 ± 0.3^{a}
10	0.895 ± 0.8^{c}	$0.452 \pm 2.7^{b, c}$	$0.729 \pm 0.7^{b, c}$	0.791 ± 0.8^{b}	0.045 ± 0.4^{a}	0.179 ± 0.3^{a}
11	1.314 ± 0.8^{c}	$0.346 \pm 2.7^{b, c}$	$0.462 \pm 0.7^{b, c}$	0.389 ± 0.8^{b}	0.387 ± 0.4^{a}	0.038 ± 0.3^{a}
13	1.747 ± 0.8^{c}	$0.837 \pm 2.7^{b, c}$	$0.974 \pm 0.7^{b, c}$	0.619 ± 0.8^{b}	0.253 ± 0.4^{a}	0.204 ± 0.3^{a}
15	1.482 ± 0.8^{c}	$0.826 \pm 2.7^{b, c}$	$0.977 \pm 0.7^{b, c}$	0.699 ± 0.8^{b}	0.129 ± 0.4^{a}	0.281 ± 0.3^{a}
17	0.727 ± 0.8^{c}	$1.302 \pm 2.7^{b, c}$	$0.99 \pm 0.7^{b, c}$	1.32 ± 0.8^{b}	0.237 ± 0.4^{a}	0.207 ± 0.3^{a}
19	1.183 ± 0.8^{c}	$0.964 \pm 2.7^{b, c}$	$0.962 \pm 0.7^{b, c}$	1.063 ± 0.8^{b}	0.069 ± 0.4^{a}	0.119 ± 0.3^{a}
24	1.293 ± 0.8^{c}	$0.530 \pm 2.7^{b, c}$	$1.06 \pm 0.7^{b, c}$	0.552 ± 0.8^{b}	0.452 ± 0.4^{a}	0.045 ± 0.3^{a}
25	1.009 ± 0.8^{c}	$0.633 \pm 2.7^{b, c}$	$0.305 \pm 0.7^{b, c}$	0.368 ± 0.8^{b}	0.139 ± 0.4^{a}	0.302 ± 0.3^{a}
26	1.141 ± 0.8^{c}	$0.583 \pm 2.7^{b, c}$	$0.35 \pm 0.7^{b, c}$	0.356 ± 0.8^{b}	0.178 ± 0.4^{a}	0.023 ± 0.3^{a}
34	1.447 ± 0.8^{c}	$0.308 \pm 2.7^{b, c}$	$0.777 \pm 0.7^{b, c}$	0.746 ± 0.8^{b}	0.192 ± 0.4^{a}	0.058 ± 0.3^{a}
35	1.717 ± 0.8^{c}	$1.093 \pm 2.7^{b, c}$	$1.211 \pm 0.7^{b, c}$	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 withinRows are significantly different at $p<0.05$.

Table5: Growth at Different pH

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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 $p<0.05$.

Table6:Growth at Different Temperature

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	Temperature (^{o}C)/ growth (O.D)					
ISOLATE CODE	25	30	32	37	40	44
1	1.043 ± 11^{b}	1.136 ± 15^{b}	1.198 ± 11^{b}	$0.474 \pm 12^{a, b}$	$0.277 \pm 19^{a, b}$	0.278 ± 12^{a}
4	1.031 ± 11^{b}	0.360 ± 15^{b}	0.598 ± 11^{b}	$0.487 \pm 12^{a, b}$	$0.786 \pm 19^{a, b}$	0.375 ± 12^{a}
7	1.487 ± 11^{b}	1.006 ± 15^{b}	1.175 ± 11^{b}	$0.701 \pm 12^{a, b}$	$0.431 \pm 19^{a, b}$	0.206 ± 12^{a}
17	0.953 ± 11^{b}	1.129 ± 15^{b}	1.147 ± 11^{b}	$0.947 \pm 12^{a, b}$	$0.858 \pm 19^{a, b}$	0.380 ± 12^{a}
19	0.843 ± 11^{b}	1.143 ± 15^{b}	1.123 ± 11^{b}	$1.049 \pm 12^{a, b}$	$1.368 \pm 19^{a, b}$	0.885 ± 12^{a}

Values are the means$\pm$ standard deviation of determinations on three replicate growths. Means with different superscript within

Rows are significantly different at $p<0.05$.

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Table7: **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 Orange, 2 = Medium Orange, 3 = Good Orange
4 = Pineapple and Orange, 5 = Spoilt Pineapple, 6 = Good pineapple

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4.1 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 is in agreement with an earlier report by Meghana) 2014)

Isolates however showed variation in terms of utilization of ten different sugars . 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. *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) (2012who reported earlier that some species of microorganism select their carbon sources in which they grow.

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 of Patil*et al*. (2016) and Mir naiman and Mohammed (2014).

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

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isolate that can tolerate up 44 °C temperature. It is important to note that the results of the thermotolerance analysis for this study is less 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.

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 (Uriah, 2003). 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 obsure (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.* (2007) reported that ethanol accumulation in fermenter inhibits specific growth rate, specific ethanol production rate, cell viability and substrate consumption. Tikka *et al.*

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(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*. (2006) 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 form 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.

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 yeast. In this study, yeasts were able to grow at 20% sodium chloride concentration. This agrees with Osho (2005) who reported maximum of 20% sugar tolerance for *S. cerevisiae* BSOSU 0269. Mir naiman and Mohammed (2014) and Kusumawadee (2015) 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*. (2005) 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

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*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 analysed. 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*. (2010) who Recommended Dietary allowances (RDA) of equal mixture of oranges and pineapple juice to be 12.15%. 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.

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S. cerevisiae produced better yield when used singly (92%) and also *K. marxianus*(40%). There was however a reduction when they were used in combination (20%). This is contrary to an earlier reports by workers such as Brooks (2008); Querol *et al.* (2013); Kusumawadee (2015); Sathees*et al*? (2017 and 2018). This may be due to inhibitory activities of the yeasts against themselves especially when it comes to production. The reason for this in this work cannot be ascertained as this is not the focus of this work (Adeyemo *et al.*, 2020b In press).

CONCLUSION: It was concluded from the study 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 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.

The environmental impact of the study is a positive one, the researchemphasized the production of bioethanol as a waste management option In Nigeria.It also encourages the reduction of environmental pollution. The potential economic implication is that it can be used to turn waste to wealth. Decaying oranges and pineapple are a major environmental nuisance which can be converted to wealth creation. The methods for isolation of the isolates and screening of starters are simple, safe and ecofriendly.Our findings could be applied in the area of waste management, turning waste to wealth, reduction of environmental pollution, climate

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change and study encouragesone of the ways of the reducing global warming. The use of fossil fuels increases global warming and the effect of climate change, it alsoincreases environmental pollution because the concentration of carbon monoxide in the environmental is increased. The use of eco-friendly bioethanol gives rise to a sustainable , safer and healthier environment to live in .

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