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Fermentation Process of Vinegar: Microbiological and Biochemical Analysis

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ABSTRACT

Vinegar is the product made from the conversion of ethyl alcohol to acetic acid by a genus of bacteria *Acetobacter*. This work is based on the ability of vinegar to be produced from *Garcina kola* and *Acer pseudoplatanus*. The production of vinegar from bitter kola and sycamore to avoid waste or spoilage of the fruits which can serve for preservation and food preparation was the essence of the study carried out. It is a useful means to help ensure that losses incurred with fruits are reduced and the vinegar produced can help to properly preserve some foods against spoilage. Bitter kola and sycamore were processed, cut and eventually blended to evaluate the production and quality of the vinegar being produced. The bitter kola and sycamore were fermentation was carried out with added inoculant and naturally by indigenous inoculant for 7d at 30°C. Results showed that pH, alcohol content and specific gravity were 4.0, 0.5 and 1.001g/cm^3 respectively. The acetic acid yields of the vinegars produced were within the range of 0.43%-1.84% due to the use of monoculture which was indigenous in the fruit and Braggs vinegar with mother. Microbiological and biochemical analysis was carried out during alcoholic and vinegar fermentation. The antimicrobial potential of the vinegars was also tested and found effective on clinical pathogens. The test proved that the *G. kola* had the most antimicrobial properties against the bacterial isolates than the *A. Pseudoplatanus* which had the lowest.

Keywords: Vinegar, Fermentation, Antimicrobial, *Acetobacter* Spp, *Garcina kola* and *Acer pseudoplatanus*.

INTRODUCTION

The word Vinegar is derived from the French *Vin* (wine) and *Aigre* (sour). Vinegar is a sour and sharp liquid used as a condiment and food preservative. Vinegar can be defined as an acetic acid liquid produced through fermentation from a suitable raw material of agricultural origin which contains starch or sugars as a carbon source also fit for human consumption, The raw materials used for vinegar production include rice, grapes, malt, apple, honey, potatoes, whey or any other sugary food [1]. Acetic acid is the significant flavor and antimicrobial component in vinegar). In human history, vinegar appears at the beginning of agriculture with the discovery of alcoholic fermentation from fruits, cereals and vegetables. The genesis of vinegar can hardly be distinguished from the origin of wine. Although vinegar has always been considered among the lowest quality products of fermented foods, it has also been used as a food condiment, as a preservative agent and, in some countries as a healthy drink [2].

The vinegar is introduced over the world before 10,000 previous years [3]. From nearly about 5000 years' vinegar is comes in differential flavors as well existing in market as profitable product. The references from Hippocrates and Old Testament show that the wounds were cured medically by the use of vinegar. Sung Tse [4], who had used a vinegar and sulfur as a hand sanitizer for prevention of various infections.

Vinegars are commonly used for pickling of fruits and vegetables and in the preparation of mayonnaise, salad dressings, mustard, and other food condiments. Although useful as a food ingredient for flavor and functional properties, the potential health benefits of vinegar varieties are leading researchers to further consider this long used food product [5]. Regular consumption of bioactive substances is promoted by many nutritional researchers and the functional food properties of vinegar have been reported in a variety of scientific and lay publications. With documentation of the health benefits of vinegar, a concurrent increase in demand for fruit vinegar production has occurred [6]. Functional therapeutic properties of vinegar described include antibacterial activity, blood pressure reduction, antioxidant activity, reduction in the effects of diabetes, prevention of cardiovascular disease, and increased vigor after exercise [7].

In [7], it was written that the scientist Dobereiner proposed a theory of production of acetic acid from ethyl alcohol. Initially, to form acetaldehyde, Dehydrogenation of alcohol occurs where two ions of hydrogen and two electrons are lost. After which hydrogen ions are combined with oxygen to form water (H_2O) to form aldehyde due to hydration of acetaldehyde. Aldehyde dehydrogenase converts acetaldehyde to acetic acid thereby releasing two ions and two electrons [8].

Some examples of vinegar are cane vinegar, apple cider vinegar, rice wine vinegar and wine vinegar. Further processing of vinegar include filtration, distillation and pasteurization at 165.2^{0}F (74^{0}C) before it is bottled [9]. The microorganisms involved in vinegar production are the yeast which acts in the first stage fermentation converting simple sugar to ethanol, then the Acetic acids bacteria which is involved in the second stage fermentation for the conversion of the ethanol into Acetic acid.

Saccharomyces cerevisiae has potential to optimize production in anaerobic condition. Starter culture of *Saccharomyces* species influence sensory characteristics including flavor and also flavor retentions of final product [10]. [11] studied that for cider production, the strains commonly used *Saccharomyces cerevisae* or *Saccharomyces bayanus* and choice of yeast strains a starting culture could highly impact the flavor of fermented beverages.

AAB are characterized by their ability to oxidize carbohydrates, alcohols, and sugar alcohols (polyhydric alcohols or polyols) into their corresponding organic acids, aldehydes, or ketones, in a process termed "oxidative fermentation," from which they gain energy [12]. [13] conducted studies on the acetous fermentation of ethanol into acetic acid by acetic acid bacteria belonging to the family *Acetobacteriaeae* and the genera *Acetobacter* and *Gluconobacter*. The chemical reaction was as follows:

$CH_3CH_2OH + O_2 \rightarrow CH_3COOH + H_2O + 493kj$

Ethanol oxygen acetic acid water

Vinegar production methods range from traditional employing wooden casks (Orleans process) and surface culture (Generator process) to submerged fermentation [14].

AAB are generally considered to be fastidious microorganismsbecause of their poor recovery on laboratory media. This trait has been observed in AAB samples isolated from environments with high levels of acetic acid [15]. The poor recovery on culture media has also been associated with the lack of a suitable synthetic media, as not all synthetic media equally support the growth of AAB and could even be selective among strains [16].However, among these culture media, GY and GYC media are the most widely used to recover AAB strains from grape must, wine [17].

This work is aimed at the production of vinegar from raw materials in the environment that tend to be waste and to enumerate microbial population as well as the antimicrobial potential of the vinegar.

MATERIALS AND METHODS

Materials

All reagents used were of analytical grades.

Study area

Anambra State (Uli Town Ihiala Local Government Area) is one of 36 States that make up the Federal Republic of Nigeria. It is located in South East geopolitical zone of Nigeria. It has geographical coordinates of 6.2758°N, 7.0068°E. It falls within the tropical rainforest zone of Nigeria.

Samples Collection and Processing

Whole fruits such as Bitter kola "*Garcina kola*" (n=5), Sycamore "*Acer pseudoplatanus*" (n=1) were obtained from different market vendors at a village market called eke market Uli Town, in Ihiala Local Government area, Anambra State. They were authenticated in the Herbarium section of the Department of Biological Science, Chukwuemeka Odumegwu Ojukwu University, Anambra State by a botanist. It was taken to Microbiology Departmental laboratory Uli campus at Chukwuemeka Odumegwu Ojukwu University, for analysis. Thus, Braggs unfiltered with mother was bought from a super market also in Awka, Anambra State."

Methods

Inoculum Development for Production of Vinegar

The ripe fruits (Bitter Kola and Sycamore) about 4kg were thoroughly disinfected with cotton wool soaked in ethanol before peeling or were washed thoroughly with using distilled water, some were peeled using knife to bring out the core while some were left with the peels (i.e with the back) then chopped into small sizes separately.

Alcoholic Fermentation

250gram of the four samples (with core and peel) were weighed and soaked in distilled water 650ml while. 0.3g of yeast nutrient (ammonium sulphate and potassium dihydrogen phosphate)

was prepared in 100ml of water and sterilized by autoclaving. 10 ml of the mixture was added into the bottles. Addition of yeast and sugar in separate Erlenmeyer flask. A control solution was also prepared in a separate Erlenmeyer flask similarly without the addition of yeast and sugar. They were allowed to ferment naturally at room temperature 30℃. This was done in order to convert the sugars to ethanol by the action of the yeasts present in the samples (naturally or added). Isolation and identification of Yeast was done at this stage to recorded the yeast that might be responsible. During this period, the mixture ferments into alcohol. The mixture was blended at the end of the seven days' fermentation. Some of the samples were thereafter boiled at 100°C for 30mins. allowed to cool and introduced into a sterile bottle while unboiled one are left as control. The mixtures were then transferred into 250 ml of conical flask, part of it was inoculated with industrial produced Apple Cider Vinegar with mother (a slimy membrane composed of cells of microorganisms found on the surface of alcoholic liquids undergoing acetous fermentation and can be added to cider or wine to produce vinegar), while the other without Acetic acid serves as a control.

This was covered with cheese cloth and the bottles were placed in the dark at 28°C.The fermentation was allowed for 28days and then the products was filtered using a tea strainer to remove the produced slime before chemical analysis and sensory evaluation. During this period of fermentation, physical observations like color, aroma, taste, pH, Specific gravity and alcohol analysis were conducted and proper changes noted on the samples daily, until the desired strength is reached.

The methods described by [18] slightly modified by [19] was adopted in the production of vinegar.

Figure 1: weighed bitter kola and sycamore in jars

Figure 2: Alcoholic fermentation

Figure 3: Homogenized samples for vinegar fermentation

Determination of pH of the fermenting samples and vinegar

An automated pH meter was used to measure the pH of the sample and vinegar during the fermentation. It was sterilized using ethanol. The electrode was dipped into the samples and the pH was read and recorded.

Determination of the alcohol content of the fermenting samples

An alcohol meter was used to measure the alcohol content of the samples during fermentation. The meter was sterilized using ethanol. The meter was dipped into the fermenting samples and alcohol content was read and recorded

Determination of specific gravity

The specific gravity was measured using a hydrometer for estimation of density in the vinegar. It was dipped into the samples and reading were recorded.

Determination of titrable acidity

The assay of acetic acid was carried out every seven days after addition of the apple cider vinegar and mother. 5mls of the vinegar were added to 20ml of distilled water in a 250ml conical flask and mixed with 5 drops of phenolphthalein. The mixture was titrated against 0.5N sodium hydroxide till the appearance of pale pink colour in the flask. The volume of sodium hydroxide consumed during the titration was measured and the percentage of acetic acid in the vinegar was calculated using the formula.

$$\frac{\text{Mass of Acetic Acid}}{\text{Mass of Vinegar}}$$

% Acetic acid = X 100

Isolation and Characterization of yeast and bacteria

5fold dilution was carried out for the samples respectively. Then, after few minutes the suspension (10^{-3}) was used to inoculate on sterilized SDA (Sabaraud Dextrose Agar) and PDA (Potato Dextrose Agar) and it was kept for 24 hrs at 37^0C.

Lactophenol cotton blue staining

A drop of 70% ethanol was used to clean the glass slide. Immerse the specimen of yeast in the drop of alcohol. Add one or two drops of Lactophenol cotton blue stain. Place a cover slip gently avoiding air bubbles. Then slide was observed microscopically under X40 objective [19].

Biochemical test for yeast

The isolated colonies were identified on the basis of morphology by performing biochemical test; Test for sugar fermentation using Glucose, Lactose, Maltose, Fructose, Sucrose, IMViC Test, Catalase test, Oxidase test.

Enumeration /Determination of microbial population in the vinegar.

GYC medium (Glucose, yeast extract powder and calcium carbonate) was prepared and allowed to cool. 5-fold serial dilution was carried out and samples were taken from the third test tubes was used for inoculation. Using spread plating,0.1ml was spread and allowed to diffuse and then inverted for growth. The CFU/ml was calculated.

Biochemical Tests

The AAB isolates were gram stained and standard biochemical tests were used to identify the AAB (oxidase and catalase).

Measurement of Antimicrobial Activity of the Vinegar samples

The antimicrobial activity was carried out by Mueller-Hinton agar well plate diffusion method. The extract was tested against four pathogenic bacteria isolated from clinical sample. These organisms were *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa*. Different concentrations of the extracts were added into the wells. The concentrations vary from 100%, 75%, 50% and 25% for each organism. Each microbe will be swabbed evenly onto plates containing MHA. A cork borer of 6mm was used to bore holes on the agar plates that have already been seeded with the various organisms. Using micropipette 0.1mls of the vinegar samples was poured into in the holes bored. The antimicrobial drug ciprofloxacin was used as a control. The principle of this method is based on the fact that the antimicrobial agent diffuses in the medium and it radially expands, with its concentration decreasing as distance from the edges of the cylinder increases [20]. If the bacteria are susceptible to the action of the tested antimicrobial agent, it will not grow in the zone of its action. Therefore, after incubation, the zones of absence of growth are observed around the cylinder, so called the inhibition zones. The zones of growth inhibition were measured using a ruler and pair of dividers and the sensitivity of the bacterial strain to the tested vinegar sample were determined. Petri plates were incubated for 24 hours at a temperature of 37˚C. Two repetitions were made for each bacterial culture and the mean value for each bacterial culture was calculated.

Mueller Hinton broth was prepared and 2mls of the prepared broth was added to the test tube and sterilized. The MHB was then cooled and 2mls of diluted concentrations of 100%, 75%, 50%, 25% was added into the broth with a stabbed inoculation of test organism and incubated for 24 hours at 37^oC.

Mueller Hinton Agar was prepared and allowed to cool, the plates were divided into four sections and samples from the incubated tubes were streaked on the plates and was incubated for 24hours at 37^0C. Positive and negative results were recorded for clear zones of our vinegar samples.

RESULTS AND DISCUSSION

The yeast was isolated using Saboraud dextrose agar and potato dextrose agar. Ciprofloxacin an antibiotic proved to inhibit the growth of bacteria. *Saccharomyces cerevisae* was present and is known to survive, grow, metabolize and multiply by making use of the dextrose sugar.

When viewed under a microscope, a transparent septate hyphae was observed.

Table 1: Biochemical characterization of AAB isolate

Samples	Gram stain	Catalase	Oxidase
1A Boiled	-	+	-
3B ACV	-	+	-
3B Natural	-	+	-
4A ACV	-	+	-
4A Natural	-	+	-
4B Boiled	-	+	-

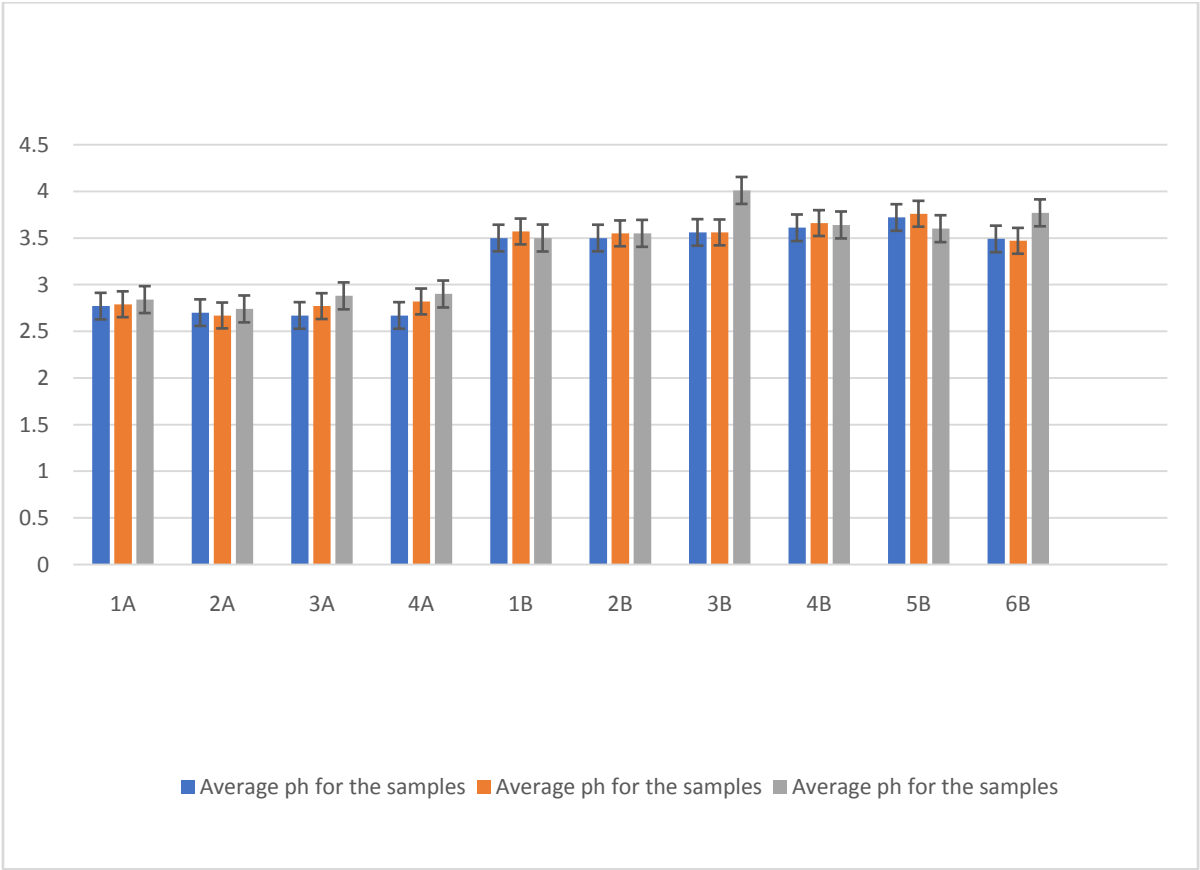

Figure 4: graphical representation of pH values during vinegar fermentation

Figure 5: fungal growth on PDA

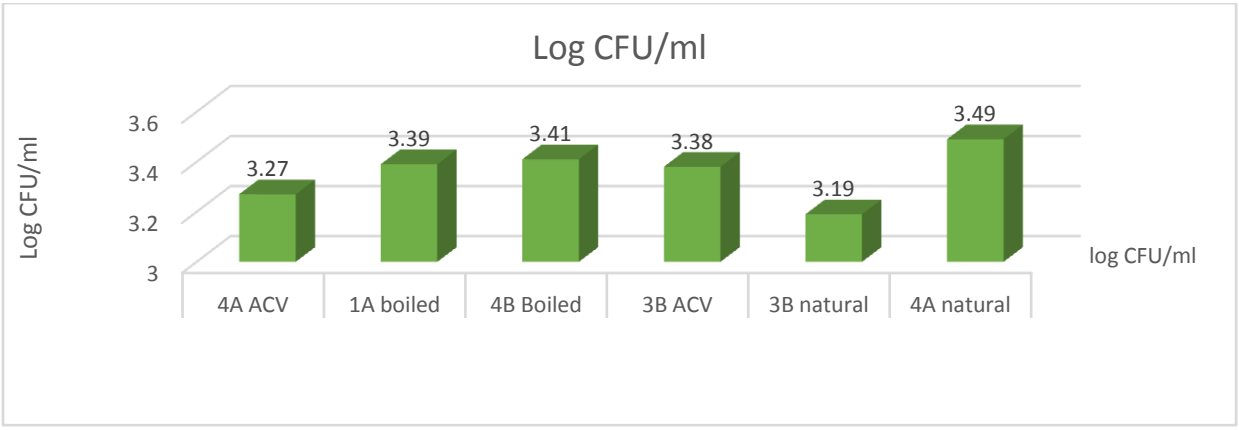

Figure 6: Total viable count of AAB

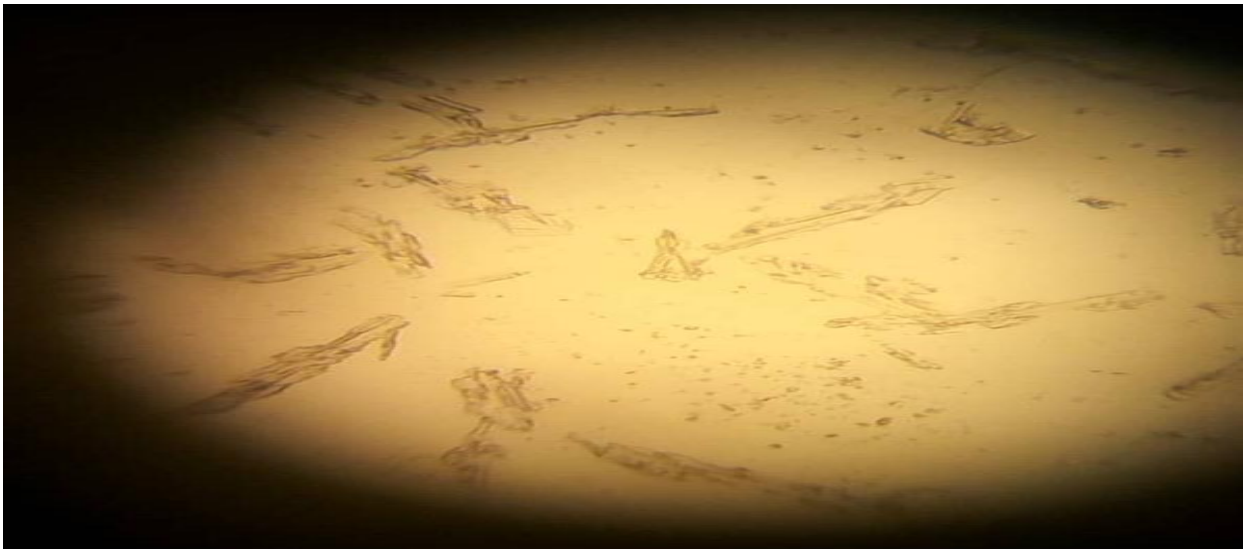

Plate 1: Transparent septate hyphae of fungi

Figure 7: Positive result for methyl red test on yeast

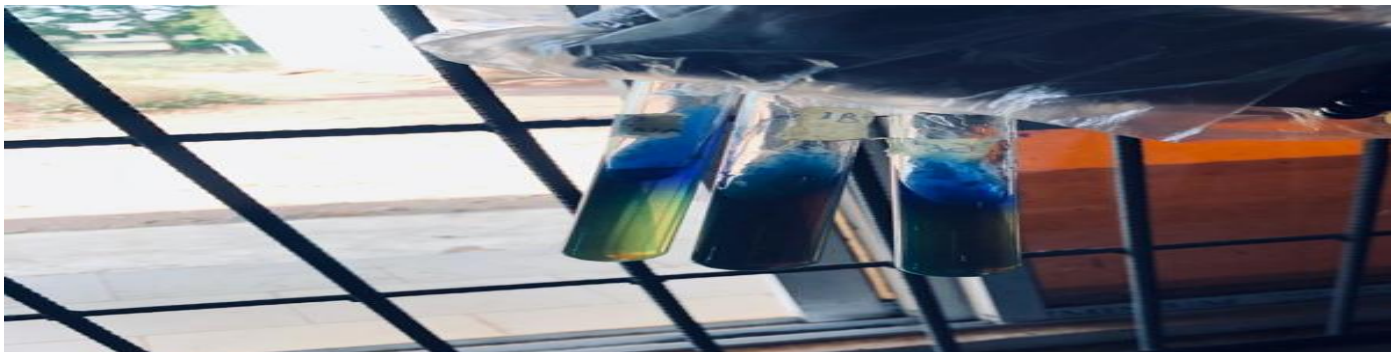

Figure 8: Positive result for citrate test for yeast.

Table 2: Morphology of fungi colony

Color	Form	Surface	Elevation	Odor	Feeding mechanism
White	Filamentous	Fuzzy	Raised	Fermented like palm wine odor	Mycelium

Table 3: Mean zone inhibition of antimicrobial test.

	E.coli				***S.aureus***				***P.aeurginosa***				***B.subtilis***			
Samples	Concentration of vinegar samples (%)															
	100	75	50	25	100	75	50	25	100	75	50	25	100	75	50	25
3B ACV	1 ± 1.0	_	_	_	_	_	_	_	_	_	_	_	5 ± 0.0	2 ± 2.0	5 ± 5.0	1 ± 1.00
3B Natural	_	_	_	_	_	_	_	_	_	_	_	_	2 ± 2.0	4 ± 3.0	4 ± 4.0	_
1A Boiled	6 ± 5.0	3 ± 2.0	_	_	4 ± 3.0	8 ± 2.0	5 ± 4.0	9 ± 9.0	6 ± 1.0	11 ± 3.0	_	_	4 ± 1.0	6 ± 3.0	_	_
4A Natural	6 ± 5.0	3 ± 2.0	4 ± 3.0	1 ± 0.0	4 ± 4.0	3 ± 2.0	8 ± 2.0		_	_	_	_	2 ± 2.0	3 ± 3.0	4 ± 2.0	4.5 ± 4.0
4B Boiled	_	_	_	_	_	_	_	_	3 ± 6.0	4 ± 4.0	3 ± 2.0	_	5 ± 1.0	6 ± 3.0	2 ± 2.0	_
4A ACV	10 ± 9.0	_	_	_	_	_	_	_	_	_	_	_	4 ± 4.0	_	2 ± 1.0	_

Table 4: Biochemical test results for yeast

Sample	Indole	Lactose	Glucose	Fructose	Maltose	Sucrose	Catalase	Oxidase	Citrate	Voges praskeur	Methyl red
B	-	-	+	+	+	-	+	-	+	+	-
3A	+	-	+	-	-	-	+	+	-	-	+
1B	+	-	+	-	-	+	+	-	-	+	+
5B	-	+	+	+	-	+	+	+	-	+	-
6B	+	+	+	+	-	+	+	+	+	-	+
3A	-	-	-	+	+	-	+	-	-	+	+
1A	-	-	-	+	-	-	+	-	-	-	-
4B	-	-	+	-	+	-	+	-	-	+	-
2B	+	-	+	-	+	+	+	+	-	+	+
2A	-	-	-	-	-	-	+	-	+	-	-
3B	+	-	-	+	-	-	+	-	-	-	+

DISCUSSION

Diverse works have been written on the production of vinegar from fruits or fruit wastes and the isolation and characterization of acetic acid bacteria. This work began with the use of the yeast *Saccharomyces cerevisae* to begin the fermentation process. The best known alcohol producing yeast organism is *Saccharomyces cerevisiae* which was isolated from our samples after 7d of alcohol fermentation. It was found both in those with added inoculum and without. There was presence of indigenous strains of *Saccharomyces* sp. The rate of alcohol content in our sample was within 0.1-0.5%. The yeast was capable of fermenting sugars in *Garcina kola* and *Acer pseudoplatanus* into alcohol. The yeast culture was removed after the alcohol fermentation and acetic acid production follows this was in correspondence to [21]. Several authors have worked on isolation and characterization of acetic acid bacteria from sugary and starchy substrates and oriental fermented foods [22]. According to Ndoye *et al.*, [19] acetic acid bacteria are gram negative strictly aerobic and commonly found in nature on different plants (fruits, grains, herbs etc.). AAB are fastidious organisms and lose a feature such as the ability to produce high concentrations of acetic acid upon sub culturing. During the cause of the vinegar fermentation samples were collected to isolate acetic acid bacteria. These bacteria are difficult to isolate and culture so GYC medium was used to support the growth of the organisms. Maintenance of AAB from fermenting cultures are not easy since they can go into viable but culturable state (VBNC) quickly. This is mainly due to lower oxygen available and drop in pH due to continuous acetic acid production which is the primary metabolite of AAB. The $CaCO_3$ in the medium relieves the physiological stress from the AAB which drives them into VBNC, this was further explained by [20]. Comparing the vinegars produced with yeast and that of natural it was observed that the vinegar produced with yeast

yielded a little bit higher acetic acid value when compared to the vinegar produced without yeast. Acetic acid value in general for the vinegar production was low within the range of 0.43-1.84%. The acetic acid concentration obtained upon using the AAB isolates in monoculture fermentation ranged from 0.4% to 1.2%. Thus, ACV production using a pre-formulated mixed starter culture was found to be more effective as compared to both-monoculture fermentation as well as traditional natural fermentation with no added inoculum [21]. Amongst the vinegar produced the vinegar produced from boiled alcohol has higher yield although it's within the range of 0.46%-1.87%. The use of starter cultures for vinegar fermentation gives a more controlled process that is easily to reproduce and gives a standardized product. The cost of production of starter cultures are high compared to the use of seed cultures from previous batch cultures from previous fermentation. The factors deterring the availability of starter cultures are difficulties in culturing the AAB and preserving their acid forming potential in the laboratory [18]. The use of starter cultures for vinegar fermentation gives a higher yield of acetic acid because their ability to produce acid are being preserved.

The vinegar samples were tested for their antimicrobial effect against clinical specimens *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa* and their zone of inhibition were recorded and mean zone of inhibition was calculated. The results of the antimicrobial test showed the vinegar with yeast and boiled that is 1A boiled has the highest inhibitory against these clinical pathogens. Vinegar produced indigenous organisms both during alcohol and vinegar fermentation. However this vinegar was not effective against *P. aeruginosa.* This means that *E. coli, S. aereus, P. aeuroginosa B. subtilis* were susceptible to 1A boiled vinegar at concentrations of 100%, 75% but *S. aereus* was susceptible at all concentrations of the vinegar sample.

The antimicrobial/antibacterial effect was expressed as measured of the diameter of the inhibition of growth in millimeters. The mean zones of inhibitions were represented along with the different concentrations of the vinegar samples. The test proved that the *G. kola* had the most antimicrobial properties against the bacterial isolates than the *A. pseudoplatanus* which had the lowest. The bitter kola was very active against *P. aeruginosa* with zone of inhibition from 11.00± 3.00mm, *S. aureus* ranging from 8.00± 2.00 to 9.00± 9.00mm, *E. coli* at 10.00± 9.00mm and least active against *S. aureus* at 100% 1.00± 0.00 to 25% *B. subtilis* at 1.00±0.00mm. This result is similar to that of [22] who showed the antimicrobial activities in vitro against both Gram-positive and Gram-negative organisms. The sycamore fruit had a low range of 1.00±1.00 to 1.00± 1.00mm against *E. coli* and *B. subtilis*. All this could be seen on Table 3.

CONCLUSION

Vinegar has been produced worldwide from different raw materials using different production method. The use of vinegar is vast both in medicine, therapeutic and food industry. This work has shown and proven that vinegar can be produced from bitter kola and sycamore, with antimicrobial potentials. Production of vinegar using bitter kola should be further exploited industrially in order to discover the active potential of it therapeutically and food industries likewise sycamore. Further studies on the health effect of vinegar should be carried out and ways of maintaining AAB in the laboratory should be also provided.

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