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INDIGENOUS ETHANOL TOLERANT *SACCHAROMYCES CEREVISIAE* ISOLATES FOR *MANGIFERA INDICA* AND *ANANAS COMOSUS* WINES PRODUCTION

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Abstract: Yeasts isolated from mango (*Mangifera indica*) and pineapple (*Ananas comosus*) fruits were sequentially inoculated into potatoes dextrose agar (PDA) containing increasing ethanol concentration from 5 to 15%. Yeasts isolates from both fruits showed a maximum ethanol tolerance of 14% and were identified as *Saccharomyces cerevisiae* strains. The yeasts were used to ferment mango and pineapple musts as well as a combination of mango and pineapple musts at a ratio of 1:1. The mango wine, pineapple wine and mango-pineapple wine produced had total soluble solids of 5.0 ^{o}Brix, 6.0 ^{o}Brix and 7.0 ^{o}Brix; pH values of 3.7, 3.5 and 3.8; titratable acidity of 1.02%, 1.35% and 1.07% and reducing sugar values of 1.5%, 2.7% and 3.8% respectively. The alcohol content of the wines produced from mango, pineapple and a combination of the musts were 11.5%, 10.9% and 10.1% respectively. The pineapple wine had the best clarification followed by mango-pineapple wine and then the mango wine. Hedonic test showed that the pineapple wine was the most acceptable followed by the mango-pineapple wine and finally the mango wine. All the wines had good aroma and taste.

Keywords: *Mangifera indica, Ananas comosus*, yeast, must, wine, pineapple.

INTRODUCTION

Wine can be defined as the product of alcoholic fermentation of juice of any fruit. The composition and quality of wine are influenced by the species of yeast used. In wine making, optimal conditions are created for the yeast to thrive, multiply and convert sugar to alcohol. Wine yeasts originated from the surface of fruits. Musts from freshly crushed grape fruits yield yeast population of 10^3 to 10^5 cfu/ml (Doyle *et al*., 2013). Yeasts have also been isolated from orange juice (Boboye and Dayo-owoyemi, 2009). Generally, fast growing, fermentative, low apiculate yeasts belonging to the genera *Kloeckera, Hanseniaspora* and *Saccharomyces* are known to colonize fruit surfaces (Abranches *et al.,* 2001).

Good wine yeasts tolerate higher alcohol levels (Berry, 1987), a characteristic of *Saccharomyces cerevisiae* which is usually the wine yeast. Yeast can also be induced to tolerate high alcohol percentage through the phenomenon of cross stress protection and adaptive stress response. Cross stress protection is the response of yeast to a mild dose of

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stress resulting in the acquisition of higher resistance to a stressor in a subsequent treatment (Stanley *et al.,* 2010).

Mango and pineapple fruits are produced in large quantities in some parts of Nigeria. Unfortunately, only a low percentage is usually consumed while the excess is wasted due to the highly perishable nature of these fruits coupled with inadequate storage and preservative approach. Consequently, converting the juices of these fruits into wines will help solve this problem. Since wine yeasts originated from surface of fruits, it is expected that mango and pineapples fruits would as well have yeasts associated with them. Yeasts that would be isolated from these fruits are expected to be well adapted and very efficient in fermentation of the fruits musts. Furthermore, yeasts isolated from the fruits will invariably be readily available and cheaper to obtain than the commercially available ones.

This research work is therefore aimed at isolating ethanol tolerant yeasts from fruits of mango and pineapple and using them to produce wines from these fruits. Unlike pineapple wine, mango wine is known to have problem with self clarification. A combination of mango and pineapple musts will hopefully aid self-clarification of mango-pineapple wine.

MATERIALS AND METHODS

Isolation of yeasts

Mango (*Mangifera indica*) and pineapple (*Ananas comosus*) fruitsobtained from Jimeta Market, Nigeria were properly washed and peeled. Juices were extracted from the fruits by blending their pulp with blender (Masterchef Crown* star model No MC-42BL), and filtering with muslin cloth. The juice extracts (musts) were kept in sterile conical flasks at 4°C in a refrigerator. Dilutions were made with sterile distilled water in test tubes up to $10^{-5}$for both mango and pineapple musts. 0.1ml of 10^{-5} dilution from either mango or pineapple juice was inoculated onto PDA plates using the spread plate method. The plates were incubated at room temperature (25 to 30°C) for 4 days. Discrete yeast colonies from the agar plates were then observed and examined with a high power objective of microscope (Olympus CHB 513301).

Isolation of ethanol tolerant yeasts

Potato dextrose broth (9.5 ml) was dispensed into each of two sterile test tubes stoppered with cotton wool and autoclaved at 121^{o}C for 15 min. 0.5 ml of absolute ethanol was then pipetted into each test tube. Yeast isolated from mango was inoculated into one test tube using sterile wire-loop while the yeast isolated from pineapple was inoculated into the other test tube. The test tubes were covered with cotton wool and incubated at room temperature for 3d. Then, samples from each of the test tubes were inoculated onto PDA plates using sterile wire- loop

and incubated at room temperature for 3 d. The yeast colonies observed were deemed 5% ethanol tolerant.

To obtain 7.5 % ethanol tolerant yeast, the 5 % ethanol tolerant yeast isolates from PDA were inoculated into sterile potato dextrose broth (PDB) containing 7.5 % ethanol (i.e. 0.75 ml absolute ethanol in 9.25 ml broth) in test tube and incubated as described for 5 % ethanol tolerant yeast isolates. Similarly, the steps were repeated for the 10 % ethanol tolerant yeasts (i.e. 1 ml absolute ethanol in 9 ml broth), 12.5 % ethanol tolerant yeasts (i.e. 1.25 ml absolute ethanol in 8.75 ml broth), 14 % ethanol tolerant yeast (i.e. 1.4 ml absolute ethanol in 8.6 ml broth) and 15 % ethanol tolerant yeast (i.e. 1.5 ml absolute ethanol in 8.5 ml broth). There was no yeast growth in 15 % ethanol broth.

Identification of yeast isolates

The yeasts from both *Mangifera indica* and *Ananas comosus* isolated at 14% ethanol were then identified based on alcohol tolerance, colony/cultural characteristics, microscopy of wet mount preparation, Gram staining, glucose assimilation and germ tube tests as described by Barnett *et al.* (2000) and Owuama, (2015).

The identified yeast species were inoculated onto PDA plates and incubated at room temperature for 2 d. Thereafter, yeast colonies were transferred to PDA slants and incubated at room temperature for two d and then kept in a refrigerator at temperature of 4^{o}C until required.

Yeast build-up and fermentation of musts

Yeast isolates from mango and pineapple on agar slants were sub-cultured on PDA plates and incubated for 48 h at about 30^{o}C. A wire loop was used to pick a colony from mango yeast culture and inoculated into 50ml of yeast extract peptone dextrose (YEPD) broth in a 250ml flask. The broth culture was incubated at room temperature for 24 h. After 24 h, the inoculum was transferred to 200ml of mango must previously (heated at 80°C for 10 minutes and allowed to cool), in a 250ml conical flask and incubated at room temperature for 48 h. A similar treatment was given to yeast isolates from pineapple and pineapple yeast mixed with mango yeast. The built-up yeast was used as inoculum for the fermentation.

Ripe mango fruits (*Mangifera indica*) obtained from Jimeta market were weighed, washed and peeled to remove the skin. The pulp was then removed from the seeds and 978.5 g of the pulp was blended in a blender (Masterchef Crown*Star model No MC-42BL). The juice obtained was filtered with sterile muslin cloth and then diluted with sterile distilled water at a ratio of 1:4. Similarly, ripe fruits of pineapple were weighed, washed, peeled and

washed and 1406.7 g then blended. The juice was also diluted with sterile distilled water at the ratio of 2:1. 600ml of the mango must was heated at 80^{o}C for 10 min to pasteurize the must (Ameryapoh *et al*., 2010). A similar treatment was given to the pineapple must. Similarly, 600 ml of mango must plus pineapple must were mixed at a ratio of 1:1 and was heated to 80^{o}C for 10 min. Thereafter, sugar (sucrose) was added to various musts (mango, pineapple and mango plus pineapple) to achieve a specific gravity of 1090.

The specific gravity, total soluble solids, pH, titratable acidity, reducing sugar and protein content of the three musts were determined before and after the addition of sugar. Total soluble solids (TSS) were determined using refractometer (REF 503)(Cheesbrough, 1987). The reducing sugars in the musts and wines were determined using the method described by Lane and Eynon (Egan *et al*., 1981). The protein content was determined in the musts and wines using formol titration method as described in Egan *et al*.(1981).

The three different musts were transferred into three different sterile 3-litre fermentation bottles. Yeast nutrients comprising 0.45g of $(NH_4)_2SO_4$ and 0.45 g of $(NH_4)_2HPO_4$ (Berry, 1987) were added to the contents of each fermentation bottle. 100ml of built-up yeast inoculum was transferred to the appropriate fermentation bottle making 700ml must in each bottle. Each fermentation bottle was stoppered with plastic cork with a hole through which a plastic pipe was inserted and the other end inserted into a beaker containing distilled water. Fermentation was carried out at room temperature.

The rate of fermentation was monitored based on the amount of gas bubbles escaping through the end of the pipe inserted in distilled water inside the beaker. The total soluble solids, pH, titratable acidity reducing sugars, specific gravity and percentage alcohol were determined at 48 hourly intervals during the primary fermentation period of 10 d. The wines were racked after 10 d of fermentation and kept in screw capped airtight bottle and allowed to undergo secondary fermentation at room temperature for two months after which the wines were again racked.

RESULTS

Yeasts isolated from *M. indica*and *A. comosus* fruits were identified to be *Saccharomyces species* based on their cultural characteristics, microscopic appearance, Gram stain reaction, germ tube test, glucose assimilation and alcohol tolerance.

Table 1: Analyses of musts before the addition of sugar

Must	TSS(°Brix)	SG	pH	TA (%)	RS (%)	PR (%)
MM	5.0	1020	3.3	0.39	5.0	0.51
PM	11.0	1040	3.6	0.40	7.5	0.68
PMM	8.0	1030	3.7	0.42	6.1	1.02

Key: TSS = Total Soluble Solids, SG= Specific gravity, pH = Hydrogen ionconcentration, TA= Titratable acidity, RS = Reducing sugar, PR = Protein MM = Mangomust, PM = Pineapple must and PMM = Pineapple-Mango must.

Table 1 above shows the total soluble solids(TSS), specific gravity(SG), pH, titratable acidity(TA), reducing sugar(RS) and protein content(PR) of the mango must, pineapple must and pineapple-mango must.

Table 2: Analyses of musts after the addition of sugar

Must	TSS(Brix)	SG	pH	TA (%)	RS (%)	PR (%)
MM	18.0	1090	3.1	0.33	16.1	0.51
PM	19.6	1090	3.5	0.32	16.3	0.68
PMM	20.2	1090	3.6	0.35	16.2	1.02

Key: TSS = Total Soluble Solids, SG= Specific gravity, pH = Hydrogen ion concentration; TA= Titratable acidity, RS = Reducing sugar, PR = Protein, MM = Mango must, PM =Pineapple must; PMM = Pineapple + Mango must.

Table 2 above shows the total soluble solids (TSS), specific gravity (SG), pH, titratable acidity (TA), reducing sugar (RS) and protein content (PR) of the mango must, pineapple must and pineapple-mango must.

Figure 1 below shows changes in soluble solids of the three must during active fermentation. Generally, the total soluble solids decreased in the musts as the days of primary fermentation increased. Prior to fermentation, total soluble solids of pineapple + mango must was 20.2^{o}Brix, that of pineapple must was 19.6^{o} Brix and for mango must 18.0^{o} Brix. The decrease in total soluble solids was remarkable during the first four days of fermentation and thereafter gradually decreased with time. At the end of primary fermentation, pineapple wine had the value7.7^{o} Brix, pineapple-mango wine 7.3^{o} Brix and then mango wine 7.0^{o} Brix.

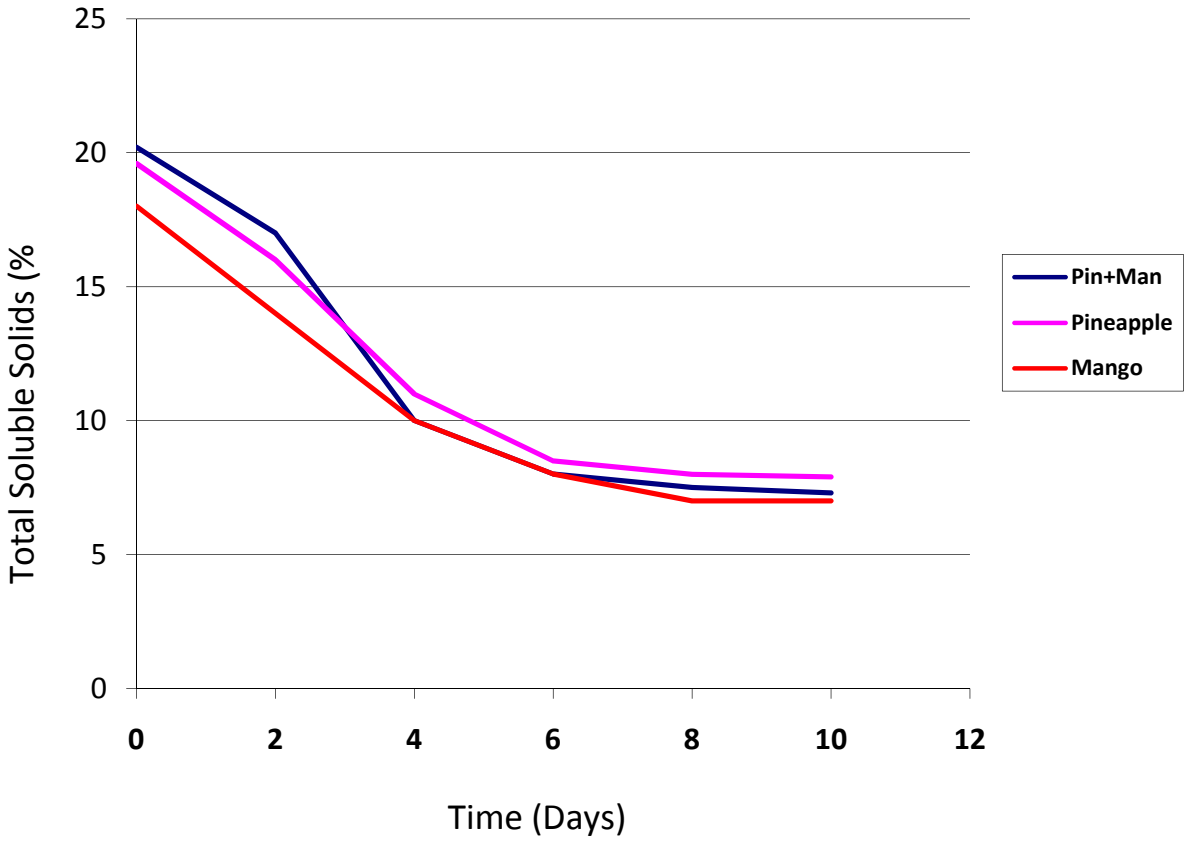

Figure 1: Changes in total soluble solids of the mango, pineapple and pineapple + mango musts with time during primary fermentation

Figure 2 showed that titratable acidity increased as the number of days of fermentation increased in all the musts. At the inoculation time, titratable acidity of a mixture of mango and pineapple must was higher (0.35 %), followed by that of mango must (0.33 %) and then pineapple must (0.32 %). Titratable acidity increased with fermentations days and at the end of primary fermentation, pineapple wine had the highest value (1.15%), followed by mixture of pineapple and mango wine (0.98 %) and then mango wine (0.95 %)

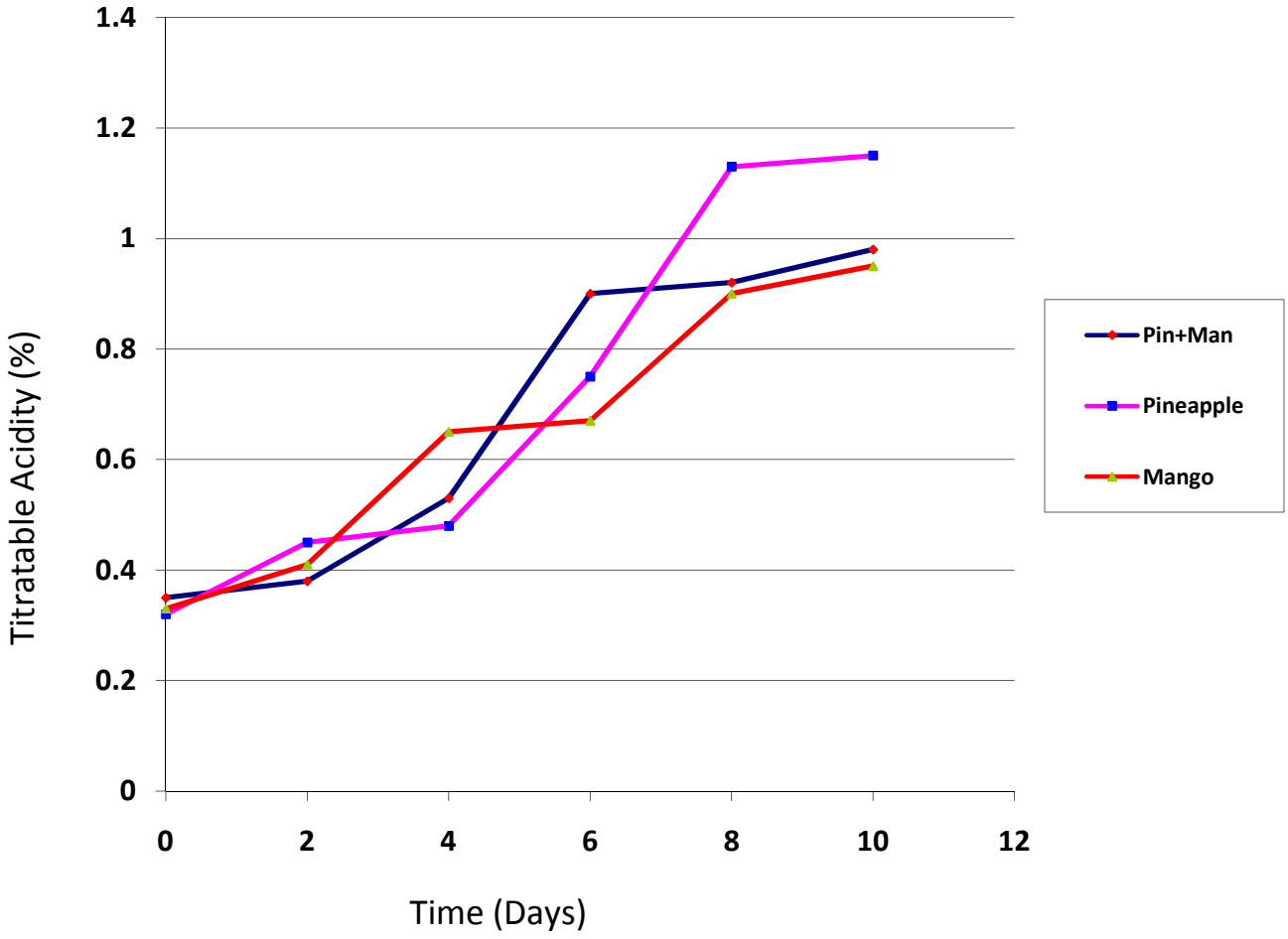

Figure 2: Changes in titratable acidity of the mango, pineapple and pineapple + mango musts with time during primary fermentation

Figure 3 showed that the pH continued to decrease in all the musts as fermentation progressed. At the onset of fermentation, the mixture of pineapple and mango musts had higher pH value (pH 3.6), followed by the pineapple must (pH 3.5) and lastly mango must (pH 3.1). The pH gradually decreased throughout the primary fermentation. The pH value of pineapple wine was higher (pH 2.7), followed by that of the mixture of pineapple and mango wine (pH 2.6) and finally that of mango wine (pH 2.4).

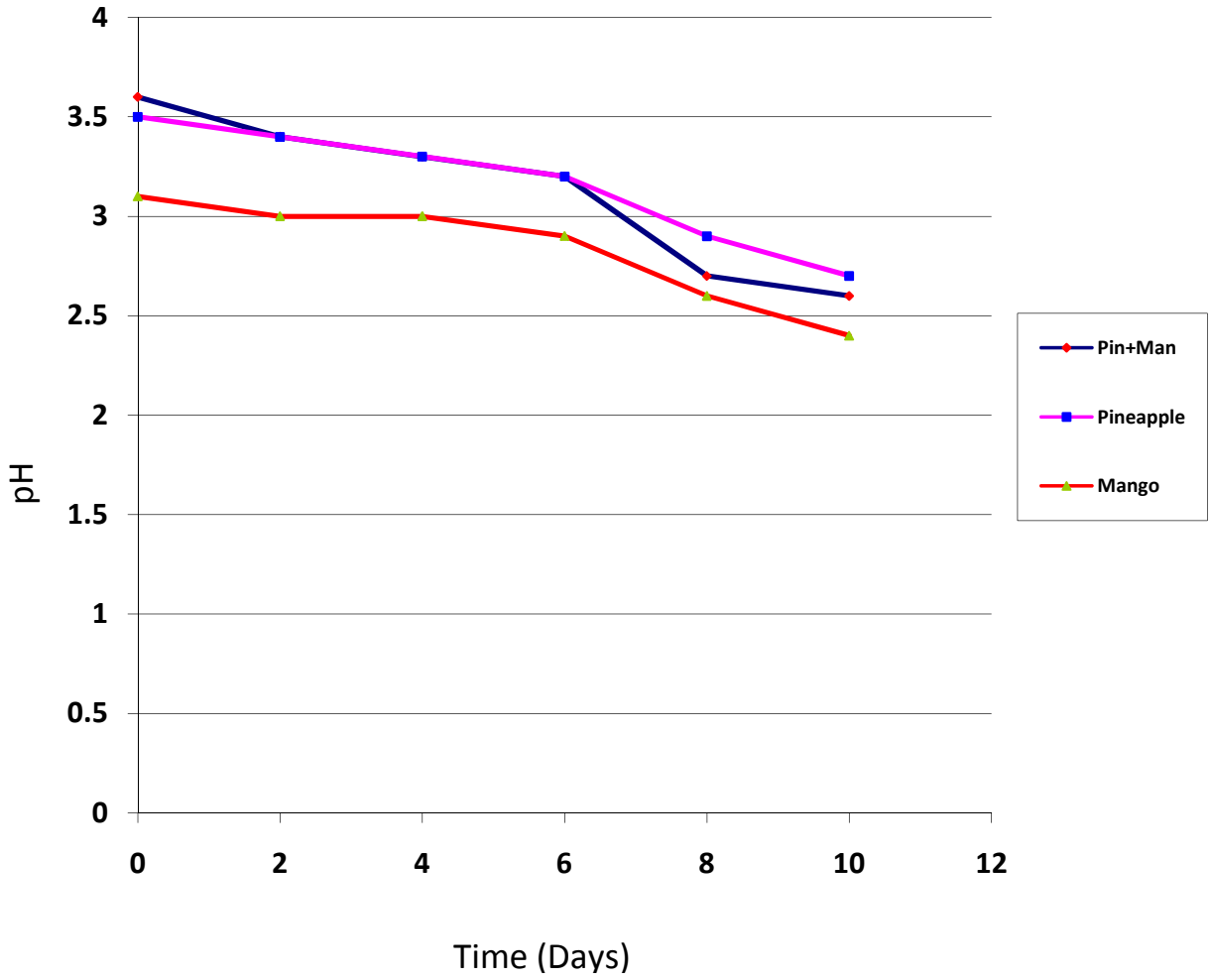

Figure 3: Changes in pH of the mango, pineapple and pineapple + mango musts with timeduring primary fermentation

Figure 4 shows the pattern of reducing sugar during primary fermentation. The reducing sugars in all the musts at the beginning of the fermentation were almost the same because of the adjustment of the respective specific gravities of the different musts. The reducing sugars decreased sharply within the first four days of fermentation and slowly declined till the end of primary fermentation that lasted for 10 days. At the end of primary fermentation, reducing sugar content of a mixture of pineapple and mango wine was the highest (4.1%), followed by that of pineapple wine (3.2 %) and then that of mango wine (3.1%).

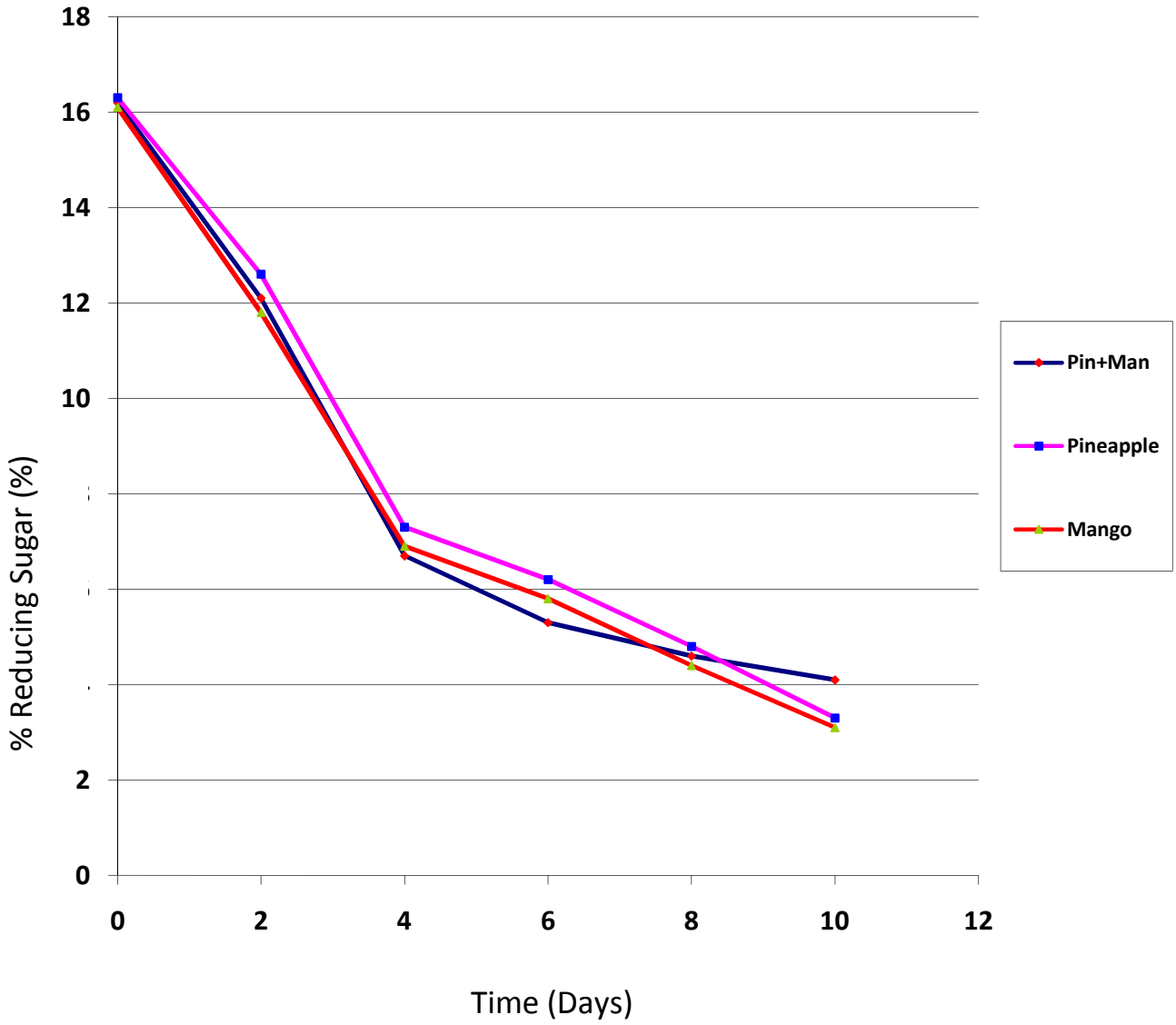

Figure 4: Changes in reducing sugars of the mango, pineapple and pineapple mango musts with time during primary fermentation

In Figure 5, the percentage alcohol production increased with days of fermentation. Percentage alcohol production was faster during the first four days of fermentation and then became gradual for the remaining days. At the end of primary fermentation, percentage alcohol production was highest in mango wine (10.5%), followed by pineapple wine (10.2%) and lastly by the mixture of mango and pineapple wine (9.8%).

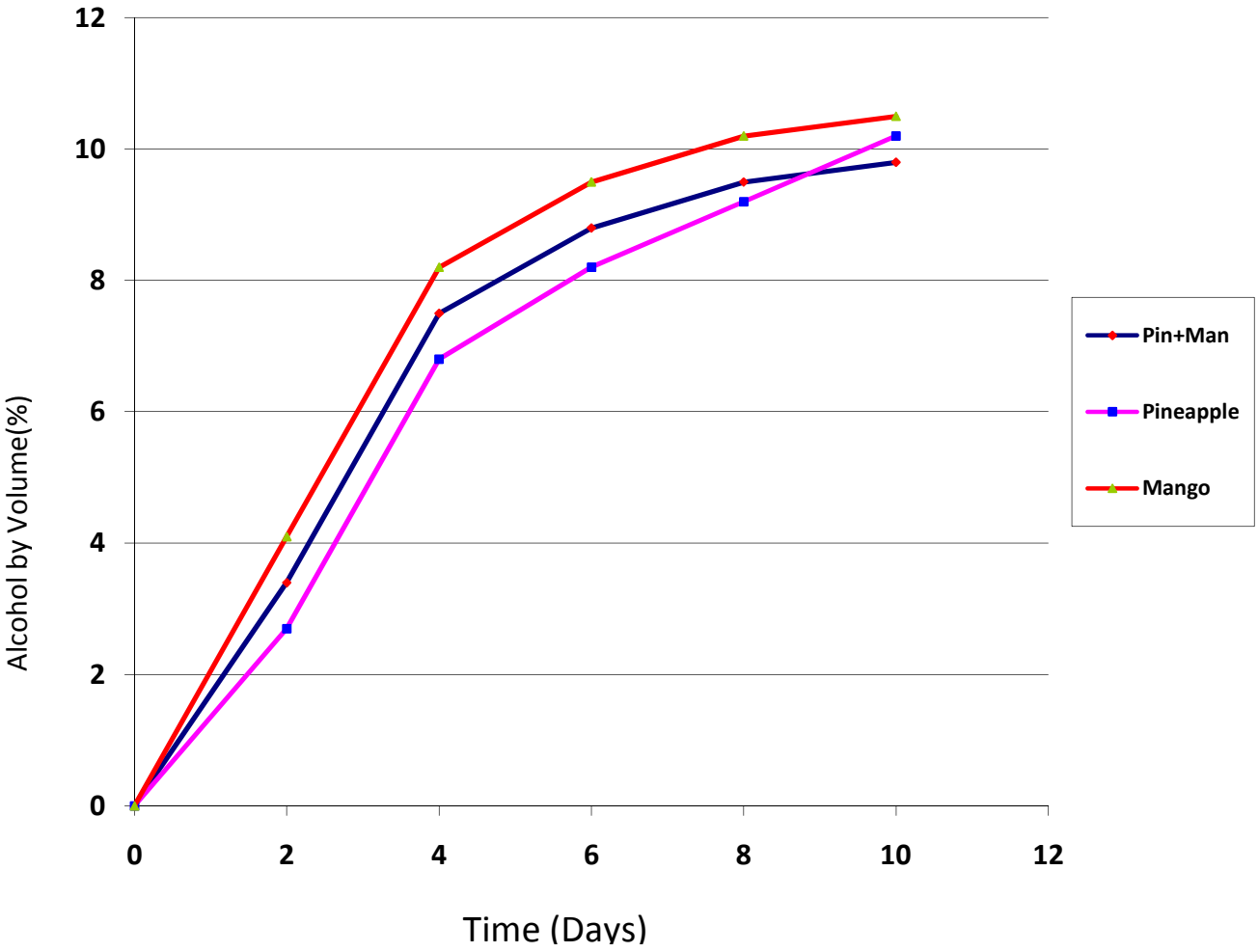

Figure 5: The percentage alcohol production with time during primary fermentation of the mango, pineapple and pineapple + mango musts.

Analysis of the wines

Table 3 showed the total soluble solids, specific gravity, pH, titratable acidity, reducing sugar and protein content in the wines measured at the end of primary fermentation. The analysis indicated that the soluble solids in the wines were still high and the highest was that of pineapple wine (7.7^{o}Brix), then mixture of pineapple and mango wine (7.3^{o}Brix) and lastly mango wine (7.0^{o}Brix). The specific gravity readings were1012, 1015 and 1018 for mango wine, pineapple wine and a mixture of pineapple and mango wine respectively indicating, that not all the sugar was fermented by the yeast. The pH values of the wines were generally lower than those of the musts. Titratable acidity values also were higher than the values in the must before fermentation. The reducing sugar values showed that not all the sugars were

converted to alcohol, part was still left. The protein content of the wines was higher than that of the respective musts.

Table 3: Analyses of the mango, pineapple and pineapple-mango wines at the end of primary fermentation

Wine	TSS(Brix)	SG	pH	TA (%)	RS (%)	PR (%)
MW	7.0	1012	2.6	0.95	3.1	2.38
PW	7.7	1015	2.7	1.15	3.3	2.04
PMW	7.3	1018	2.6	0.98	4.1	2.21

Key: TSS = Total Soluble Solids, SG = Specific Gravity, pH = Hydrogen ion concentration, TA = Titratable Acidity, RS = Reducing Sugar, PR = Protein, MW = Mango wine, PW =Pineapple wine; PMW = Mineapple + Mango wine

Table 4 showed the total soluble solids, specific gravity, pH, titratable acidity, reducing sugar and protein content measured after secondary fermentation for two months. Analyses of the wines revealed that, there was a reduction in soluble solids, specific gravity, and reducing sugars. On the contrary, the pH, titratable acidity and protein content increased slightly when compared to results obtained at the end of primary fermentation. The percentage alcohol of the mango wine was 11.5 %, that of pineapple wine 10.9 % and a mixture of mango and pineapple wine was 10.1 %.

Table 4: Analyses of the mango, pineapple and pineapple + mango wines after secondary fermentation

Wine	TSS(^{o}Brix)	SG	pH	TA (%)	RS (%)	PR(%)	%Al
MW	5.0	1005	3.7	1.02	1.5	2.55	11.5
PW	6.0	1010	3.5	1.35	2.7	2.21	10.9
PMW	7.0	1016	3.8	1.07	3.8	2.28	10.1

Key: TSS = Total Soluble Solids, SG = Specific Gravity, pH = Hydrogen ion concentration, TA = Titratable Acidity, RS = Reducing Sugar, PR = Protein, AL = Alcohol, MW = Mango wine, PW =Pineapple wine and PMW = Pineapple-Mango wine

Organoleptic analysis

The organoleptic assessment using hedonic scale by five persons based onappearance (clarity), aroma and taste showed that pineapple wine was the clearest, followed by pineapple-mango wine and then mango wine. The aroma of pineapple wine was also rated the

highest, followed by pineapple-mango wine and then mango wine. However, mango wine had the most acceptable taste.

DISCUSSION

The isolation of *Saccharomyces species* from ripe fruits of *Mangifera indica* and *Ananas comosus* is consistent with the earlier reports that yeasts are usually associated with fruits surfaces. Wine yeasts such as *Saccharomyces cerevisiae* and other yeasts such as *Brettanomyces bruxellensis, Hansemiaspora uvarum, Saccharomyces rosei, Pichia fermentans* and *Hypopichia burtoni*have been isolated from grapes and oranges((Doyle *et al.,*2013; Boboye and Dayo-Owoyemi, 2009).

The isolation of 14% ethanol tolerant *Saccharomyces cerevisiae* from mango and pineapple up indicates that good wine yeasts can be obtained from the fruits. Good wine yeasts are known to tolerate high alcohol levels (Berry, 1987). *Saccharomyces cerevisiae* can tolerate up to 14 % ethanol concentration (Doyle *et al*., 2013). Adaptation of yeast to high alcohol concentration apparently occurs due to cross stress protection and adaptive stress response. Perhaps, the phenomenon of cross-stress protection occurs as a consequence of the general stress response mechanism which is activated under mild stress conditions (Stanley *et al.,* 2010). Four endogenous *Saccharomyces cerevisiae* genes *INO1, DOG1, HAL1* and *MSN2* have been identified as over expression targets eliciting improved tolerance to ethanol (Hong *et al.*, 2010). Yeast cells exposed to ethanol are known to synthesize a range of heat shock proteins HSPs which include HSP104 and HSP12. HSP104 acts as a remodeling agent in the disaggregation of denatured protein whereas HSP12 is a membrane-associated protein that can protect liposomal membrane integrity against desiccation and ethanol (Stanley *et al*., 2010).

The percentage alcohol of the mango, pineapple and mango-pineapple wines of 10.1 to 11.5 % (Table 4) closely resembles fruit wines alcoholic content of 10.0 to 11.5 % as reported Owuama and Saunders(1990), Reddy and Reddy (2006), Li *et al.* (2010), Akubor (1996), Obisanya *et al*. (2008) and Kumar *et al*. (2009). The variations in percentage alcohol produced in different reports may be attributed to the fruit variety and the strains of yeast used.

. The mango must had protein content of 0.51 % and pH of 3.3 while pineapple must showed protein content of 0.68 % and pH of 3.6 (Table 1). These values are closely related to earlier reports on mango juice with protein content of 0.50 % and pH range of 3.8-4.7 and pineapple juice with protein content of 0.54 % and pH range of 3.3-3.7 (Chia and

Wanitprapha, 2008, Williams *et al*., 1987 and Beuchant, 1987). The pH of 3.3 and 3.6 for mango and pineapple musts are closely resemble the pH of grape (3.0-5.5) as reported by Frazier and Westhoff (2013).

During fermentation, the total soluble solids (Fig. 1), reducing sugars (Fig. 4) decreased progressively. Total soluble solids are part of the reducing sugars and as such the reduction in reducing sugars resulted in decrease in soluble solids. This agrees with the observation in wine production with blood plum (*Haematostaphisbarteri*) (Owuama and Habu, 1991) and yellow plum (*Xiemenia americana*) (Owuama and Mhay, 1992). The titratable acidity (Fig. 2), pH (Fig. 3) and percentage alcohol production (Fig. 5) increased with time in all the musts. The decrease in specific gravity and reducing sugars occurred because of the conversion of reducing sugars to alcohol by yeast activities. As more sugars were converted to alcohol, there was a drop in hydrometer reading from 1090 to 1012 in the fermenting mango must, 1090 to1015 in pineapple must and 1090 to1018 in mango-pineapple must at the end of primary fermentation. The pH and titratable acidity increased because more organic acids were produced alongside alcohol and carbon dioxide during fermentation (Alais and Linden, 1999). The protein content at end of fermentation showed a substantial increase for mango wine from (0.51 %-2.38 %), for pineapple wine from (0.68 %-2.04 %) and for mango-pineapple wine from (1.02-2.21 %) (Table 3). This may be attributable to the death of some yeast cells which may have released protein into the fermenting medium resulting in an increase in the protein content of the wines. This agrees with the observation that, although, *Saccharomyces cerevsiae* is ethanol tolerant, relatively high ethanol concentration inhibit cell growth, viability and consequently cell death (Stanley *et al*., (2010).

The results of organoleptic characteristics or sensory properties of the three wines produced using hedonic scale differ markedly. The result obtained indicated that the pineapple wine was the clearest, followed by mango-pineapple wine then mango wine. Pineapple wine was the clearest probably because of the presence of a proteolytic enzyme, bromelain in pineapple (Taussig and Batkin, 1988) which breaks down the protein in the pineapple must thus decreasing the soluble solids and increasing the clarity of the pineapple wine. The level of clarity in pineapple-mango wine is apparently because of the same proteolytic enzyme which may have contributed to the breaking down of the protein in the mixed musts. Clarity in mango wine was the least apparently because of lack of hydrolytic enzymes. This agrees with the observation that mango wine have problem with self-clarification, as it appears slightly cloudy and would invariably require treatment with

clarifying agents (Owuama, 2011, pers. communi). However, all the wines had good aroma and taste and were found acceptable.

CONCLUSION

The results from this study reveal that fruits of *Mangifera indica* and *Ananas comosus* are good sources of high ethanol tolerant yeast suitable for wine production. The sensory analysis of the wines produced revealed pineapple wine to be the clearest, followed by pineapple-mango wine and lastly mango wine. This is an indication that mango wine has problem with self- clarification, thus combining mango must with that of pineapple will help to solve this problem. Also further work should be done towards the commercialization of wine production using mango and pineapple fruits to ameliorate huge wastage and economic losses usually incurred annually.

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