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Research Article

Microbial Populations in Sweet Sorghum Juice during Fermentation

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Abstract

The specific objective of this study was to investigate the effect of different levels of yeast inoculation and different times of yeast inoculation on the microbial populations and ethanol yield in sweet sorghum juice. Targeted microorganisms included Total Plate Count, lactic acid bacteria, *coliforms*, *E. coli* and *yeast*. Sweet sorghum was pressed with a small-scale roller press, and the resulting juice was fermented at room temperature in 0.5 L vessels. Yeast was inoculated at levels of 0.13 and 0.26 g/l, and inoculation time was varied from 3 to 48 hr after pressing. Samples were plated at intervals of 12, 24 and 48 hr after inoculation onto petrifilms containing selective media for the organism of interest. Results indicated that growth characteristics of the microorganisms were not affected by yeast inoculation time. Yeasts, and especially lactic acid bacteria, increased irrespective of the time of inoculation. *Coliforms* generally decreased during the fermentation process, regardless of inoculation factors. Growth characteristics of the microorganisms were unaffected by the level of yeast inoculation. Initial levels of bacterial populations did vary with harvest date, but it is hypothesized that varying environmental conditions (temperature, humidity and soil conditions) during harvest and the way the harvest is conducted contributes to the initial differences in levels of microflora. Juice inoculated soon after pressing yielded more ethanol than the juice inoculated later in time. But statistical analysis showed no significant difference in the ethanol yield between the two levels of yeast inoculum tested.

Keywords: Sweet sorghum; ethanol; *yeast*; microorganism; fermentation.

Introduction

Renewable fuel represents a positive alternative to fossil fuels. In addition to being renewable, biofuelsare also reported to produce lower emissions [1]. Ethanol is the most widely used alternative transportation fuel [2]. Corn is the major source of fuel ethanol in the US at present, but extensive use of corn ethanol has caused some controversy. Recently, sweet sorghum has been identified as a feedstock for ethanol production. There are several characteristics that make sweet sorghum favorable for fuel ethanol production [2]: the presence of directly fermentable sugars, its ability to grow on all continents (tropical, sub-tropical and temperate regions), its ability to grow in marginal cultivable land, poor quality soils and semi-arid regions, and relatively low input requirements. The directly fermentable sugars in sweet sorghum represent an important advantage for ethanol production because the pressed juice is simply inoculated with yeast and allowed to ferment under anaerobic conditions. However, this advantage is also linked to a disadvantage. The directly fermentable sugars are unstable, and will deteriorate rapidly after harvest if not processed immediately.

The presence of numerous different types of microorganisms in unsterilized sweet sorghum juice has been documented by others [3]. Contaminating microflora can affect ethanol production by reducing carbon available for conversion to ethanol, competing for nutrients needed by yeast cells, and producing toxic byproducts like lactic and acetic acid [3]. Lactic acid bacteria are the primary bacterial contaminants in the fermentations for producing fuel ethanol [4]. Tolerance to high temperature, low pH and their rapid growth rate make lactic acid bacteria the most bothersome [5]. In the natural ecosystem, lactic acid *bacteria* and *yeast* are often encountered together, which could be in competition for the same nutrients [6]. Previous sweet sorghum juice fermentation studies also suggest that delayed *yeast* inoculation time will negatively affect ethanol yield, and it was hypothesized that this was due to changes in growth of other microbial populations during the delay in inoculationn time. In order to better understand this deterioration process, studies must be conducted to evaluate the native microflora of the juice and to analyze how they behave during the period of time immediately after harvest through yeast inoculation.

The main objectives of this study were to evaluate the microbial

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populations in freshly pressed sweet sorghum juice and to determine the effects of harvest conditions, yeast inoculation time, and yeast inoculation level on the microbial populations and ethanol yields.

Material and Methods

Sweet Sorghum Juice

Sweet sorghum variety M81 was manually harvested from the Stillwater Cow Creek Bottom site and the juice was obtained by pressing the stalks using a small scale roller press described elsewhere [7]. Table 1 lists the harvest events used for microbial testing, including planting dates and harvest dates. The juice was collected in a clean five gallon container and immediately taken to the laboratory for treatment. In the laboratory, juice was distributed into 500ml polypropylene containers (Lab-Tek Multi-Purpose Lab Containers, VWR) with airtight lids. Each container was filled with 430 mL of sweet sorghum juice. Clear vinyl tubing (0.60 cm outer diameter and 0.40 cm inner diameter, Watts) was inserted through the airtight lid making sure that the end did not touch the juice and the other end was inserted into a beaker of water to form an airlock. Each container was sampled every 12 hr for microbial analysis and pH measurement. A sterile 10 mL syringe (BD, Luer-Lok™ Tip) and stainless steel needle 15.2 cm in length were used to mix and draw 10 mL of each sample into clean sample tubes for pH measurement (Oakton pH 110 series pH meter, EUTECH instruments, Singapore) and plating on to petrifilm for enumeration.

Table 1: Harvest events used for microbial testing.

Harvest Number	Plot Number	Date Planted	Date Harvested
1	1	04/24/09	09/03/09
2	2	05/19/09	09/23/09
3	2		11/25/09
4	3	06/15/09	11/05/09
5	3		11/25/09

Experimental Design

Two containers were labeled as controls and remained uninoculated while two containers (replicates) each were labeled for inoculation at 3, 12, 24 and 48 hr after pressing, respectively. Each container contained 430 mL of the juice. A similar set was labeled for 2nd level of yeast inoculation at 3, 12, 24 and 48 hr. Each pair of containers was sampled every 12 hr after inoculation with yeast for microbial populations and pH measurement. A sterile 10 mL syringe (BD, Luer-Lok™ Tip) and stainless steel needle 15.2 cm in length were used to mix and draw 10 mL of each sample into clean sample tubes for pH measurement (Oakton pH 110 series pH meter, EUTECH instruments, Singapore) and plating on to petrifilm for enumeration.

Yeast

Super Start Distillers yeast (Crosby & Baker, ALLTECH) was used for fermentation. *Yeast* stored at 4^{0}C was weighed into sterile polypropylene centrifuge tubes. The first level of yeast inoculation was 0.13 g/L and the second level was 0.26 g/L. The *yeast* was hydrated with 15 ml warm water for 15 minutes before inoculating the juice. The samples for end product analysis were taken no sooner than 5 days after inoculation, ensuring that all samples had been allowed to proceed to completion. The amount of ethanol in the uninoculated juice and the fermented juice was analyzed using an Agilent 1100 Series Liquid Chromatograph with BIORAD HPLC Organic Acid Analysis Column and Aminex HPX-87H Ion Exclusion Column (Agilent Technologies, Santa Clara, CA).

Microorganisms

The organisms of interest were Total Plate Count, lactic acid bacteria, yeast and coliforms/*E.coli*. Petrifilms from 3M microbiology (www.3M.com/microbiology) were used for enumerating the microbial populations. After the pH of the samples was measured, sweet sorghum juice was serially diluted (1: 10 dilution). One mL of juice from each sample tube was transferred into a test tube consisting of 9 ml peptone water and homogenized, representing a 10^{-1} dilution. Serial dilutions of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} were also prepared. One mL of each diluted suspension was pipeted onto a 3M Petrifilm plate and evenly spread using a plastic spreader. Petrifilms were incubated in a horizontal position in stacks of no more than 20 plates at 35^{0}C ± 1^{0}C for 48 hr according to the manufacturer's instructions (3M Corporation Headquarters, St. Paul, MN). All Petrifilms were interpreted and the colonies enumerated using a QUEBEC dark field colony counter from LEICA (Buffalo, New York).

Statistical Analysis

Statistical analysis of the data was performed using two sample t-tests, one way ANOVA, and Tukey's multiple comparison tests. The level of significance used for analysis was 0.05 (α=0.05).

Results and Discussion

Initial Microbial Populations in Sweet Sorghum Juice

The largest microbial counts were found in total plate count, followed by *yeast* and lactic acid bacteria, and the lowest counts were found in coliforms. Comparisons were made to determine whether variations in initial microbial level were affected by planting date, level of maturity, or harvest date. When comparing the three different planting dates, yeast levels were significantly different between 2 of the 3 dates, but all other microbial levels were not significantly different. This suggests that different planting dates don't likely result in significantly different microbial levels. Juice that was harvested from three different plots at similar levels of maturity was also compared (Table 2). Significant differences in *yeast*, *lactobacilli* and *coliform* counts resulted, but total bacteria were not different.

Interestingly, when microbial populations were compared for samples harvested on the same day, even though they were from

Table 2: Microbial counts from plots 1, 2 and 3, at similar levels of maturity.

Organism	Mean Microbial Populations* (Log_{10}CFU/mL)		
	Plot 1 - 138 Days After Planting **Planted: 04/24/09** **Harvested: 09/03/09**	**Plot 2 - 128 Days After Planting** **Planted: 05/19/09** **Harvested: 09/23/09**	**Plot 3 – 144 Days After Planting** **Planted: 06/15/09** **Harvested: 11/05/09**
Total aerobic bacteria	6.9 ± 0.01[a]	7.0 ± 0.08[a]	7.0 ± 0.02[a]
Lactobacilli	5.5 ± 0.34[a]	6.0 ± 0.01[ab]	6.6 ± 0.09[b]
Yeast	5.1 ± 0.12[a]	6.1 ± 0.10[b]	6.1 ± 0.22[b]
Total coliforms	5.4 ± 0.04[a]	5.0 ± 0.01[b]	4.5 ± 0.04[c]

** n=2 for each data point

*Means followed by the same letter within a row are not significantly different (p>0.05)

two different planting dates and two different levels of maturity, no significant differences were found (Table 3). This suggests that the harvest event is an important factor in determining microbial levels in fresh sweet sorghum juice. Environmental conditions during harvest such as temperature, humidity, and soil condition can affect the microbial load. These factors can impact the amount of soil picked up by the plant entering the press, and hence, contaminant loads in the press. The number of coliforms, especially *E. coli,* in sugarcane juice has been reported to be highly influenced by the amount of soil picked up on the cane after cutting, before transportation and while pressing [8]. Such changes in conditions during the harvest could have contributed to the variation in the number of microorganisms in the juice from different harvest events.

Fermentation of Sweet Sorghum Juice with Different Yeast Inoculation Times

Changes in pH During Fermentation: Changes in the pH of sweet sorghum juice inoculated at various times with level 1 (0.13 g/L) inoculation are shown in Figure 1. The initial pH was 5.12, 3.76, 3.56 and 3.42 for the juice inoculated at 0, 12, 24 and 48 hr after harvest, respectively. It can be clearly seen that the pH curves for all samples are nearly identical, regardless of yeast inoculation time. The final pH was 3.48, 3.42, 3.39 and 3.29 for the juice inoculated at 0, 12, 24 and 48 hr, respectively, after harvest. A similar pH curve was obtained for the level 2 (0.26 g/L) *yeast* inoculation.

Microbial Populations of the Sweet Sorghum Juice during Fermentation with Yeast Inoculation at Different Time Intervals

Total Plate Count: The pattern of growth observed in each inoculated sample agrees with the general bacterial growth curve, showing an initial lag phase followed by an exponential phase and a stationary phase in the case of the samples inoculated immediately after harvest and 12 hr after harvest. The growth patterns appear unaffected by the time of inoculation. A similar initial lag phase or low numbers of bacteria was also observed in the study by Daeschel (1981) while determining microbial changes in sweet sorghum juice. The average intial pH of juice inoculated at both levels of yeast was 5.14. The mildly acidic pH enabled the growth of certain bacteria other than lactic acid bacteria which are *Acinetobacter, Enterobacter, Erwina* and *Pseudomonas* [9]. The highest number of bacterial count was observed 24 hr after harvest in all the samples. The average pH of the juice samples 24 hr after fermentation was found to be 3.59, which favors the growth of most lactic acid bacteria species. This high number was a result of the dominant species *L. mesenteroides*, whose rapid multiplication was enabled by the availability of sugar and a favorable pH [9].

Lactic acid Bacteria

Figure 3 shows the lactic acid bacteria counts for sweet sorghum juice inoculated at various times. The different inoculation times did not have an effect on the lactic acid bacteria counts. Lactic acid bacteria counts increased consistently up to 24 hr after harvest, after which the counts remained constant. The same pattern of growth and numbers of the lactic acid bacteriapopulation was observed for the first level (0.13g/L) of yeast inoculation. Lactic acid bacteria are acid tolerant microorganisms. They have the ability to grow in a wide range of pH in the presence of organic acids. Their mechanism of acid-tolerance is not completely known [10]. The average initial pH of the inoculated juice samples was 5.14. Not all species of lactic acid bacteriagrow in this pH, which

Table 3: Microbial counts from plots planted on different days and harvested on the same day.

Organism	Mean Microbial Populations (Log_{10}CFU/mL)	
	Plot 2 **Planted: 05/19/09** **Harvested: 11/25/09**	**Plot 3** **Planted: 06/15/09** **Harvested: 11/25/09**
Total aerobic bacteria	7.9 ± 0.04[a]	7.8 ± 0.01[a]
Lactobacilli	6.2 ± 0.37[a]	5.9 ± 0.23[a]
Yeast	6.6 ± 0.05[a]	6.5 ± 0.06[a]
Total coliforms	5.0 ± 0.29[a]	5.3 ± 0.40[a]

**n=2 for each data point

*Means followed by the same letter within a row are not significantly different (p>0.05).

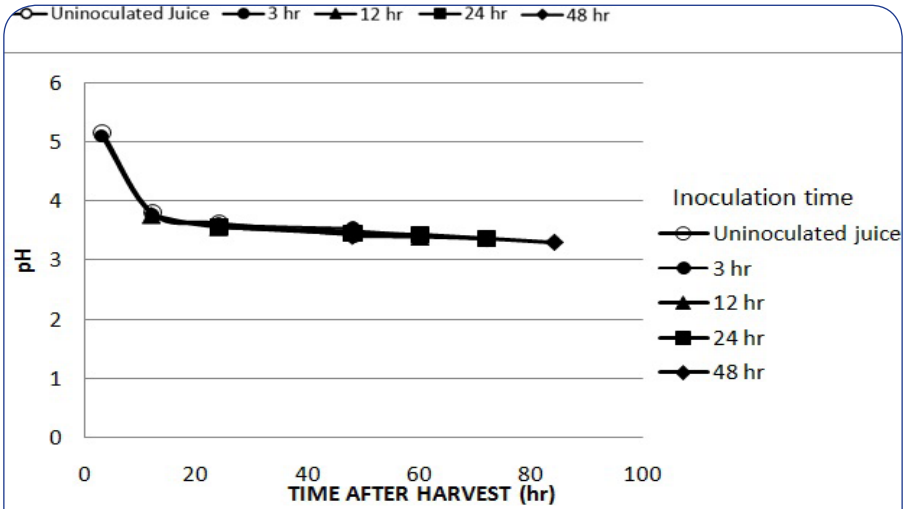

Figure 1: pH changes during fermentation of sweet sorghum juice with different inoculation times (3, 12, 24 and 48hr) after harvest with level 2 yeast (0.13g/L). Harvest event 4, n=2.

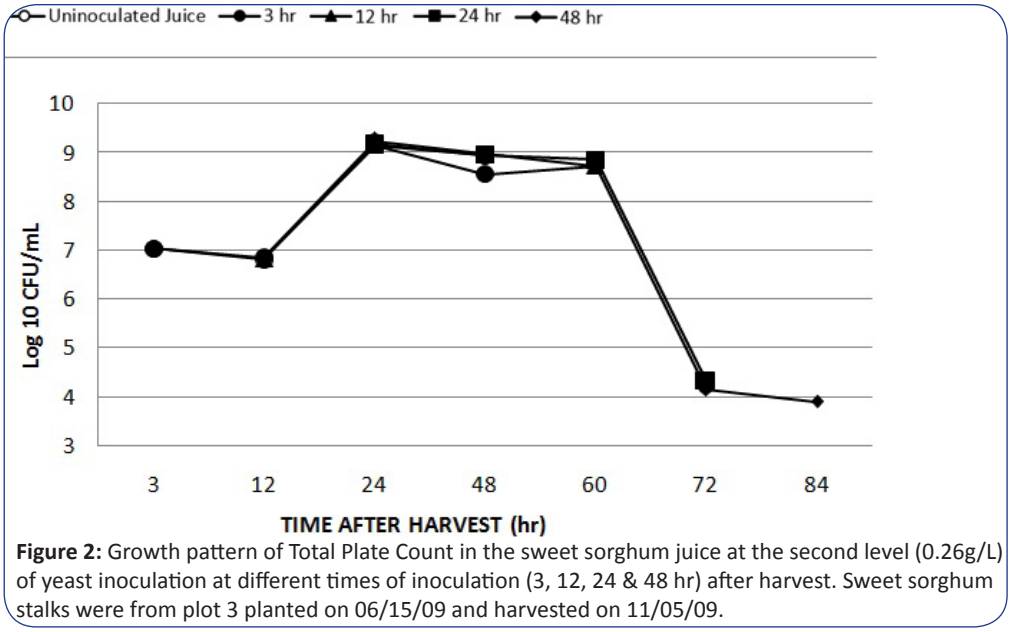

Figure 2: Growth pattern of Total Plate Count in the sweet sorghum juice at the second level (0.26g/L) of yeast inoculation at different times of inoculation (3, 12, 24 & 48 hr) after harvest. Sweet sorghum stalks were from plot 3 planted on 06/15/09 and harvested on 11/05/09.

is the reason for the low initial count of lactic acid bacteria in the fresh sweet sorghum juice. This initial pH facilitates the growth of *L. mesenteroides* which grows early during the fermentation process [10]. The highest number of lactic acid bacteria occurred 24 hr after the harvest when the average pH of the juice samples was 3.59. This population is maintained until the end of the fermentation process. This pH enabled the growth of other lactic acid bacteria species such as *L. brevis, L. fermentum*, and *L. cellobiosus.* The average final pH of the inoculated juice samples was 3.3. At this pH the most predominant organism, *L. plantarum,* likely grows during the final stages of fermentation [10].

Yeast

Figure 4 shows the yeast counts for sweet sorghum juice inoculated at various times. The growth of *yeast* inoculated at the second level (0.26 g/L) showed a consistent increase irrespective of the time of inoculation. A similar growth pattern of the *yeast* population was observed for the first level (0.13 g/L) of yeast inoculation. The most dominant species in the juice is likely *C. intermedia* followed by *C. krusei*, *S. cerevisiae, S. montanus, Cryptococcusspp., Pichia membranaefaciens, and Rhodotorula spp* [9]. The naturally present yeast also plays a significant role in the manufacture of liquor from sugarcane products [11]. Although natural yeasts are present, little

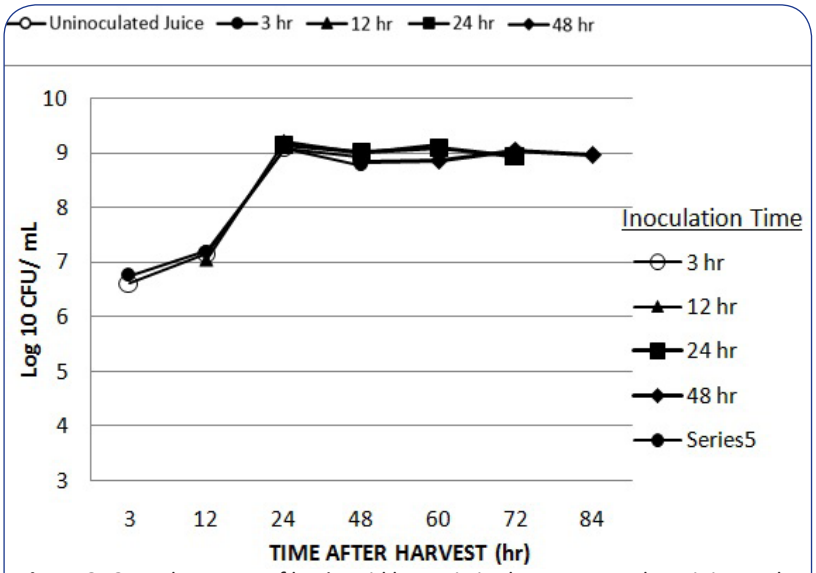

Figure 3: Growth pattern of lactic acid bacteria in the sweet sorghum juice at the second level (0.26g/L) of yeast inoculation at different times of inoculation (3, 12, 24 & 48 hr) after harvest. Sweet sorghum stalks were from plot 3 planted on 06/15/09 and harvested on 11/05/09.

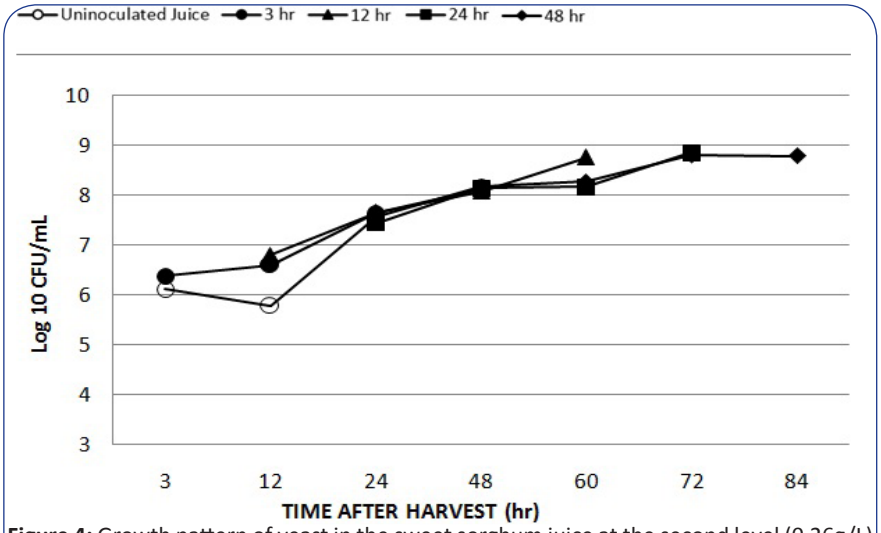

Figure 4: Growth pattern of yeast in the sweet sorghum juice at the second level (0.26g/L) of yeast inoculation at different times of inoculation (3, 12, 24 & 48 hr) after harvest. Sweet sorghum stalks were from plot 3 planted on 06/15/09 and harvested on 11/05/09.

is known about their fermentation efficiency to produce the desired products in the required large quantities, which is the reason for the addition of external *yeast* sources to obtain high fermentation efficiency and consistency.

Total Coliforms

Figure 5 shows the total *coliform* counts for sweet sorghum juice inoculated at various times. The coliforms showed a consistent drastic decrease in their population at both levels of *yeast* inoculation (0.13 and 0.26 g/L) regardless of the time of inoculation. In addition, *E. coli* were present in numbers that were too low to count at both levels of *yeast* inoculation, regardless of inoculation time. The initial high *coliform* numbers were due to the mildly acidic pH and the presence of nutrients providing a favorable environment for their growth. Decrease in the number of coliforms was due to the production of organic acids by the lactic acid bacteria that began increasing 12 hr after the juice was harvested. The organic acids caused a rapid reduction in the pH creating an environment selective against the growth of less-acid-tolerant organisms like coliforms. *E.coli* occurred in numbers that were too low to count or simply did not occur in comparison to the coliforms that were in the countable range. It is assumedthat most of the coliformsthat occur in the sweet sorghum juice should be of *Aerobacter* types [9].

Fermentation of Sweet Sorghum Juice with Different Levels of *Yeast* Inoculation

Changes in pH with Different *Yeast* Inoculation Levels: Figures 6 shows pH changes in juice inoculated with level 2 *yeast.* The change in pH was generally consistent for all treatments. The average initial pH of the fresh sweet sorghum juice from all harvest events was 5.14. The average final pH was 3.3 , for juice inoculated with 0.13 g/L (first level) and 0.26 g/L (second level) *yeast*..

Microbial Populations of the Sweet Sorghum Juice During Fermentation with Two Levels of *Yeast* Inoculation

Figure 7 shows the total plate counts for samplesinoculated with two levels of *yeast*. Comparison of the total plate count shows similar trends in growth pattern and number in the sweet sorghum juice inoculated with level 1 and level 2 of yeast 12 hr after harvest. This similarity in growth pattern and number between the two yeast inoculation levels was observed in the juice samples from both the harvest events 2 and 4. Similar results were observed for lactic acid bacteria, *yeast* and *coliforms*. Level of yeast inoculation had no significant impact on the microbial populations in the sweet sorghum juice.

inoculation 12 hr after harvest. Harvest 4 was from plot 3 planted on 06/15/09.

Ethanol Content in the Sweet Sorghum Juice After Fermentation

Figure 8 shows the effect of inoculation time and inoculum level on ethanol yield for harvest 1. Results showed higher ethanol yield from samples inoculated immediately after pressing. Samples with higher level (0.26 g/L) of *yeast* yielded more ethanol than the samples inoculated with the lower level of *yeast* (0.13 g/L). The control (no *yeast* added) had very low ethanol.

Results from the ethanol analysis reveal that juice samples inoculated immediately after harvest provides the optimum conditions for maximum ethanol yield. The high sugar content of the fresh juice, optimum pH (5.14) and temperature provided yeast ideal fermentation conditions to produce a large amount of ethanol. At this pH yeast could compete sooner with lactic acid bacteria and other bacteria for carbohydrates and other nutrients. This is the initial lag phase for other native microflora, which are

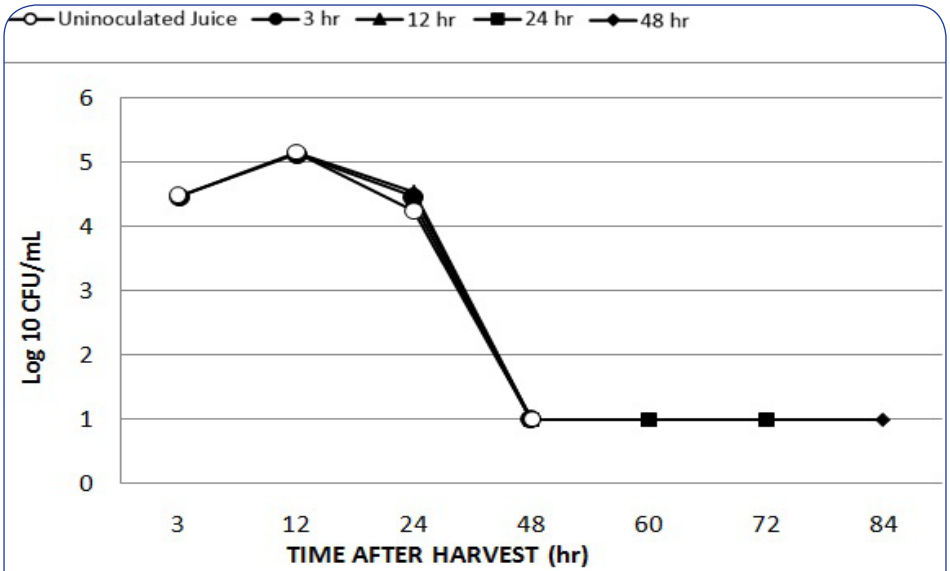

Figure 5: Growth pattern of coliforms in the sweet sorghum juice at the second level (0.26g/L) of yeast inoculation at different times of inoculation (3, 12, 24 & 48 hr) after harvest. Sweet sorghum stalks were from plot 3 planted on 06/15/09 and harvested on 11/05/09.

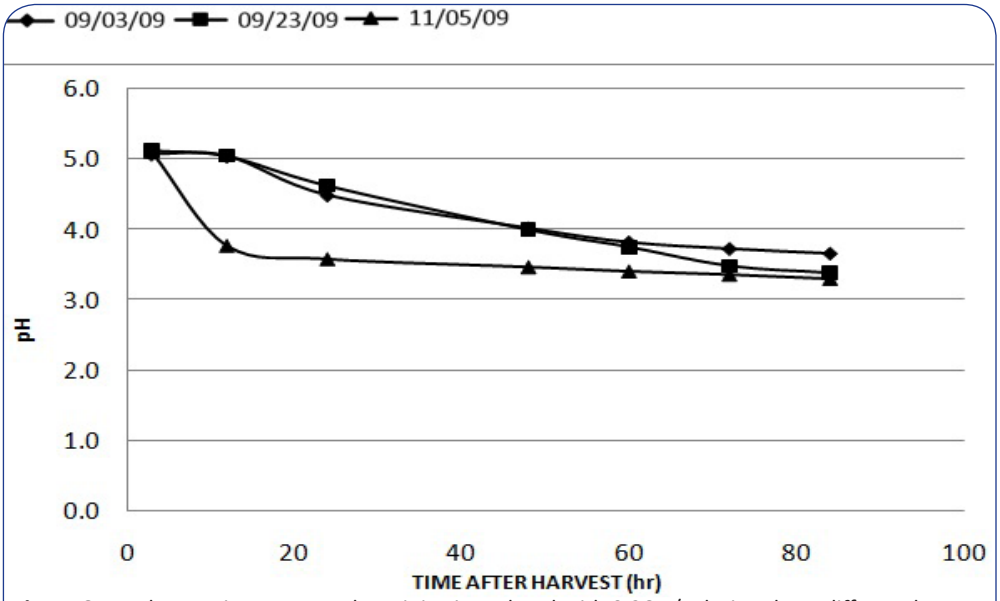

Figure 6: pH changes in sweet sorghum juice inoculated with 0.26 g/L during three different harvest events.

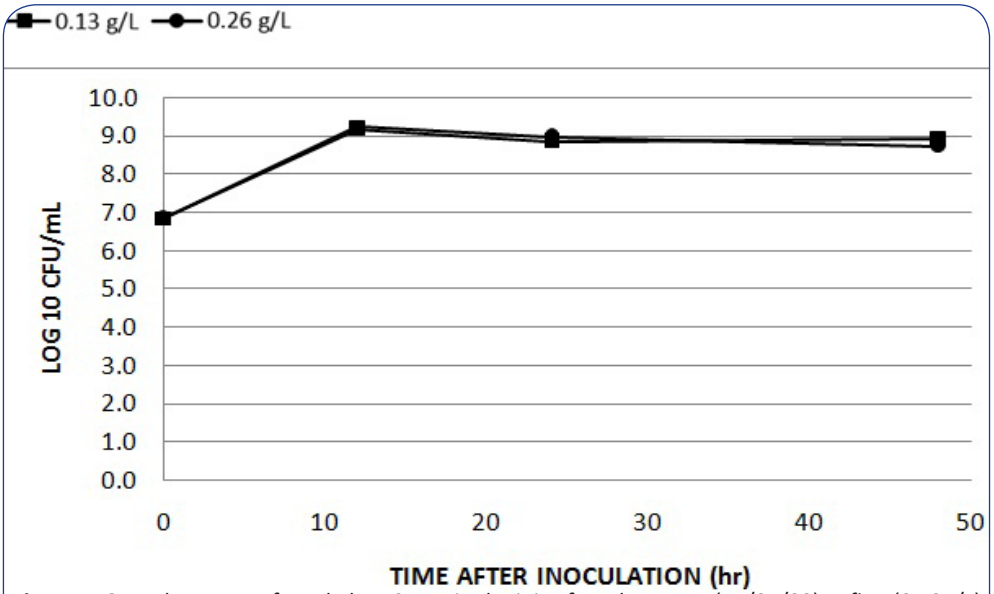

Figure 7: Growth pattern of Total Plate Count in the juice from harvest 4 (11/05/09) at first (0.13g/L) and second level (0.26g/L) of yeast inoculation 12 hr after harvest. Harvest 4 was from plot 3 planted on 06/15/09.

still in the preparatory stages of growth. The external source of yeast that was reconstituted with warm water and inoculated was ready to grow, multiply and metabolize, and hence took advantage of these conditions to ferment the sugars in the fresh juice to ethanol and carbon dioxide. 12 hr after harvest, the decrease in ethanol is due to the fall in pH and the competition for carbohydrates and other nutrients from *L. mesenteroides* that appears early during the fermentation [10]. As the pH decreased further down to around 4.0 the other lactic acid bacteriaspecies (*L. plantarum, L. brevis, L. fermentum*, and *L. cellobiosus*) began to grow, which further increased the competition for sugars and nutrients [9]. This competition decreased the growth and metabolism of yeast. As fermentation neared its final stages around 48 hr after inoculation, *L. plantarum* usually terminates the fermentation, dominates the microbial population and competes heavily with yeast that has already reduced its rate of metabolism and growth [5]. This high population of lactic acid bacteria likely resulted in the accumulation of lactic acid that in turn resulted in increased acidity, lower yeast concentration, reduced carbohydrate utilization, and reduced ethanol yields [12].Table 4shows the mean ethanol content in samples from harvest 1 inoculated with level 2 yeast at different times. The highest ethanol yield was obtained when the juice was

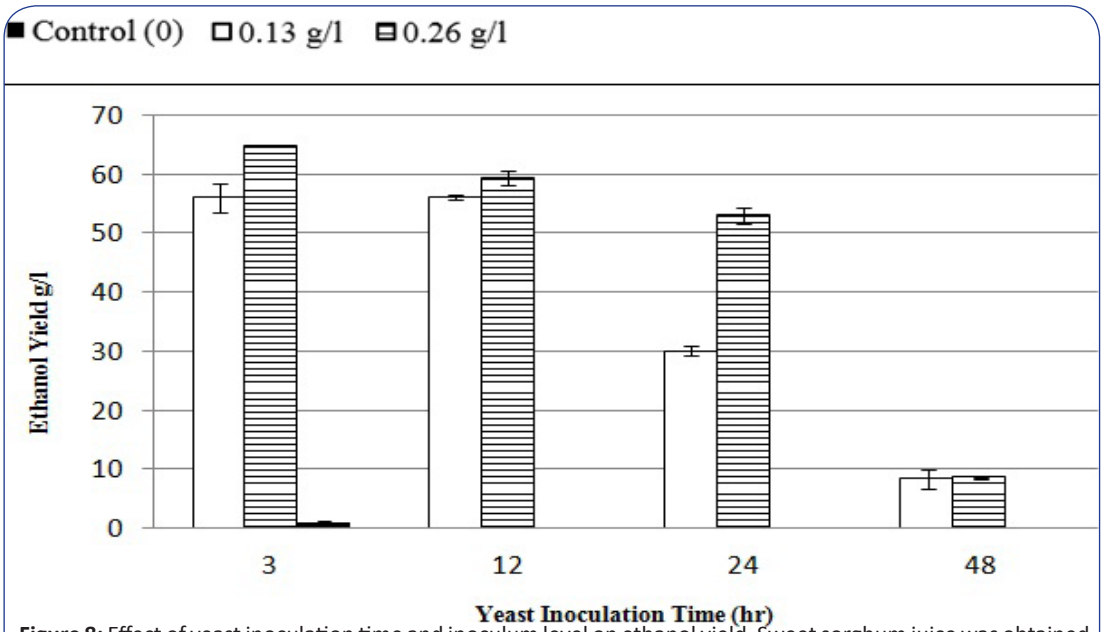

Figure 8: Effect of yeast inoculation time and inoculum level on ethanol yield. Sweet sorghum juice was obtained during harvest 1 (09/03/09) from Plot 1 planted on 04/24/09.

inoculated immediately after harvest. Statistical analysis showed significant differences in the amount of ethanol obtained when sweet sorghum juice was inoculated at different times.

Table 4: Mean ethanol content (g/L) in sweet sorghum juice from harvest 1 when inoculated with level 2 (0.26 g/L) yeast at different times.

Time of Inoculation After Harvest (hr)	[1]Mean Ethanol Content (g/L)
3	64.62a
12	59.33b
24	52.94c
48	8.54d

n=2 for each data point

Means followed by the same letter within a column are not significantly different (p>0.05).

Conclusion

During fermentation of unsterilized sweet sorghum juice, ethanol yield can be affected by time of yeast inoculation. It was therefore hypothesized that changes in yeast inoculation time were affecting microbial population in the juice. That hypothesis was disproven in this work, and clearly showed that microbial populations in sweet sorghum juice are not affected by *yeast* inoculation time. pH changes in the juice were not affected by yeast inoculation time nor inoculation level. Growth characteristics of the microorganisms were not affected by yeast inoculation time. Yeasts, and especially lactic acid bacteria, increased irrespective of the time of inoculation. Coliforms generally decreased during the fermentation process, regardless of inoculation factors. Growth characteristics of the microorganisms were also unaffected by the level of yeast inoculation. However, there were significant differences in ethanol yield at each different inoculation time.Juice inoculated soon after pressing yielded more ethanol than the juice inoculated later. There were no significant differences in the ethanol yield between the two levels of yeast inoculum tested.

The largest microbial counts were found in total bacteria, followed by *yeast* and *lactobacilli*, and the lowest counts were found in coliforms. Comparisons were made to determine whether variations in microbial level were affected by planting date, level of maturity, or harvest date. When comparing the three different planting dates, yeast levels were significantly different between 2 of the 3 dates, but all other microbial levels were not significantly different. This suggests that different planting dates don't likely result in significantly different microbial levels. Juice that was harvested from three different plots at similar levels of maturity was also compared. Significant differences in yeast, *lactobacilli* and *coliform* counts resulted, but total bacteria were not different. Interestingly, when microbial populations were compared for samples harvested on the same day, even though they were from two different planting dates and two different levels of maturity, no significant differences were found. This suggests that the harvest event is an important factor in determining microbial levels in fresh sweet sorghum juice. Environmental conditions during harvest such as temperature, humidity, and soil condition can affect the microbial load. These factors can impact the amount of soil picked up by the plant entering the press, and hence, contaminant loads in the press.

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