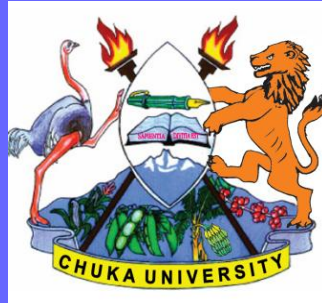


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Research manuscripts should preferably range from 2,000 to 6,000 words. Pages should be centrally numbered at the bottom. Each manuscript should contain the following parts:

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- Introduction
- Materials and Methods
- Results and Discussion, with supportive Tables, Figures/Illustrations, where applicable

- Conclusions and Recommendations
- References

8.4.1. Title Page

The title page should bear the title of the manuscript, followed by authors' names and addresses. The title should be succinct, descriptive of the research reported and not exceed fifteen (15) words. The corresponding author should be indicated with a note.

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Each research article must have an abstract that is a non-critical informative digest of the contents, conclusions and recommendations of the article. Each manuscript should have an abstract written in a single paragraph of 300 words maximum. Below the abstract, indicate keywords of up to eight for indexing purposes.

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The introduction should indicate the importance, define the problem, state the hypotheses and objectives, and give a brief survey of the relevant literature. It should follow the abstract after skipping only one space.

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This section describes the methodology employed to conduct the research and hence it should clearly describe in prose format, not as a list, the individuals/items/materials used, their sources, conditions, research design, and steps or procedures followed in experimentation to enable others adopt or double-check findings.

8.4.5. Results and Discussion

Results support or reject the hypotheses, or answer the questions stated in the introduction. The discussion section interprets the data and draws conclusions, inferences and recommendations for adoption and further research.

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Table 1. Effect of tomato varieties on fecundity and longevity of *Tetranychus evansi*

Variety	Fecundity (average eggs laid per female)	Average longevity (days)
Cal J	32.0 ^b	7.5 ^b
Onyx	23.0 ^c	7.2 ^b
Roma	23.9 ^c	6.7 ^b
Riogrande	48.6 ^a	8.2 ^b
Money Maker	52.4 ^a	12.8 ^a
Eden Fl	31.3 ^b	7.6 ^b
Anna	33.2 ^b	7.4 ^b
Wild type	9.1 ^d	4.8 ^c
Mean	31.94	7.78
SE	1.48	0.34
CV (%)	19.03	13.8

Means within a column followed by the same letter are not significantly different according to Tukey HSD test, $P = 0.05$. SE = Standard Error. CV = Coefficient of Variation

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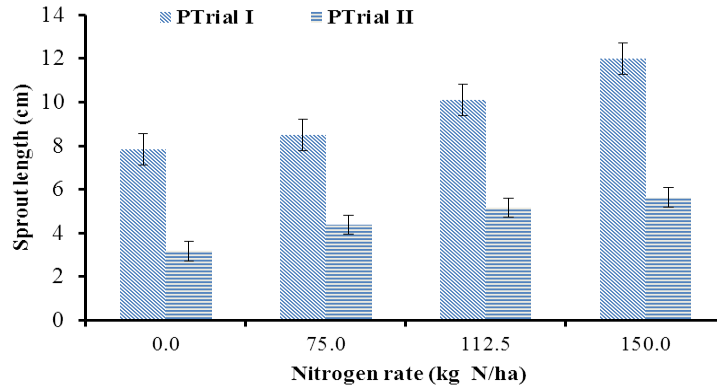


Figure 1: Effect of nitrogen rate on seed potato sprout length at 90 DAS

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Plate 1. Tomato plants grown in two types of soil: treated with 8% Lippia + 8% Ocimum showing clean healthy roots (A), and not treated (control) showing galls (B)

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-Oswell, F.N., Rufaro, M.M., Susan, K. and Arnold, B.M. 2007. Indigenous knowledge of the traditional vegetable pumpkin (*Cucurbita maxima/moschata*) from Zimbabwe. *African Journal of Agricultural Research*, 2(12):649-655.

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Footnotes must be avoided in the main body of the manuscript.

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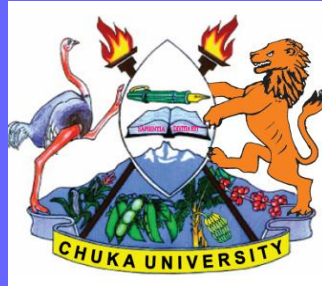
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Journal of Environmental Sustainability Advancement Research

Contents	Volume: Year: Pages

YIELD MAXIMIZATION OF ETHANOL BY METABOLISM OF UNFERMENTED SUBSTRATE IN COCONUT PALM SAP WINE

Okal, E.J.¹, Chimbevo, M.L.*¹, Kahindo, J.¹ and Agoi, L.K.¹¹Department of Pure and Applied Science, Technical University of Mombasa, P. O. Box 90420-80100, Mombasa³Department of Biochemistry, Mt. Kenya University, P. O. Box 342-01000, Thika

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Abstract

Palm wine (*Mnazi*) tapping is a socio-economic activity among the Mijikenda community at the Kenyan coast. It is prepared for economic, religious and cultural purposes. It contains lots of fermentable compounds. However, not all is fermented to generate ethanol as the end product by the naturally occurring wild yeast. This study determined the level of fermentable compounds, unfermented substrates and ethanol yields of both natural and controlled fermentation. Fresh palm wine (*Mnazi*) samples tapped overnight were collected from Chumani, Rabai and Mtwapa in Kilifi County into sterile bottles and placed in a cool box at below 4°C. The samples were fermented under natural and controlled conditions using *A. niger* and *S. cerevisiae* for six days. Substrate levels before and after fermentation were then quantified. The student's t-test was used to compare differences between fermented substrates, soluble solids, pH and ethanol content in fresh *Mnazi* and natural and controlled fermented *Mnazi*. Fresh *Mnazi* had high percentages of fermentable substrates (10.1% sucrose, 2.3% glucose and 0.6% fructose), soluble solids (14.6%) and pH 6.0. Ethanol increased while percentages of unfermented substrates, pH and soluble solids in *Mnazi* decreased after both natural and controlled fermentation. The levels of fermented substrates, total soluble solids and pH differed significantly ($P < 0.05$) after both natural and controlled fermentation. Thus controlled fermentation using *A. niger* and *S. cerevisiae* generated higher ethanol levels than natural process and can be simultaneously used in industrial ethanol production from *Mnazi*.

Key words: Fermentation; Fermentable substrates, Ethanol; Palm wine

INTRODUCTION

Palm wine (*Mnazi*) tapping is a common socio-economic activity among the Mijikenda community at the Kenyan coast (Kadere et al., 2009). The wine is prepared for economic, religious and cultural purposes (Waijeng et al., 1993). It contains lot of fermentable compounds such as sugars, proteins, vitamins, phytochemicals and other organic compounds (Kadere et al., 2004; Eze and Ogan, 1988). However, not all the sugars in the palm sap are fermented to generate ethanol; the end product by the natural occurring wild yeast.

Unlike wine made from grape, the fermentation of palm wine is not controlled, leading to wide variability in the ethanol produced. Further fermentation converts the ethanol into acetic acid, lactic acid and tartaric acid, a transition that makes it to have sour test (Kadere and Kutima, 2012). If the fermentation is allowed to continue further for 24 hours, vinegar is produced and *Saccharomyces cerevisiae*, *Sachioschromyces probe*, *Lactobacillus plantunum*, *Leuconostoc spp.* and *Leuconostoc mesenteries* are responsible for this fermentation (Kadere et al., 2008). Palm wine may also consist of pathogenic bacteria e.g. *Serratia*, *Micrococcus* and

Klebsiella; and probiotic bacteria e.g. *Lactobacillus* and *Pediococcus* (Kadere et al., 2008). The theoretical alcohol yields lies in the range of 9-10% with natural fermentation process of the palm wine of about 5%-6% (Kumuthini et al., 1988).

It is therefore clear that most of the sugars in the palms sap are not converted to ethanol, a product which is of great industrial significance. The lower level of ethanol obtained by the traditional method could be associated with the natural process and the microbes involved. The method also accounts for 1.0%-2.0% alcohol loss by injudicious handling (Kadere et al., 2009).

Most of the *Mnazi* prepared in the Kenyan coast is done by small-scale tappers with the intention of making the local brew on a small-scale level (Gachanja et al., 2007), and yet large amount of the sugars in the palm wine remains unfermented. Moreover, the wine goes bad a few days after tapping, thus it cannot be prepared locally on large scale. Furthermore, there are no preservation methods in place, thus a lot of the wine goes to waste.

With the current great demand for ethanol to meet the energy demand of alternative liquid fuel for automobiles and industrial use, production of industrial ethanol from fresh tapped palm sap would be suitable alternative. *Mnazi* has been associated with irresponsible behaviour and poor health, especially among men and youths in the Kenyan coast (Mwachiro and Gakure 2011; Kadere et al., 2009). Mechanisms to ensure unfermented sugars are utilized to make important products need to be put in place through metabolism of the unfermented substrates using microorganisms such as *A. niger* and *S. cerevisiae* in controlled fermentation. This will lead to production of palm sap on large-scale for commercialization activities such as production of industrial ethanol.

Production of other useful products from this important resource will be a major step in curbing the negative effects of *Mnazi* brewers. This will enhance economic growth and minimize the social and health problems associated with excessive *Mnazi* drinking. The present study aimed at determining the level of total sugars, solid content of fresh palm sap, quantity of the unfermented substrates and ethanol yields of both natural and controlled fermentation.

MATERIALS AND METHODS

Sample Collection and Storage

Fresh palm wine (*Mnazi*) samples tapped overnight were collected from Chumani, Rabai and Mtwapa (Kilifi County) and placed in a cool box at below 4°C to prevent natural fermentation by the naturally occurring yeasts and bacteria. It was transported to the laboratory of Pure and Applied Science Department at the Technical University of Mombasa and stored below 4°C to prevent fermentation.

Fermentation of the Palm Sap

In natural fermentation, samples of fresh palm wine obtained from tappers were fermented under natural conditions for six days. No chemicals or substances were added into the samples. Ethanol content, pH, sucrose and soluble solid content were quantified after fermentation. In *controlled fermentation*, strains of *S. cerevisiae* and *A. niger* were obtained from the Microbiology Laboratory in the Department of Pure and Applied Sciences of the Technical University of Mombasa. The organisms were maintained on Potato Dextrose Agar slants at 37°C. The sap was pasteurized by boiling for 15 minutes in boiling water bath and allowed to cool before inoculation with a 5 ml of *S. cerevisiae* and

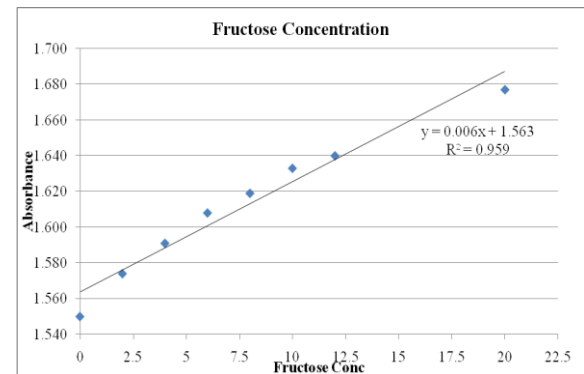
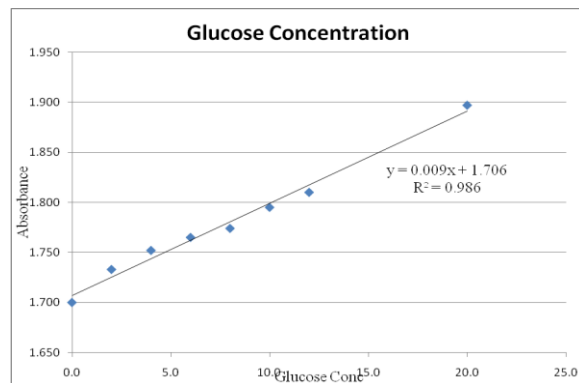
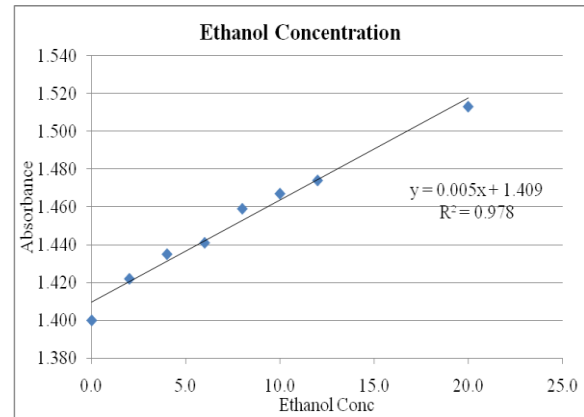
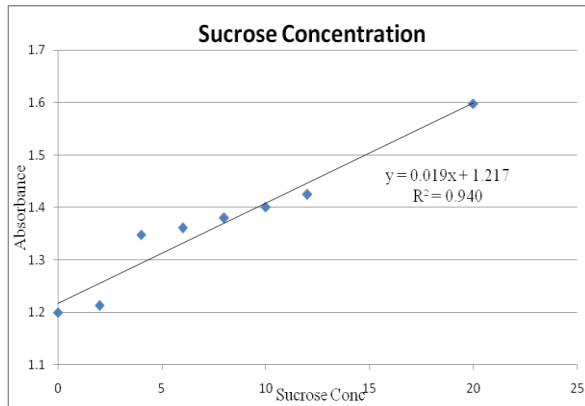
A. niger. Fermentation processes were performed in 250 ml flasks where each fermentation media was inoculated with *A. niger* and incubated at 37°C for 3 days. The *S. cerevisiae* was added in the fermenting broth and fermentation continued for 4 days. The pH of the medium was adjusted to 5.5 for each experiment using normal HCl or NaOH. Inorganic salts 0.5 g/L NH₄Cl, 0.2 g/L, MgSO₄ and 0.5 g/L KH₂PO₄ were added to serve as sources of nitrogen, magnesium and phosphorus, respectively. The flasks were made air-tight using a paraffin paper to maintain anaerobic conditions and incubation was done for six days.

Quantification of Substrate Levels Before and After Fermentation

Total content of sucrose, glucose, and fructose in both fresh and fermented samples was determined using modified dinitrosalicylic (DNS) method as described by Wilson and Walker (2000). A 5 ml aliquot of both fresh sap and fermented wine was centrifuged for 10 minutes. Dinitrosalicylic (3 ml) was added to 3 ml of sucrose, glucose and fructose in capped test tubes, heated at 90°C to develop red brown colour. Rochelle salt (potassium sodium tartarate) solution (1 ml of 40%) was added to stabilize the colour. After cooling at room temperature in a cold water bath, absorbance of the sugars was read from a spectrophotometer (Digital Model) at 340 nm (sucrose), 590 nm (glucose) and 490 nm (fructose) and standard curve used to calculate the unknown concentrations. Ethanol level in both fresh sap and fermented sap was determined using spectrophotometric method as described by AOAC (2000). *Mnazi* test samples (3 ml) and 2 ml of 0.1447 M K₂Cr₂O₇ in the presence of 6 ml H₂SO₄ solution were allowed to react in test tube for 30 minutes. Absorbance was measured at 560 nm and standard curve was used to calculate the unknown concentrations. The total amount of soluble solids in the palm sap was determined using a refractometer. Mass of 100 ml of each sample was measured on a scale and density was recorded using a hydrometer.

Data Analysis

Data was subjected to analysis of variance using statistical software (Digital Edition) and Microsoft Excel. T-test with $P = 5\%$ was used to compare differences between the recorded means of ethanol content. The standard solutions prepared were used to obtain regression graphs for calculating the unknown concentrations in test solutions.



RESULTS

Analysis of Substrates in Fresh Palm Sap Samples

The highest concentration of ethanol was 2.8%, with the lowest concentration of 2.2% in a sample from Rabai. Sucrose was the main sugar in palm sap. Rabai sample had the highest sucrose content (10.09%), while Mtwapa had the lowest content of sucrose (9.28%). Fresh sap had low levels of glucose compared to sucrose (Table 1). Samples from

Mtwapa had highest glucose content of 2.2%, while Chumani had lowest of 1.9%. Fructose levels were very low in fresh *Mnazi* compared to the other sugars with the highest recorded in Mtwapa (1.2%), while the lowest (0.61%) in Rabai samples. Soluble solids were the highest components, with Mtwapa fresh sap having lowest level (13.28%), while Rabai having highest content (14.63%) (Table 1).

Table 1. Percentage concentration ± SEM of substrates in fresh palm sap

Sample	Ethanol	Sucrose	Glucose	Fructose	Soluble solids
C1	2.80 ± 0.12	9.0807 ± 0.12	1.93 ± 0.132	1.067 ± 0.067	14.307 ± 0.098
C2	2.53 ± 0.13	9.5967 ± 0.22	2.07 ± 0.075	1.343 ± 0.346	14.023 ± 0.054
R1	2.20 ± 0.12	10.087 ± 0.10	2.04 ± 0.0367	0.61 ± 0.148	13.58 ± 0.197
R2	2.60 ± 0.12	9.46 ± 0.27	2.18 ± 0.0367	0.943 ± 0.057	14.627 ± 0.056
M1	2.27 ± 0.067	9.28 ± 0.21	2.00 ± 0.168	0.78 ± 0.147	13.28 ± 0.074
M2	2.47 ± 0.134	9.913 ± 0.037	2.29 ± 0.132	1.23 ± 0.243	14.457 ± 0.046

SEM = Standard Error of the Mean; C = Chumani, R = Rabai, M = Mtwapa, 1 = First sample, 2 = Second sample

Analysis of Substrates in Controlled Fermentation

After 6 days under controlled fermentation conditions ethanol level increased in the palm sap. Controlled fermentation generated higher ethanol level than the natural fermentation with the highest level (6.13%) from Rabai sample (Table 2), while the lowest value obtained was 5.67%. Sucrose concentration in the

fermented samples was low since it had been utilized by microorganisms in the fermentation process. The concentration of glucose and fructose was below 1% after both controlled and natural fermentations (Tables 2 and 3).

Table 2: Percentage concentration ± SEM of substrates in palm sap after controlled fermentation

Sample	Ethanol	Sucrose	Glucose	Fructose	Soluble Solid	PH
C1	5.8±0.23	2.067 ±0.1345	0.587±0.032	0.327±0.043	4.127±0.25	3.99
C2	5.73±0.067	1.87 ±0.24	0.48±0.02	0.23±0.049	4.717±0.055	3.76
R1	6.13±0.134	1.47 ±0.35	0.513±0.067	0.387±0.059	5.06±0.061	4.12
R2	5.93±0.241	1.867 ±0.067	0.557±0.067	0.423±0.059	4.913±0.464	3.97
M1	5.8±0.177	1.667 ±0.35	0.48±0.052	0.353±0.077	5.237±0.35	4.12
M2	5.67±0.35	1.667 ±0.24	0.62±0.064	0.387±0.069	5.29±0.25	4.35

SEM = Standard Error of the Mean; C = Chumani, R = Rabai, M = Mtwapa

Analysis of Substrates in Natural Fermentation

After 6 days of natural fermentation, the highest level of ethanol obtained was 5.6%, while the lowest concentration was 4.87% (Table 3). Most naturally fermented samples had higher glucose and fructose

content than the controlled fermentation. The highest concentration of sucrose was in the sample fermented naturally (2.47%), while the lowest was in the sample under controlled fermentation (Table 3).

Table 3: Percentage concentration ± SEM of substrates in palm sap after natural fermentation

Sample	Ethanol	Sucrose	Glucose	Fructose	Solid	PH
C1	4.87±0.291	2.47 ±0.07	0.44±0.029	0.38±0.059	5.323±0.091	2.98
C2	5.13±0.177	1.800 ±0.18	0.49±0.029	0.38±0.041	5.007±0.45	3.27
R1	5.21±0.116	2.27 ±0.291	0.47±0.04	0.223±0.017	6.83±0.203	3.08
R2	5.63±0.241	2.13 ±0.241	0.60±0.041	0.3±0.017	5.883±0.281	3.15
M1	5.33±0.116	2.07 ±0.35	0.60±0.077	0.41±0.076	4.877±0.25	3.03
M2	4.93±0.177	2.00 ±0.24	0.58±0.072	0.223±0.088	6.15±0.48	3.30

SEM = Standard Error of the Mean; C = Chumani, R = Rabai, M = Mtwapa

For the fresh palm sap, the lowest pH was 6.24 in samples from Chumani, while the highest 6.53 was for samples from Mtwapa (Figure 1). The pH of palm sap drastically decreased during both controlled and natural fermentation processes. The highest pH level was recorded in naturally fermented samples after the six days. Putting into consideration that sodium

hydroxide and other compounds were added in samples for controlled fermentation, the highest pH was 4.35. At 95% confidence, level of sucrose, glucose, fructose, total soluble solids and pH differed significantly ($P<0.05$) between fresh Mnazi samples, after both natural and controlled fermentation.

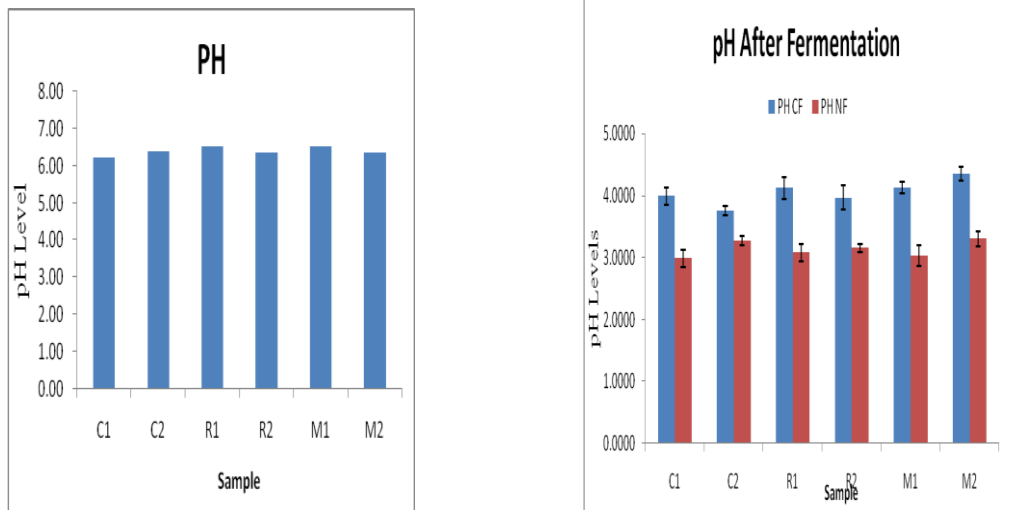


Figure 1: The pH of palm sap wine (*Mnazi*) before and after both natural and controlled fermentation

DISCUSSION

Fresh *Mnazi* contained ethanol in the range of 2.0-3.0%. Presence of ethanol in the fresh sap could have been contributed by the natural microflora of palm trees (Kalaiyarasi et al., 2013; Kadere et al., 2008), microorganisms in tapping implements the long period of tapping that was carried out overnight, or during storage period (Kadere, 2004). Samples fermented by *A. niger* and *S. cerevisiae* yielded higher ethanol than naturally fermented samples. Lower ethanol obtained from samples fermented naturally could have been due to lactic acid fermentation or acetification by the microbes in the sap (Kadere and Kutima, 2012), which further resulted in lower pH.

Sucrose was recorded as the highest soluble solid in all fresh sap collected. Thus, it was the main sugar utilized in fermentation process in all samples. Consequently after fermentation, the samples had very low levels of sucrose. Most samples fermented under controlled conditions had the lowest sucrose levels. This is because conditions employed promoted optimum metabolism of sucrose into ethanol by *A. niger* and *S. cerevisiae*. On the other hand, the higher sucrose level in naturally fermented samples could have been due to harsh conditions that hindered sucrose metabolism. Harsh conditions might have been caused by acidic pH resulting from acetic and lactic acid fermentation in the sample media (Kadere and Kutima, 2012).

Glucose and Fructose levels were very low in fresh *Mnazi*. The two are sugar molecules readily used up by microbial enzymes in the broth media, and therefore their concentrations were low. Similarly, after fermentation fructose and glucose levels were very low and almost negligible. From observation made on fresh *Mnazi*, soluble solids contributed to the high turbidity, sweet fermentative smell and sweet taste. The turbidity of palm sap greatly depends on its protein content and the polyphenolic compounds (Balange, 2009). Soluble solids were the main component of *Mnazi* that was used in fermentation. It is worth noting that of the soluble solids, some were fermentable, while others were unfermentable. The lower soluble solids content in samples fermented by *A. niger* and *S. cerevisiae* was because saccharification was enhanced by the *A. niger*. It produces enzymes involved in substrate breakdown to yield fermentable molecules that are easily fermented by *S. cerevisiae*.

Similar microorganisms in naturally fermented sap could have been out-competed by other microbes in the sample broth. In addition, if similar microbes

involved in saccharification were present in naturally fermented sap the species might have been different and inefficient. Other soluble solids content apart from sucrose, glucose and fructose that could have contributed to the total soluble solids are xylose, raffinose, celliobose, mannose, rhamnose, trehalose and dextrose (Eze and Ogan, 1988; Okafor, 1978).

Microorganisms, mainly lactic acid bacteria produce organic acids; e.g. lactic acid that increase the total acidity and decrease pH (Kadere and Kutima, 2012). The pH levels in fresh palm sap wine samples were above 6.0. Fermentation in all samples caused a decrease in pH. Naturally fermented samples had a lower pH than samples fermented under controlled conditions. The lower pH in naturally fermented media could have been due to acids generated from acetic and lactic acid fermentation. On the other hand, controlled fermentation had slightly higher pH due to addition of NaOH and MgSO₄.

CONCLUSION

From results obtained, controlled fermentation generated higher ethanol levels than natural process. Larger amounts of soluble solids were used up in controlled method than in the natural process, thus saccharification was accomplished. Most of the sugar substrates were utilized in the controlled process, thus their levels were minimal when measured in samples after fermentation. Lactic and acetic acid fermentations appear to be one of the causes of pH decrease in the natural process. The ethanol yields were greatly affected by substrate contents and pH levels. Therefore, in saccharification of unfermentable sugars in *Mnazi*, microbes and fermentation conditions employed are critical factors to consider in obtaining higher ethanol yields. *Aspergillus niger* can thus be utilized in metabolism of unfermentable substrates in palm wine to maximize industrial ethanol production. Pure ethanol for industrial use can then be recovered from *Mnazi* through distillation.

RECOMMENDATION

From findings of this study, *A. niger* and *S. cerevisiae* can be simultaneously used in industrial ethanol production from *Mnazi*. Further research should be done on these two microbes and their genomes improved to enable them carry out ethanol fermentation in *Mnazi* at higher levels. This can be achieved through genetically improving the fermentative microbe's ability to metabolize unfermentable substrates and tolerance to ethanol. Production of alternative commercial products such as industrial ethanol, acetic acid, lactic acid or other significant chemicals from palm wine during the

fermentation process should also be promoted. Furthermore, tappers should be educated on importance of using clean implements in handling of *Mnazi* since it affects ethanol yields.

ACKNOWLEDGEMENTS

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Variety	Fecundity (average eggs laid per female)	Average longevity (days)
Cal J	32.0 ^b	7.5 ^b
Onyx	23.0 ^c	7.2 ^b
Roma	23.9 ^c	6.7 ^b
Riogrande	48.6 ^a	8.2 ^b
Money Maker	52.4 ^a	12.8 ^a
Eden F1	31.3 ^b	7.6 ^b
Anna	33.2 ^b	7.4 ^b
Wild type	9.1 ^d	4.8 ^c
Mean	31.94	7.78
SE	1.48	0.34
CV (%)	19.03	13.8

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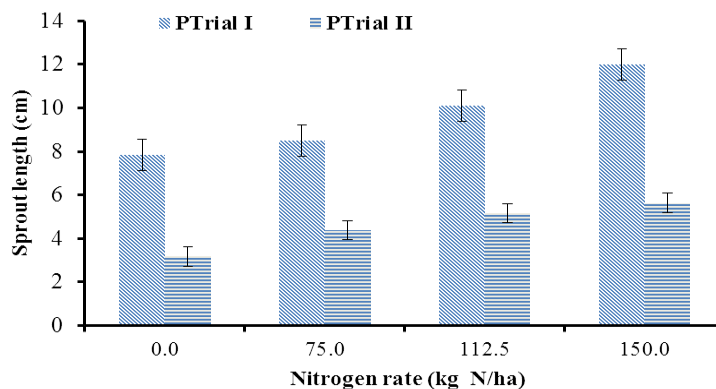


Figure 2: Effect of nitrogen rate on seed potato sprout length at 90 DAS

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I hereby kindly request you to review the attached manuscript, in accordance with the following criteria and guidelines:

QUESTION	TICK AS APPROPRIATE		
1. Write the title of the paper here:			
2. Is the content of this manuscript is original	YES	NO	
3. Does this paper address any of the subject areas listed above?	YES	NO	
4. Rate the length of this paper Ideal length is 10 single-spaced pages	TOO LONG	TOO SHORT	JUST RIGHT
5. You recommend it be:			
a	Published as it is		
b	Published subject to MAJOR corrections		
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6. Write corrections you would wish the authors to effect to improve the paper on a continuation sheet and attach, or on the manuscript.

Thank you very much for your assistance.

Editor, JESAR

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