Growth of Different Yeast Strains During Fermentation of Soursop (*Annona muricata*) Juice as Influenced by Acetic acid Bacteria (*Acetobacter aceti*)

Sunday P. Ukwo[1], Chidi F. Ezeama[1] and Nyaudoh U. Ndaeyo[2]*

[1]Department of Food Science and Technology, Michael Okpara University of Agriculture, Umudike, P.M.B. 7267, Umuahia, Abia State, Nigeria.

Department of Crop Science, Faculty of Agriculture, University of Uyo, Akwa Ibom State, Nigeria.

dr_nundaeyo@yahoo.com.

Abstract: A study was conducted to investigate the growth of different yeast strains as influenced by Acetic acid bacteria (*Acetobacter aceti*) during fermentation of soursop juice. Preliminary studies were carried out to identify the endogenous species of yeast responsible for natural fermentation of soursop juice and the effect of exogenous acetic acid and pH on growth and tolerance rate of the different yeast isolates. The growth rate of different isolates was monitored for 7th days with and without *Acetobacter aceti* (AAB). Soursop juice was inoculated with the different yeast isolates *Saccharomyces cerevisiae* (SC), *Hansenula anomala* (HA), *Canadida tropicalis* (CA) and a brewery strain *Saccharomyces pastorianus* (SP). Results showed a reduction in maximal cell concentration with samples treated with *Acetobacter aceti*. The inhibition of growth rate was higher on non-*Saccharomyces* yeasts HA and CA while the brewery strain SP indicated a higher tolerance to *Acetobacter aceti* in the fermenting juice. Addition of 0.5ml v/v of exogenous acetic acid resulted in a drop in pH from 6.8 to 4.1 and caused a reduction in cell count while 1.0ml v/v acetic acid resulted in a drop of pH from 6.8 to 3.7 and showed complete inhibition of growth of all the yeast isolates. When the medium was adjusted by 1N hydrochloric acid to pH 4.1 and 3.7 all the yeast isolates showed the same growth as the control (pH 6.8). [Nature and Science 2010;8(10):285-291]. (ISSN: 1545-0740).

Key words: fermentation, soursop juice, yeast, acetic acid bacteria.

1. Introduction

The development of fermentation technologies has been lost in the mist of history and has been regarded as one of the oldest methods of food processing as well as preservation for decades and virtually every culture has it as part of its diets (Viljeon and Heard, 1999). Today, alcoholic fermentation is quite indispensable for the production of alcoholic beverages including wine from tropical fruits such as orange, grapes, pineapple, banana, guava, soursop and sugar cane. Fermented foods according to Food and Agricultural Organization (FAO) have played important role in improving food security, enhancing livelihood and increased the nutritional status of the final products (Steinkraus 1992).

Yeasts as a group of micro-organism has been quantitatively and commercially exploited as a fermentative species needed to carry out alcoholic fermentation and this has urged many scientist to study the factors governing its growth, survival and biological activities at different ecosystem (Heard and Fleet 1985).

It has been established that the growth of yeast during fermentation depends on the media composition (substrate), the initial level of pH, temperature and dissolved oxygen (Ruiz et al, 2004).

According to Battcock and Azam-Ali (1998), other micro-organism during fermentation have the potential to influence the performance of yeast at various stages of fermentation process. He asserted that apart from their "Killer Strains" of yeast, bacteria like acetic acid and lactic acid bacteria posses a tremendious effect on the activities of yeast vis-à-vis the final products. According to Gafner (2006), most of the off –flavour encountered during fruit juice fermentation comes from acetic acid which are either produced by undersirable yeasts or acetic acid bacteria during fermentation. Studies has also shown that any condition that affect the growth of yeast cells during fermentation process posses a negative effect on the final fermentation products. According to Ayugo (1999), the amount of acetic acid produce by desirable yeast is between the range of 0.04-0.2g/l and this amount of acetic acid according to Reed and Nagodawithana, (1991), produced by yeasts are dependent on fermentation temperature and composition of the medium.

Although the important role of undissociated form of acetic acid on yeast cells have been investigated, literature on the successfully induced fermentation where both yeasts and acetic acid bacteria like *Acetobacter aceti* are inoculated together during fermentation are either non-existing

or are lacking and hence this study is directed toward that. The objective of this work is to assessed the growth rate of different yeast isolates singly and in the presence of *Acetobacter aceti* during fermentation of soursop juice. The effect of exogenous acetic acid and pH on the growth and tolerance rate of different yeast isolates is also investigated.

2. Materials and methods

2.1 Sample preparation

Fresh and fully ripe soursop (*Annona muricata* L.) fruits were purchased form Akpana-Ndem market in Uyo, Southern Nigeria. The fruits were washed with sterile water and further sterilized with 2% sodium hypochlorate solution. Under a sterile condition, the fruits were hand peeled, decored and deseeded. The pulp was blended using an electric blender (National, model MX-795N, Malaysia). Sterile water was added at the ratio of 1:2 (w/v, pulp/water) to facilitate blending process and make filtration process easier. The pulp was filtered using a sieve and Muslin cloth. The juice collected was pasteurized at 65^0C for 30 minutes and allowed to cool.

2.2 Preliminary Microbiological Studies

A preliminary experiment was carried out to identify the endogenous species of yeast mostly responsible for the natural fermentation of soursop juice as outlined by Ezeama (1999). This was done to ensure that species of yeasts to be identified were used for the fermentation studies. Healthy soursop fruit was washed with sterile water, the skin was peeled off and the pulp (mesocarp) squeezed out under aseptical condition and the juice collected. The soursop juice so collected was allow to undergo natural fermentation for 3 days. The fermenting sample was then collected for this study.

2.3 Micro-Organisms

Yeast strains used for this study was isolated from soursop juice. A 0.1ml of the juice prepared after 10 fold serial dilution was seeded on PDA (Potato infusion 200g/l, Dextrose 20g/l and Agar 15g/l) at pH 5.6±0.2 at 20^0C was fortified with 0.25g/l of chloramphenicol to inhibit bacterial growth. The plates were incubated aerobically at room temperature (28 ± 2^0C) for 3-5 days for colony development. Discrete colonies were streaked on fresh YPD-Agar (Yeast extract 10g/l peptone 20g/l, glucose 20g/l and agar 20g/l) (Kapsapoulou *et al.*, 2005) and incubated for 3-5 days the isolate stored at 4^0C. The ability to utilize certain sugar for growth and assimilation was use for identification (Lee *et al.*, 2006). A pure culture of brewery stain of *Saccharomyces pastorianus* was obtained from Champion Breweries, Uyo and maintained in YPD-agar and stored at the same temperature.

Acetic acid bacteria was isolated from a fermented palm wine dreg. A 0.1ml of dreg was seeded on glucose-yeast extract containing 100g glucose, 10g of yeast extract, 10g of $CaCO_3$ and 20g of agar in 1 litre and incubated aerobically at 28 ± 2^0C for 2-3 days (Lisdiyanti *et al.,* 2001) colonies were randomly picked and subcultured using the same media.

2.4 Preparation of Inoculum

Yeasts inoculum were prepared by inoculating each species of yeasts identified into 25ml YPD without Agar and water to prepare the inoculum was substituted by soursop juice to condition the yeast and were allowed for 24 hours at 28 ± 2^0C before inoculum.

Acetobacter aceti inoculum was from a 24 hours culture that grew on slant into 1% D-glucose, 0.5% ethanol 0.3% acetic acid, 1.5% bactopeptone and 0.8% yeast extract and the pH adjusted to 3.4 using citric acid (Lisdiyanti *et al*., 2001) and stored for 24 hours before inoculation.

2.5 Fermentation studies

Fermentation was carried out by inoculating 1% of the actively growing yeast cells (5.0×10^5cfu/ml) into 500ml sterile soursop juice in 1 litre conical flask capped with cotton wool. Fermentation was carried out at room temperature (28 ± 2^0C) for 7 days without agitation.

To study the effect of *Acetobater aceti* on yeast performance and fermentation rate, 1% of actively growing cell (1.0×10^6 cfu/ml) was inoculated into the fermenter. Samples were taken daily and analysed for the determination of yeast growth. All determinations were carried out in triplicate and each value used in figure and tables were the arithmetic mean.

2.6 Enumeration of Yeasts Growth

The growth rate of yeasts were determined by the plate count technique (Kapsopoulou *et al*., 2005). Samples were taken at 24 hours intervals and serially diluted and spread-inoculated (0.1ml) on plates of YPD agar containing the following per litre: Yeast extract 10g, peptone (oxoid), 20g glucose (BDH) 20g and agar 20g and was fortified by 0.25g/ℓ of chloramphenicol. The plates were incubated at room temperature (28 ± 2^0C) for 3-5 days.

2.7 Effect of exogenous Acetic acid and ph on the growth and tolerance rate of yeast isolates

This was a pure culture experiment. The method outlined by Bechem *et al* (2007) was adopted with minor modification. To investigate the growth and tolerance rate of different yeast isolates to exogenous acetic acid, and pH, the inoculum used for fermentation experiment in section 3.5 were grown on a synthetic media prepared in 5 places as follows: 1.0g peptone (oxoid), 10.0g of glucose (BDH), 1.0g of yeast extract and 2.0g of agar all in 100ml into which 0ml, 0.5ml and 1.0ml of exogenous acetic acid were added to each of the media while the last two their pH were adjusted with IN HCl to pH of level of 4.1 and 3.7 before autoclaving. A 0.1ml of the inoculum were transferred into each plate and the media with various concentrations of acid were added swirled and incubated aerobically at room temperature for 5 days followed by observation for growth and the level of tolerance of the isolates to acetic acid and pH.

3. Results

3.1 Characterization and Identification of Yeast Isolates

The results obtained from the study identified three (3) endogenous species of yeast mostly responsible for the natural fermentation of soursop juice. The ability of the different yeast isolates to utilizes certain sugars for growth was further used for identification as indicated in the Table 1 below.

Table 1: Biochemical, assimilation tests and morphology of 3 yeast isolates obtained from soursop

Tests	SC	HA	CA
Fructose	+	+	+
Sucrose	+	+	+
Xylose	-	-	+
Maltose	+	+	+
Glucose	+	+	+
D-Raftinose	+	+	+
Lactose	-	-	-
Nitrate	+	+	+
Hydrogen perioxide	+	+	+
Methanol	-	+	+
Cell Morphology	Smooth, spherical cremish	Whitish to cream colony	Elevator and cremish
Cell shape	Round, oval with thick cell wall ellipsoidal	Cylindrical, spherical hat shape or Saturn shape	Enlongated cell with spores. True Mycelum
Identification	*Saccharomyces cerevisiae*	*Hansenula anomala*	*Candida tropicalis*

\+ positive
\- negative

3.2 Effect of Acetic Acid Bacteria (*Acetobacter aceti*) on Yeast Growth

They include; *Saccharomyces cerevisiae* (SC) *Hansenula anomala* (HA) and *Candida tropicalis* (CA). The growth pattern of *Saccharomyces* yeast isolates which include *Saccharomyces cerevisiae* (SC) and a brewer strain *Saccharomyces pastorianus* (SP) inoculated into soursop juice without *Acetobacter aceti* (AAB) was characterized by a very high concentration of cells which reached 8.5 and 8.4 log (cfu/ml) for SC and SP respectively as indicated in Figure 1. The non-*Saccharomyces* yeast isolates; *Hansenula anomala* (HA) and *Candida tropicalis* (CA) inoculated into a similar medium without *Acetobacter aceti* (AAB) showed a similar growth pattern (Figure 2). Furthermore, the cells concentration of *Saccharomyces cerevisiae* (SC) showed higher viability and decreased much more slowly to a level of 7.0 log (cfu/ml) after 7 days of fermentation.

However, the growth of yeast isolates inoculated together with *Acetobacter aceti* (AAB) was characterized by lower concentration of cells as indicated in Figures 1, 2, 3. There was no exponential phase of fermentation. The non-*Saccharomyces* yeast, HA and CA showed a higher sensitivity to the presence of AAB and were therefore strongly inhibited (Figure 2) while the *Saccharomyces* yeast SC and SP showed minimal cell concentration with SP giving 6.7 log (cfu/ml) which was the highest compared (Figure 1) to the other.

However, strain of *Saccharomyces pastorianus* showed a high tolerance to the presence of *Acetobacter aceti* during fermentation especially when inoculated together with other yeasts as indicated during the mixed culture fermentation.

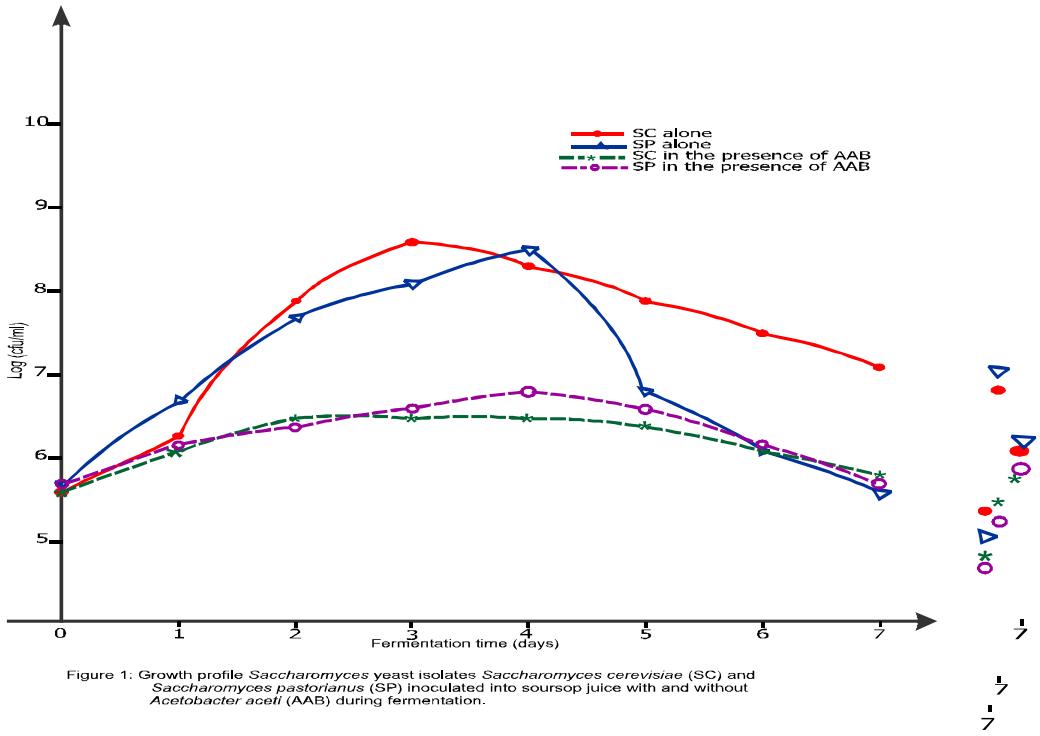

Figure 1: Growth profile *Saccharomyces* yeast isolates *Saccharomyces cerevisiae* (SC) and *Saccharomyces pastorianus* (SP) inoculated into soursop juice with and without *Acetobacter aceti* (AAB) during fermentation.

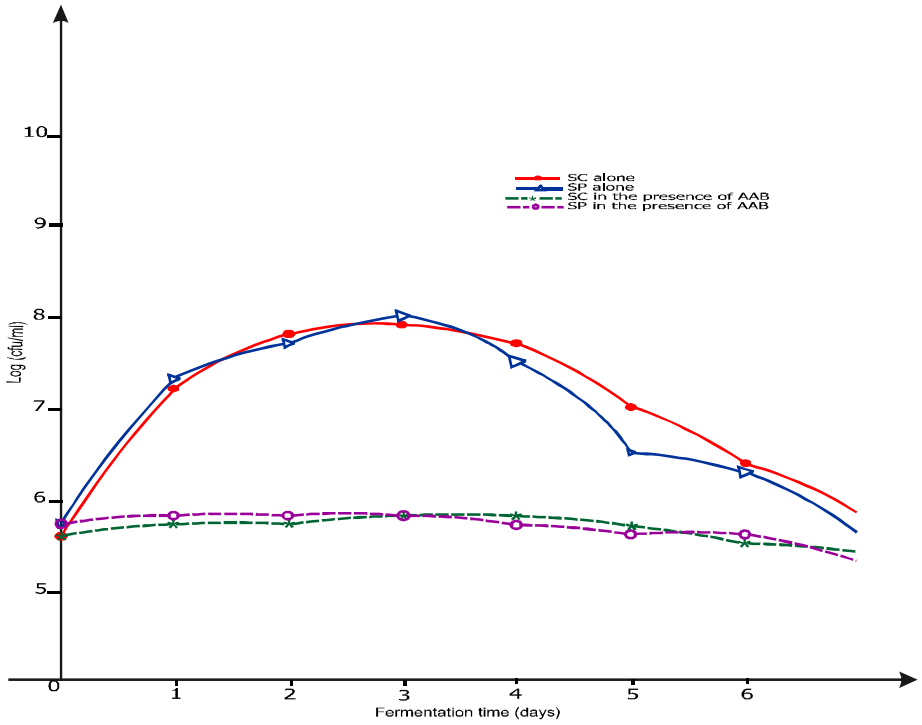

Figure 2: Growth profile *of* Non-*Saccharomyces* yeast isolates *Hansenula anomala* (HA) and *Candida tropicalis* (CA) inoculated into soursop juice with and without *Acetobacter aceti* (AAB) during fermentation.

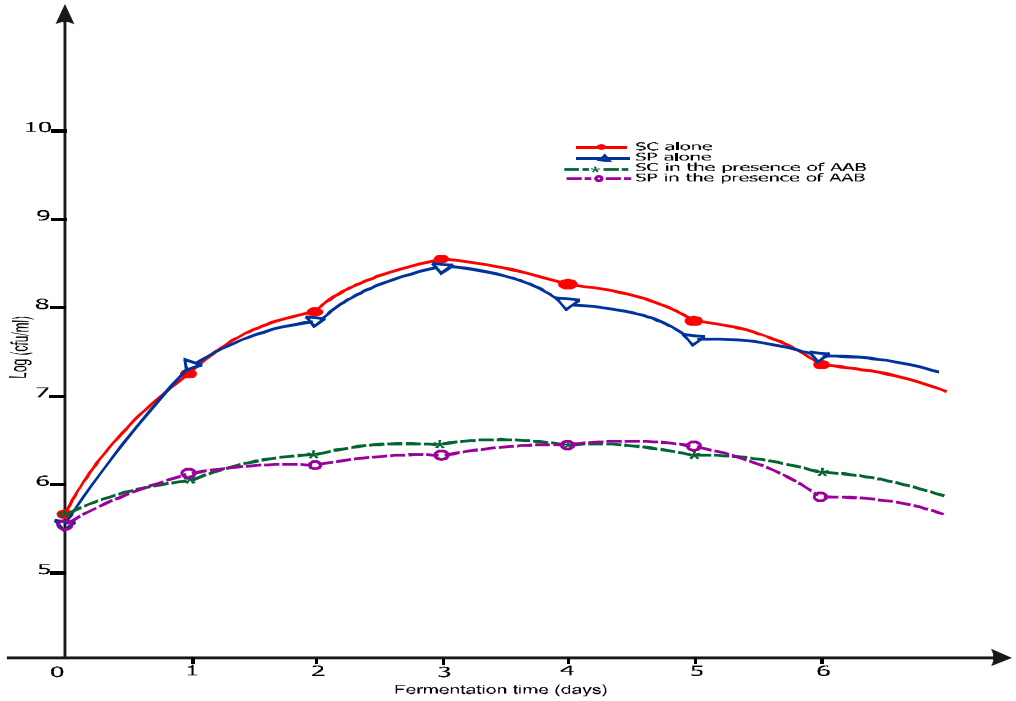

Figure 3: Growth profile of combined strain of yeast isolates inoculated into soursop juice with *Acetobacter aceti* (AAB) during fermentation.

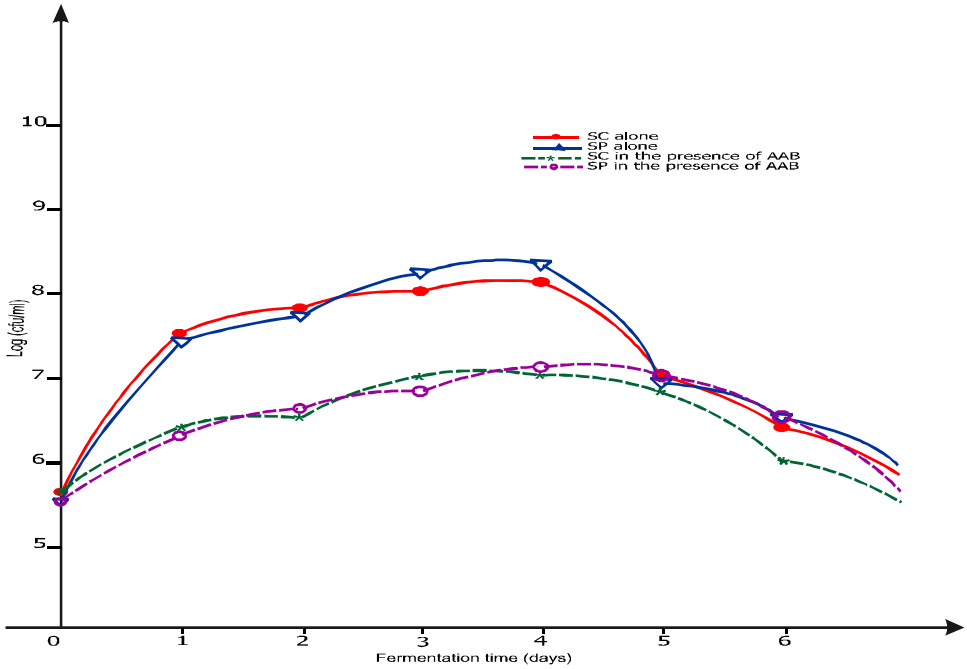

Figure 4: Growth profile of combined strain of yeast isolates inoculated into soursop juice with *Acetobacter aceti* (AAB) during fermentation.

3.3 Effect of Exogenous Acetic acid and pH on growth and tolerance rate of Yeast Isolates

The addition of 0.5ml v/v of acetic acid to the test medium resulted in pH reduction from 6.8 to 4.1. The cultivated yeast isolates showed that *Saccharomyces cerevisiae* (SC) and *Saccharomyces pastorianus* (SP) exhibited a comparable slight decrease in growth and cell counts a little lower than that of the control medium while that of the non- *Saccharomyces* yeasts *Hansenula anomala* (HA) and *Candida tropicalis* (CA) exhibited a much lower cell count and decrease in growth.

This shows that acetic acid at 0.5 ml or 0.5% concentration can slightly inhibit growth of yeast by causing loss of viability after inoculation. The medium added with 1.0 ml v/v of acetic acid resulted in pH reduction from 6.8 to 3.7. There was no growth at all in all the test medium containing the yeast isolates after 5 days (Table 2).

Table 2: Yeast growth and tolerance rate as affected by exogenous acetic acid and pH

		Yeast Count cfu/ml			
Sample	p^H	SC	SP	HA	CA
0ml v/v (acetic acid)	6.8	3.0×10^5	3.2×10^5	3.2×10^5	3.1×10^5
0.5ml v/v (acetic acid)	4.1	2.8×10^5	2.3×10^5	5.0×10^4	4.0×10^4
1.0ml v/v (acetic acid)	3.7	ND	ND	ND	ND
pH 4.1 with IN HCl	4.1	3.1×10^5	3.3×10^5	3.0×10^5	3.1×10^5
pH 3.7 with IN HCl	3.7	3.2×10^5	3.2×10^5	3.2×10^5	3.0×10^5

N.D = Non detectable.
Values are means from 3 determinations

To investigate the effect of acetic acid on growth reduction and inhibition of yeast isolates was not from low pH caused by adding acetic acid, experiment were carried out by adjusting the pH of the synthetic media by IN HCl to the same pH level (pH 4.1 and 3.7). Results showed that the growth and rate of tolerance of yeast isolate were not different compared to the control medium (Table 1). This indicates that the low pH did not inhibit the growth of yeast isolates but acetic acid as reported by Phowchinda *et al*., (1995).

4.0 Discussion

This study has shown a rapid growth of yeast cells during fermentation followed by decrease in cells concentration after 5 days to a level of 5.5, 5.8, and 5.6 log (cfu/ml) for SP, HA and CA respectively. This could probably be due to combined influence of alcohol and anaerobic condition caused by yeast growth. Furthermore, the cells concentration of *Saccharomyces cerevisiae* (SC) showed higher viability and decreased much more slowly to a level of 7.0 log (cfu/ml) after 7 days of fermentation. A similar result was observed by Kapsopoulou *et al*., (2005) and Mora *et al.,* (1990) during their individual studies on growth and fermentation characteristics of wine yeasts.

Acetobacter aceti has shown that it is responsible for oxidizing ethanol to acetic acid in wine and the effect of this acid has also been reported to have an inhibitory effect on yeast growth and metabolism during fermentation processes. The inhibitory effect of *Acetobacter aceti* on yeast growth could therefore be as a result of oxidation of ethanol to acetic acid (Battcock and Azam-Ali, 1998). The acetic acid causes a reduction in maximal cell concentration of yeast cells during fermentation process (Phowchinda et al, 1995). It is likely that, the undissociated acetic acid produced by *Acetobacter aceti* diffused into yeast cells and caused decrease in the pH of cytoplasm which may have inhibited the activities of key enzymes. The higher cell count observed in this mixed culture of SP with HA and CA with *Acetobacter acid* may be due to relative tolerance of SP to AAB as was found by Limtong, (2000) that some strains of *Saccharomyces* yeasts showed higher tolerance rate to acetic acid than non-*Saccharomyces* yeasts.

In the findings of the effect of exogenous acetic on yeast growth and tolerance rate of yeast isolates, addition of 1.0ml v/v of acetic acid resulted in pH reduction from 6.8 to 3.7, there was no growth in all the test medium indicating strong inhibition at this concentration of acetic acid. These findings agrees with the report of Bechem *et al*. (2007) on tolerance rate of different isolates of *Saccharomyces* spp on acetic acid solution and that of Ferrari *et al*., (1992) on the inhibitory effect of acetic acid on xylose fermentating yeasts. When the pH was adjusted by IN HCl , to 4.1 and 3.7, there was normal growth compared to the control medium. This shows that the pH of the medium did not inhibit the growth of isolates but acetic acid influence

5.0 Conclusions

This study investigated the effect of (*Acetobacter aceti*) on the growth of different yeast strain during fermentation of soursop. The study showed that the growth pattern of *Saccharomyce* yeasts *Saccharomyces cerevisiae* (SC) and *Saccharomyces pastorianus* (SP) and non-*Saccharomyces* yeasts *Hansenula anomala* (HA) and *Candida tropicalis* (CA) inoculated either simply or in mixed culture into soursop juice without *Acetobacter aceti* was similar to other studies on spontaneous fermentation of fruit juice by yeasts. However, the growth pattern of sample treated with *Acetobacter aceti* was characterized my minimal cell concentration for *Saccharomyces cerevisiae* (SC) while *Saccharomyces pastorianus* was less sensitive to the presence of *Acetobacter aceti*. But the non - *Saccharomyces* yeasts (*Hansenula anomela* (HA) and *Candida tropicalis* (CA) indicated higher sensitivity to the presence of *Acetobacter aceti* and therefore was strongly inhibited. The inhibition of growth rate of yeast during fermentation was proved to be the result of acetic acid form by the oxidation of ethanol by *Acetobacter aceti* and not from the low pH as indicated by the use of hydrochloric acid.

Correspondence to:
Nyaudoh U. Ndaeyo
Department of Crop Science
Faculty of Agriculture
University of Uyo, P. M. B. 1017,
Uyo, Akwa Ibom State, Nigeria.
Cellular Phone: +2348023285242, +2348027519138
Email: dr_nundaeyo@yahoo.com

References

[1] Viljeon BC, Heard GM, *Saccharomyces cerevisiae* In: Encyclopedia of food Microbiology. Academic Press 1999; 3: 1918-1925.

[2] Steinkraus K, Lactic Acid Fermentation. In: Application of Biotechnology to Traditional Fermented Foods. National Academy Press, Washington D.C. 1992; 43-50.

[3] Heard G, M, Fleet G, H, Growth of natural yeast flora during the fermentation of inoculated wines. Applied and Environmental Microbiology 1985; 50: 727-728.

[4] Ruiz L, Guerra N, Roses R, Factors affecting the growth of *Saccharomyces cerevisiae* in Batch culture and solid state fermentation. Electric Journal of Environmental, Agricultural and Food Chemistry. 2004

[5] Battcock M, Azam-Ali S, *Fermented Fruits and Vegetables. A Global perspective*, Food and Agricultural Origination (FAO) Rome, Italy. 1998; Bulletin No. 134.

[6] Gafner J, Origin of Acetic acid and increase acidity in wine; Stressed Fermentation. 2006; 31st Annual New York Wine Industry Workshop: USA.

[7] Ayugo TE, Evaluation of the performance of a yeast isolate from Nigeria palm wine in wine production from pineapple fruits. Bioresources Technology 1999; 69: 189-190.

[8] Reed G, Nagodawithana TW, Wine yeasts, In: Yeast Technology. Van Nostrand Reinhold. New York. 1991; 151-224.

[9] Ezeama C F, Effect of potassium metabisulphite on growth of some endogenous microflora of pineapple and soursop juices. J. Agric., Biotech, Environ., 1999;1: 60-67.

[10] Kapsopoulou K, Kapaklis A, Spyropoulas H, Growth and fermentation characteristics of a strain of wine yeast Kluyveromyces thermotolerans isolated in Greece. World Journal of Microbiology and Biotechnology 2005: 21: 1599-1602.

[11] Lee C, Lo P, Yu R, Selection, Characterization and Identification of several wine yeasts from Black Queen grapes in Taiwan. Institute of Food Technology (IFT), Annual Meeting and Food Exposition, Orlando, Florida, USA, 2006.

[12] Lisdiyanti P, Kawagaki H, Seki T, Yamada Y, Uchimura T, Komagata K, Identification of *Acetobacter* strain from Indonesian Sources. Journal General and Applied Microbiology 2001;47:119-131.

[13] Bechem E E, Omoloko C, Nwaga D, Titanji V, P, Characterization of palm wine yeasts using osmotic ethanol tolerance and the Isozyme polymorphism of alcohol dehydrogenase. African Journal of Biotechnology 2007; 6 (14): 1715-1719.

[14] Phowchinda O, Delia-Dupuy M, L, Strechaiano P, Effect of acetic acid on growth and fermenative activity of *Saccharomyces cerevisiae*. Biotechnology Letters, 1995; 17 (2): 237-242.

[15] Mora J, Barbas I, Mulet Growth of yeast species during the fermentation of musts inoculated with *Kluyveromyces thermoterance* and *Sacchaomyces cerevisiae*. *American Journal of Enology and Viticulture* 41, 1990; 156-159.

[16] Limtong S, Sumpracht T, Vichien K, Manee T, Seki T, Yoshida T, Effect of acetic acid on growth and ethanol fermentation of xylose fermenting yeast and *Saccharomyces cerevisiae;* Kasetsart Journal of Natural Science, Thailand 2000; 34: 64-73.

[17] Ferrari M., Neirotti E, Albornoz E, Saucedo E, Ethanol Production from eucalyptus wood lemicellulose hydrolysate by Pichia stipitis. Biotechnology Bioengineering, 1992; 40: 735-759.

11/09/2010