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Studies on Proliferation of Acetic Acid Bacteria during Soursop Juice Fermentaion

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Abstract

A study was conducted to investigate the proliferation of acetic acid bacteria during various stages of soursop juice fermentation. Preliminary experiment was carried out to identify the genera and species of acetic acid bacteria associated with soursop fruits. The effect of pH, temperature and momentary aeration on the growth of acetic acid bacteria and its effect on the growth of Saccharomyces cerevisiae were studied. Soursop juice undergoing spontaneous fermentation was monitored for 10 days. Results showed that acetic acid bacteria were present at all stages of fermentation. A succession of acetic acid bacteria indicated that Gluconobacter oxydan was found to be associated with unspoiled fruit and at the early stage of fermentation. Acetobacter aceti was prevalent throughout fermentation days while the presence of Acetobacter pasteurianus was noted at the later stage of fermentation. These species exhibited rapid growth on short exposure of the fermenting vessel to atmospheric oxygen which resulted in a strong correlation between the growth of acetic acid bacteria and concentration of acetic acid in the fermenting sample. Higher temperature and elevated pH favoured the growth and metabolism of acetic acid bacteria. There was a reduction in maximal cell concentration of Saccharomyces cerevisiae inoculated with Acetobacter aceti as compared to species not treated with the bacteria.

Key words: Fermentation, Soursop juice, Aceti acid bacteria.

Introduction

Fermentation is an energy regenerating metabolic process of which degradation products of organic substrates serve as hydrogen donor as well as hydrogen acceptor. It is a step-wise process initiated and controlled by yeasts, bacteria or an exogenous enzymes. Schlegel (2002) noted that when the process proceed in the presence of oxygen it is regarded as oxidative fermentation and the end-products include alcohols, organic acids such as acetate, gluconate, keto acid etc. which is accompanied by gradual decrease in pH. According to Zehnder, (1998) the acetic acid bacteria share the ability to form acids by incomplete oxidation of sugars or alcohols and to excrete these acids either transiently or into the medium as non-utilizable products. Battcock and Azam-Ali (1998) noted that yeasts and acetic acid bacteria exist together in form of commensalism during fermentation of fruit juices. He asserted that acetic acid bacteria have the potential to influence the performance of yeast at various stages of fermentation process vis-à-vis the final products.

Acetic acid bacteria include gram-negative rods with limited motility by virtue of peritrichous (Acetobacter) or polar (Gluconobacter) flagellation. The natural habitat of acetic acid bacteria are plants and whenever sugar containing sap or secretions occur. Acetic acid bacteria can be found in association with yeasts (Schlegel, 2002). Their ability to efficiently convert ethanol through acetaldehyde to acetic acid is utilised in culinary and medical vinegar production. However in the wine industry this capability constitutes spoilage (Fleet, 1993). Wines spoiled by acetic acid bacteria have characteristic volatility, a vinegar-like sourness on the palate and a range of acetic acid aromas which is often a reduction in fruity characters. (Bartowsky et al; 2003). Such wines have low commercial value. However, this can be improved by blending or treatment by a reverse-osmotic process to lower acetic acid content.

In recent years, researchers have shown concerned about the increased levels of acetic acid encountered during fermentation of fruit juice and this has provided the basis of this study. The objective of this work is to study the proliferation of acetic acid bacteria during different stages

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of soursop juice fermentation. The relationship between growth of acetic acid bacteria and the formation of acetic acid during fermentation and the impact of Acetobacter aceti on the growth Saccharomyces cerevisiae during fermentation of soursop juice is also investigated.

Material and Methods

Sample Preparation. Fresh and fully ripe soursop (Annona muricata L.) fruit were aseptically harvested from the plant. Under a sterile condition, the fruits were hand peeled, decored and deseeded. The pulp was blended using an electric blender (National, Model MXN Malaysia). Sterile water was added in the ratio of 1:2 (w/v pulp/water) to facilitate blending process and make filtration process easier. The pulp was filtered using sieve and muslin cloth under a sterile condition.

Association of Acetic Acid Bacteria with Soursop Fruits. Preliminary experiment was carried out to identify the genera and the species of acetic acid bacteria prevalent to fruit juices as outlined Joyeux et al., (1984). Soursop fruits were harvested from different plants depending on the level of ripeness.

Isolation and Enumeration of Acetic and Bacteria. Acetic acid bacteria were selectively isolated by seeding 0.1ml of the sample on glucose-yeast extract agar containing glucose (BDH) England 100g/l, yeast extract 10g/l, CaCO3 20g/l and agar 20g/l. The plates were inverted and incubated aerobically at room temperature for 2-3 days (Hommel and Ahnert 1999) after 3 days isolate were randomly picked with loop and subcultured for purification using glucose. Yeast extract agar containing 1.0% D-glucose, 0.5% ethanol, 0.3% acetic acid, 1.5% Bactopeptone, 0.8% yeast extract and 0.3% agar. The medium was fortified withy 0.1ml of 0.5% Pimaricin solution to inhibit the growth of yeasts and mould while 0.1ml of 0.25% penicillin solution was added to inhibit the growth of lactic acid bacteria. The pH of the medium was adjusted to 3.4 using citric acid and the plates were inculated at 250C for 4 to 8 days after purification (Lisdiyanti et al, 2001). Distinct colonies based on colonial morphology were purified to obtain pure cultures that were subjected to routine biochemical tests. The isolates were then identified according to the scheme of Buchanan and Gibbons (1974). The concentration of acetic acid, lactic acid and citric acid as well as glucose concentration were determined by methods outlined by James (1995).

Growth of Acetic Acid Bacteria on Soursop Juice during Fermentation. To investigate the proliferation of acetic acid bacteria at different stages fermentation, samples were taken on the, 0 2nd, 4th, 6th, 8th and 10th days of fermentation for analysis. The method outlined by Joyeux et al (1984) was adopted. The levels and species of acetic acid bacteria found at different stages of fermentation were recorded.

Growth of Acetic Acid Bacteria and Acetic Acid Formation. The relationship between the growth the acetic acid bacteria and the formation of acetic acid during fermentation of soursop juice was determined. The method outlined by Joyeux et al., (1984) was adopted with minor modification. 50ml of the fermenting sample at pH 3.5, ethanol 10% was taken and incubated at 100ml conical flask at 190C to provide for aerobic condition. Growth of acetic acid bacteria was determined by plate count technique (Lisdiyanti et al., 2001) while acetic acid production was determined by method outlined by James (1995).

Effects of pH, Temperature and Momentary Aeration on Growth of Acetic Acid Bacteria. The effects of pH, temperature and momentary aeration on acetic acid bacteria growth were determined by taking the fermenting samples on the first day of fermentation, adjusted the pH to 3.7, 4.1 and stored at 100C or 180C. The samples were stored at 100ml completely filled conical flask to yield an anaerobic environment. After 4 days of storage, the contents of the flasks were exposed to air for 3 mins by pouring them into the beakers and returning it to the flasks. Analysis was done 3 days after this aeration (Zoecklein et al., 1989).

Impact of Acetic Acid Bacteria on the growth of Saccharomyces cerevisiae during fermentation. Experiment was carried out to investigate the effect of acetic acid bacteria on the growth of Saccharomyces cereivisiae during fermentation of soursop juice. Freshly prepared soursop juice was pasteurized at 650C for 30 mins and allowed to cool. Acetobacter aceti and Saccharomyces cerevisiae were simultaneously inoculated in the juice (Jussier et al., 2006). The growth of Saccharomyces cerevisiae was determined by plate count technique as outlined by Kapsopoulou et al., (2005).

Results

Identification and differentiation of acetic acid bacteria strains. The major distinguishing features between Acetobacter and Gluconobacter is the ability to oxidise acetic acid to CO2. Acetobacter species are able to oxidise ethanol to acetic acid and then to CO2 and H2O whereas Gluconobacter species do not have a complete citric acid cycle and cannot oxidise ethanol further than acetic acid. Gluconobacters are sometimes referred to as under-oxidisers while Acetobacters are overoxidisers, because of their difference in oxidative potential. Acetic acid bacteria have been successfully identified to genus and species levels according to an array of morphological physiological and biochemical tests as shown in table 1 and 2 below:

Table1. Distinguishing AAB genera

	Acetobacter	*Gluconobacter*
Motility and flagellation	Peritrichous/non motile	Polar/non motile
Oxidation of ethanol to acetic acid	+	+
Oxidation of acetic acid to CO_2 and H_2O	+	-
Oxidation of lactate to CO_2 and H_2O	+	-
Growth on 0.35% acetic acid medium	+	+
Growth on 30% glucose medium	-	-
Acid production from glycerol	+	+
Acid production from mannatol	-	-
Acid production from Raffinose	-	-

Table 2. Distinguishing Acetic acid bacteria isolates

	Acetobacter			*Gluconobacter*
	aceti	*pasteurianus*	*tropicalis*	*oxydans*
Growth on carbon sources				
Glycerol	+	+	+	+
Ethanol	+	Variable	-	+
Sodium acetate	+	+	-	Variable
Acid production from				
D-glucose	+	+	Variable	+
D-mannose	+	-	-	+
D-galactose	+	Variable	-	+
D-xylose	+	+	-	-
Ketogenesis from				
Glycerol	+	-	-	+
Sorbutol	+	-		+
Mannitol	Variable	-	-	+
Formation from D-glucose of				
2-keto-D-gluconic acid	+	Variable	+	+
5-keto-D-gluconic acid	+	-	-	+
Nitrate reduction	-	+	+	

- = negative
+ = positive
Variable = 11-89% of strains positive

Association of Acetic Acid Bacteria with Soursop Fruits
Studies were conducted with soursop fruits harvested from different plantations in Uyo Southern Nigeria. It shows that the density of acetic acid bacterial was always found to be linked to the degree of ripeness of the fruit. Freshly extracted juice from the soursop fruits at the early harvest contain an average 102cfu/ml of acetic acid bacteria.
The level of acetic acid bacteria increase during storage from 105 to 107cfu/ml and keep on increasing as the fruit got spoilt. Gluconobacter oxydan was the acetic acid bacteria found on sound, unspoiled soursop fruits. Acetobacter aceti became more prevalent as the fruit became spoiled. These two species accounted for 75-85% acetic acid bacteria found on the harvested soursop fruits.
Growth of Acetic Acid Bacteria on Soursop Juice during Fermentation. The levels and species of acetic acid bacteria found at different stages of fermentation of soursop juice are represented in the Table below:

Table 3. Occurrence of Aceti acid bacteria during fermentation of soursop juice

Fermentation Time (days)	**Acetic acid bacteria (cfu/ml) ($x10^4$)**	**Micro organism (%)**
0	1640	*G. oxydans* 90, *A. aceti* 10
2	120	*G. oxydans* 50, *A. aceti* 50
4	100	*A. aceti* 80, *G. oxydans* 10 *A. pasteurianus* 10
6	80	*A. aceti* 50, *A. pasteurianus*50
8	50	*A. pasteuranus* 60, *A. aceti* 40
10	40	*A. pasteuranus* 80, *A. aceti* 20

* Values are means from 3 determinations

Freshly prepared soursop juice contain 90% of G. oxydans. The population progressively decreased during alcoholic fermentation, and at the end of fermentation (10th day) no colony of G. oxydans was detected. Also, Acetobacter aceti

was dominant all through the fermentation days as it rose from 10% in the freshly prepared juice to the peak at the 4th day of fermentation and fall to 20% at the 10th day of fermentation. A. pasteuranus was also seen to be present during the late stages of fermentation from the 4th day to 10th day of fermentation.

Growth of Acetic Acid Bacteria and Acetic Acid formation. The relationship between the growth of acetic acid bacteria and the formation of acetic acid during fermentation was investigated. At the bacteria counts consisting of mixed population of A. aceti and A. pasteuranus progressively increased from the initial level of 3.0 x 104cfu/ml into 2.3 x 106cfu/ml. At the same time the concentration of acetic acid in the wine increased from 0.72g/l to 2.10g/l. While the level of lactic acid did not change. It was also noted that between the second and third day of incubation, 100mg of acetic per litre was produced although the bacteria population increased only from 6.8 x 102 to 9.8 x 102 cfu/ml.

Effect of pH, Temperature and Momentary Aeration on Growth of Acetic Acid Bacteria. The result showed samples stored in a completely anaerobic condition for both temperature (10 and 180C). It indicated that the growth of acetic acid bacteria decreased more rapidly at pH 3.7 than at pH 4.1 while the concentration of acetic acid stayed constant. Also 3 days after the momentary aeration, the result showed little growth at 100C but the number of cells increased by 30-to-40 fold on storage at 180C. This was accompanied by a significant increase in the level of acetic acid. Slightly high counts of bacteria were observed at the higher pH of 4.1.Table 4 below summarises the result

Table 4. Effect of pH and storage temperature on growth of acetic acid bacteria and concentration of acetic acid after aeration

Storage temp. (^{0}C)	pH	Acetic acid bacteria (cfu/ml)	Acetic acid (mg/l)
10	3.7	2.0 x 10^3	380
10	4.1	2.0 x 10^3	400
18	3.7	2.5 x 10^5	480
18	4.1	3.5 x 10^5	540

* Values are means from 3 determinations

Impact of Acetic Acid Bacteria on the growth of Saccharomyces cerevisiae. The growth of Saccharomyces cerevisiae inoculated into soursop juice without Acetobacter aceti was characterized by a very high concentration of cells which reach its peak at the 4th day with 3.2 x 108cfu/ml cells (Table 5). This similar result was observed by Kapsopoulou et al., (2005) during his studies on growth and fermentation characteristics of wine yeast. However, the growth of Saccharomyces cerevisiae inoculated alongside Acetobacter aceti was characterized by lower concentration of cells. The exponential stage of fermentation was not noted but rather a sharp reduction in yeast count. There was no visible air bubble usually observed during fermentation process.

Table 5. Growth of Saccharomyces cerevisiae (Sc) inoculated into soursop juice with and without Acetobacter aceti (AAB) during fermentation of soursop juice

Fermentation Time (days)	Yeast count (cfu/ml)	
	Sc	Sc + AAB
0	3.2 x 10^5	3.2 x 10^5
2	2.0 x 10^6	1.0 x 10^6
4	3.2 x 10^8	2.0 x 10^6
6	1.6 x 10^7	1.6 x 10^6
8	2.0 x 10^6	1.0 x 10^6
10	1.0 x 10^6	3.0 x 10^5

* Values are means from 3 determinations

Discussion

This study has indicated the presence of acetic acid bacteria during spontaneous fermentation of soursop juice. Gluconobacter oxydan was acetic acid bacteria found in sound, unspoiled fruits and also occur at the early stage of fermentation. Acetobacter aceti became more prevalent as the fruit became spoiled and as a major contaminants during fermentation of soursop juice while Acetobacter pasteurianus was found at the later stage of fermentation. Acetic acid bacteria produced acetic acid through the metabolism of ethanol to acetaldehyde and to acetic acid. There are two membrane bound enzymes catalysing the reaction. Firstly alcohol dehydrogenese convert ethanol to acetaldehyde and secondly acetaldehyde dehydrogenese which convert acetaldehyde to acetic acid (Adachi et al; 1987). The intermediate metabolite acetaldehyde can contribute to the sensory spoilage of wine with distinct aroma. The growth of A. pasterianus to 4.0 x 104cfu/ml on the 10th day of fermentation is an indication of a complete change in the chemical composition of the fermenting must. At this stage there is a decrease in ethanol concentration and a substantial increase in acetic acid concentration. This findings confirms the result observed by Bartowsky and Henschke (2008) in her studies on spoilage of bottle wine by acetic acid bacteria.

Fruit juice is despite being a rich medium with high concentrations of hexoses (glucose and fructose 200-250g/L) high pH (pH 2.9-3.7) and Titratable acidity (2-10g/L as tartaric acid) provide a high selective medium for bacterial growth. Also the very harsh environment of the fermenting juice created by high ethanol concentration, low oxygen content and redox potential, low pH and depletion of nutrients, resulting from consumption by yeast during alcoholic fermentation restricted the growth of Gluconobacter oxydans (Drysdale and Fleet, 1985). In the absence of yeast, especially during juice preparation, acetic

acid bacteria populations can increase to spoil the juice by production of acetic acid, but during fermentation, the population of the bacteria tends to decrease and can fall below 102cfu/ml by the end of fermentation. Acetobacter species are better adapted to the higher ethanol concentrations and thus tend to colonised the fermented juice thereby causing spoilage (Ukwo et al., 2010).

There was a strong correlation between the growth of acetic acid bacteria and the concentration of acetic acid present at the fermenting sample. Also allowing atmospheric oxygen into the fermenting vessels resulted in rapid multiplication of these species and consequently the production of more acetic acid. This process is particularly favourable at higher temperature and elevated pH. This has shown that acetic acid bacteria being strickly aerobic would developed in wine when the fermenting vessel is partially filled. This findings agrees with the result obtained by Joyeux et al., (1984) where he noted that this could be counter productive during large scale production of wine.

The effect of acetic acid bacteria on the growth of Saccharomyces cerevisiae during fermentation of soursop juice resulted in a slow or sluggish fermentation. The presence of these bacteria induced classic symptoms of an incomplete fermentation with high residual sugar, lower ethanol, reduction in iso-amyl alcohol and glycerol with a corresponding increase in the production of acetic acid, gluconic acid, acetaldehyde and ethyl acetate (Ukwo et al, 2010). Reports have shown that acetic acid bacteria possess high oxidative enzymes such as alcohol dehydrogenase and aldehyde dehydrogenase capable of oxidizing alcohol produce by yeast to acetic acid during fermentation (Hommel and Ahnert, 1999). Studies has shown acetic acid have inhibitory effect on the growth and metabolism of Saccharomyces cerevisiae during fermentation (Battcock and Azam-Ali 1998). Phowchinda et al., (1995) also noted that acetic acid causes a reduction of maximal cell concentration of Saccharomyces cerevisiae cells during fermentation.

According to Pampulla and Loweiro-Dias (1989), the undissociated acetic acid produced by Acetobacter aceti diffuses into yeast cells and caused a decrease in the pH of cytoplasm thereby inhibiting the activities of key enzymes especially enolase, aldolase, phosphoglyceromutase and triosephosphate isomarase. This have resulted in the lower cells of Saccharomyces cerevisiae inoculated alongside acetic acid bacteria.

Conclusion

This study investigated the occurrence of acetic acid bacteria during various stages of soursop juice fermentation. The study demonstrated permanent presence of acetic acid bacteria during fermentation of soursop juice. There were changes in the type of bacteria present at various stages of fermentation. Successively, these bacteria are G. oxydan, A. acetic A. pasteurianus. Only A. aceti was prevalent all stages of fermentation. A higher temperature elevated pH, and the presence of atmospheric oxygen are encouraging factors for bacteria growth and acetic acid production which is counter productive to wine production. Prevention of acetic acid bacteria proliferation is based on an understanding that these bacteria are aerobic in their physiology and require oxygen for growth. Such growth can be prevented by practices that include blanketing wine with an inert gas such as carbon (iv) oxide, ensuring that fermenting containers are completing filled with the juice to minimise contact with the head space of air or oxygen and by the process of sterile filtration of the juice. These will provide an important tool toward reducing the incidence of wine spoilage caused by proliferation acetic acid bacteria during fermentation.

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