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*Full Length Research Paper*

# Isolation and identification of the genera *Acetobacter* and *Gluconobacter* in coconut toddy (mnazi)

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**This study investigated the occurrence and identified the dominant spoilage genera of acetic acid bacteria in coconut wine (mnazi), by plating the dilution series previously pre-enriched in a basal medium onto GYP agar, followed by physiological and biochemical tests. Both *Acetobacter* and *Gluconobacter* strains were Gram variable, oxidase negative and catalase positive. All *Acetobacter* strains over-oxidized ethanol to acetic acid and finally to $CO_2$ and $H_2O$, while *Gluconobacter* were unable to oxidize acetic acid to $CO_2$ and $H_2O$. *Acetobacter* and *Gluconobacter* alike showed positive growth at 25, 30 and 40°C and also at pH 7.0 and 4.5, while there was no growth at 45°C, pH 2.5 and 8.5. *Acetobacter* strains oxidized both lactate and acetate while *Gluconobacter* oxidized lactate only. Both genera were unable to liquefy gelatin. *Acetobacter* showed negative growth at 15°C and also in peptone medium, while *Gluconobacter* showed positive growth both in peptone medium and at 15°C. Both genera were able to ferment arabinose, xylose, ribose, glucose, galactose, mannose and melibiose and unable to ferment amylagdine, cellibiose, esculine, lactose, maltose, mannitol, melezitose, Na-gluconate, raffinose, rhamnose and salicine. The *Acetobacter* and *Gluconobacter* strains isolated in this study were found to be responsible for the spoilage of mnazi.**

**Key words:** Mnazi, palm wine, acetic acid bacteria*, Acetobacter, Gluconobacter*.

## INTRODUCTION

Mnazi is a typical tropical alcoholic beverage, produced by fementation of sugary coconut sap. The coconut sap is tapped from palm trees which grow at the coastal region of Kenya. The trees commonly used for this purpose are *Elaeis guineensis, Raphia vinifera, Cocos nucifera and Arenga pinnata.* Mnazi is a whitish, effervescent, acidic alcoholic beverage (Swings and De Ley, 1977). The tapping process of the toddy used in this study was as explained by Kadere et al. (2004). It is a product of a mixed alcoholic, lactic and acetic fermentation. As a first step, the sugar of the sap is fermented to ethanol within 8 - 12 h by yeasts and lactic acid bacteria, thus creating a highly suitable medium for the development of acetic acid bacteria. During fermentation, the acetic acid bacteria appear after 2 - 3 days. Acetic acid bacteria utilizing the glucose and/or sucrose might be present in earlier stages of the mnazi fermentation (Okafar, 1975).

Acetic acid bacteria are divided into the genera *Gluconobacter, Acetobacter and Fratueria* (Holt et al., 1994). Of these, *Gluconobacter oxydans, Acetobacter aceti, Acetobacter pasteurianus, Acetobacter liquefaciens* and *Acetobacter hansenii* are normally associated with grapes and wines (Blackwood et al., 1969; Joyeux et al., 1984a). However, according to Ruiz et al. (2000), acetic acid bacteria are divided into the genera *Acetobacter, Acidomonas, Gluconobacter* and *Gluconacteobacter*. *Acetobacter* species prefer ethanol as carbon source (De Ley et al., 1984) and usually dominate during the later stages of wine fermentation (Drysdale and Fleet, 1985; Joyeux et al., 1984a; Du Toit and Lambrechts, 2002). *Acetobacter* species were earlier isolated from palm wine (Faparusi, 1973; Faparusi and Bassir, 1972; Okafar, 1975) and from immature spadix of palm tree (Faparusi, 1973). *A. pasteurianus* was isolated from palm wine (Simmonart and Laudelout, 1951), and *A. aceti subsp.*

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*Xylinium* from the leaflets of the palm tree and the surrounding air (Faparusi, 1973). *Gluconobacter oxydans subsp. Suboxydans* was found on the floret of palm tree (Faparusi, 1973); in the tap holes and in palm sap (Faparusi, 1974).

*Acetobacter* cells and *Gluconobacter* alike are Gram-negative or Gram variable, ellipsoidal to rod-shaped, straight or slightly curved, 0.6 - 0.8 µm X 1.0 - 0.4 µm, occurring singly, in pairs or chain. Pleomorphic form occurs which may be spherical, elongated, swollen, club shaped, curved or filamentous. *Acetobacter* cells are non-motile or motile, if motile, petrichous or lateral flagella are present. However, motile strains of *Gluconobacter* have three and eight polar flagella; a single flagellum is rarely observed. In liquid media, *Acetobacter* forms a ring, film or pellicle, uniform turbidity of the medium and a cell deposit is sometimes observed (De Ley, Swings and Gosselé, 1984). Some strains produce a pink, non-diffusible pigment whereas others may produce a soluble, dark brown pigment and ɣ-pyrone. The pathway for ɣ-pyrone formation has been elucidated (Asai, 1968) and it is believed that the product of brown pigments is related to ɣ-pyrone synthesis (Rainbow, 1981).

The strains of acetic acid bacteria are useful for vinegar production; however, lack of defined pure starter cultures is due to problems in strain isolation, cultivation and preservation of vinegar bacteria (Kittleman et al., 1989; Sievers et al., 1992; Sokollek and Hammes, 1997). Acetic acid bacteria are able to produce high amounts of acetic acid from alcohol. Furthermore, these bacteria can produce other compounds, apart from acetic acid, that can influence wine quality (Drysdale and Fleet, 1989a). Earlier research has also shown that acetic acid bacteria (genera *Acetobacter* and *Gluconobacter*) were able to produce some polysaccharides such as cellulose, levan and dextran (Hibbert and Barsha, 1931; Loitsyanskaya, 1965; Hehre and Hammilton, 1953). Valla and Kjosbakken (1981) showed that cellulose-negative strain of '*Acetobacter xylinum*' obtained by spontaneous mutation produced an extra-cellular polysaccharide composed of glucose, rhamnose, mannose and glucuronic acid in a molar ratio of 3:1:1:1 (Hibbert and Barsha, 1931; Loitsyanskaya, 1965; Hehre and Hammilton, 1953).

The objectives of this study were to investigate the occurrence and identification of the dominant spoilage genera of acetic acid bacteria in mnazi tapped by traditional methods at the coastal region of Kenya.

## MATERIALS AND METHODS

### Samples

The coconut wine samples for the isolation of the acetic acid bacteria (AA) were obtained from Mtwapa and Kikambala areas of the coastal region of Kenya. The freshly tapped wine samples were collected in sterile sampling tubes. The pH of the sample was determined at the sampling site using a portable pH meter. The samples were kept at 4ºC and transported in cool boxes packed with dry ice to the Food Science and Technology Laboratory at the Jomo Kenyatta University of Agriculture and Technology (JKUAT). The samples were preserved at -20ºC in the Food Microbiology laboratory at JKUAT for a period of six hours before they were air-lifted to Okayama University, Japan, Animal Food Functions laboratory for isolation and identification of the two genera.

### Isolation and identification of acetic acid bacteria (AAB)

The strains were isolated by plating one milliliter of each dilution series previously pre-enriched in a basal medium (5% glucose, 1% yeast extract, 100 ppm cycloheximide) onto GYP agar {glucose (2% m/v), Na-acetate.$3H_2O$ (0.5% m/v), tryptone (0.5% m/v), yeast extract (0.5% m/v), potassium phosphate (0.1% m/v), Tween 80 solution (0.5% v/v) and agar (1.7% m/v)} and mannitol medium {mannitol (2.5% m/v), yeast extract (1% m/v), and agar (1.5% m/v)}. The pH for GYP was adjusted to pH 6.8 whereas mannitol medium was adjusted to pH 7.0. Growth of lactic acid bacteria was inhibited by addition of 50 mg/l primaricin and 50 mg/l nisin to GYP agar and mannitol medium respectively. The dilutions were plated in triplicate and incubated at 25, 30 and 37ºC for a period of 3 - 5 days. Representative colonies of the isolates (98 strains) were Gram stained using the conventional method. Isolates that were Gram negative/positive (variable), oxidase and catalase positive were stored on GYP agar slants at 4ºC and transferred monthly until identification. The ninety eight (98) acetic acid bacterial isolates were reduced further to 17 isolates based on physiological and morphological similarities. The 17 isolates were identified up to genera using the following biochemical and physiological tests. Oxidase test (Kovacs, 1956; Steel, 1961) was carried out using test strips (Difco). Oxidase positive colonies developed pink colour, which became successively dark red, purple and black in 5 - 10 s. A delayed positive was indicated by purple colouration within 10 - 60 s, any later reaction was regarded as negative. Growth in gelatin and gelatin liquefaction was done using the gelatin infusion broth (Gelatin – 40 g, Beef heart, solids from infusion-500 g, Tryptose - 10 g, NaCl – 5 g, distilled water - 1000 ml, pH 7.4). Any liquefaction of the medium was considered positive result for gelatin liquefaction. Peptone broth (Peptone – 10 g and distilled water – 1000 ml) was used to determine growth in peptone. Motility observation was conducted using the motility test by soft agar medium (Glucose - 0.5 g, yeast extract - 0.5 g, peptone - 0.5 g, meat extract - 0.5 g, Tween 80 - 0.05 g, agar-0.15 g, distilled water – 100 ml and pH 6.8). Incubation of previously stabbed medium was done at 30ºC for 2 - 3 days. Cultures that showed positive growth only at the stabbed areas were regarded as negative while those that showed growth all over the medium were regarded as positive growth. Oxidation of ethanol and acetic acid at pH 7.0 and pH 4.5 was determined as explained by Frateur (1950). Over oxidation of ethanol into acetic acid and finally into $CO_2$ and $H_2O$ was done at pH 4.5 and 7.0 respectively by the method explained by Carr (1968). The medium used for these tests contained 3% Difco yeast extract, 2% ethanol, 0.0022% bromocresol blue (green) and 2% agar. Oxidation of lactate was done using the method explained by Frateur (1950) using yeast water agar {yeast extract (3% m/v), calcium lactate (2% m/v), agar (2% m/v)}, while 2% sodium acetate was used instead of calcium lactate for the oxidation of acetate**.** Growth at temperatures 15, 25, 30, 37, 40 and 45ºC and that at pH 2.5, 3.0, 4.0, 7.0, 8.0 and 8.5 was also conducted using GYP broth (Frateur, 1950). This was then followed by production of a brown pigment on GYP medium (Drysdale and Fleet, 1988). Biochemical tests, included fermentation of carbohydrates using 22 different sugars. The basal medium for these tests was GYP broth incorporated with 0.5 ml of 5% tested sugar as the sole source of carbon. For Esculine 2.5% was used instead. Tests preparations were incubated at 30ºC and readings were done after 1 - 10 days of incubation.

**Table 1.** Physiological and biochemical characteristics of *Acetobacter* strains.

| Bacterial Strains | TYC4031 | TP3051 | AYC4031 | GY541 | GY542 | GY553 | GY554 | GY555 | GY557 | GY558 |
|---|---|---|---|---|---|---|---|---|---|---|
| G. Stain | v | v | v | v | v | v | v | v | v | v |
| Catalase | + | + | + | + | + | + | + | + | + | + |
| Oxidase | - | - | - | - | - | - | - | - | - | - |
| growth in gelatin | + | + | + | + | + | - | + | + | + | + |
| Gelatin Liquefaction. | - | - | - | - | + | - | - | - | - | - |
| Growth pH 2.5 | - | - | - | - | - | - | - | - | - | - |
| Growth pH 3.0 | - | - | + | - | - | - | - | + | - | - |
| Growth pH 4.5 | + | + | + | + | + | + | + | + | + | + |
| Growth pH 7.0 | + | + | + | + | + | + | + | + | + | + |
| Growth pH 8.0 | + | + | + | - | - | - | - | - | - | - |
| Growth pH 8.5 | - | - | - | - | - | - | - | - | - | - |
| Growth at 15ºC | - | + | + | - | - | - | + | - | - | - |
| Growth at 25ºC | + | + | + | + | + | + | + | + | + | + |
| Growth at 30ºC | + | + | + | + | + | + | + | + | + | + |
| Growth at 40ºC | + | + | + | + | + | + | + | + | + | + |
| Growth at 45ºC | - | - | - | - | - | - | - | - | - | - |
| Growth in peptone | - | - | - | - | w | - | - | - | - | - |
| [1]Growth in Lactate | + | + | + | + | + | + | + | + | + | + |
| [1]Growth in Acetate | - | - | - | + | + | w | + | + | + | + |
| Oxid. Ethanol at pH 7.0 | + | + | + | + | + | + | + | + | + | + |
| Oxid. Ethanol at pH 4.5 | + | + | + | + | + | + | + | + | + | + |
| Over oxid. Ethanol at pH 7.0 | + | + | + | + | + | + | + | + | + | + |
| Over oxid. Ethanol at pH 4.5 | + | + | + | + | + | + | + | + | + | + |
| Motility | + | + | + | + | + | + | + | + | w | + |
| Brown pigment on GYP | - | - | - | - | - | - | - | - | - | - |

+ = Positive result, - = negative result, v = variable, w = weak.
[1]Strains did not only show positive growth but were also able to oxidize the compounds to $CO_2$.

## RESULTS

### Identification of the genera *Acetobacter* isolated from Mnazi

All the strains that were Gram negative or Gram variable, oxidase negative, catalase positive ellipsoidal to rod-shaped, straight or slightly curved were preliminarily identified as acetic acid bacteria (Table 1) according to the biochemical and physiological tests described in materials and methods. All the isolated *Acetobacter* strains were Gram variable, oxidase negative and catalase positive. They all showed positive growth at pH 7.0 and pH 4.5. Growth at 25 and 30 and 40ºC was positive for all the *Acetobacter* strains, while that at 45ºC, pH 8.5 and pH 2.5 was negative for all. Most of them were unable to grow at 15ºC, pH 3.0 and pH 8.0. Motility on molten agar was positive, growth on peptone medium and formation of brown pigment on GYP medium were all negative. Most strains showed positive growth on gelatin, but gelatin liquefaction was negative. These strains registered positive growth on lactate, in addition they were able to oxidize lactate to $CO_2$ and $H_2O$ with deposit of $CaCO_3$ around the inoculated zones. All strains suspected to fall under the genera Acetobacter were able to oxidize acetate to $CO_2$ and $H_2O$ except TYC4031, TP3051 and AYC4031. All *Acetobacter* strains over-oxidized ethanol to acetic acid and finally to $CO_2$ and $H_2O$ in neutral (pH 7.0) and acidic conditions (pH 4.5). The acetic acid produced by *Acetobacter* strains changed the indicator from blue to yellow and upon further incubation, the acetic acid was further oxidized to $CO_2$ and $H_2O$; the indicator then reverted to the blue color.

Table 2 shows that most *Acetobacter* strains were able to ferment the following sugars: arabinose, xylose, ribose, glucose, galactose, mannose, melibiose and trehalose. All the strains were unable to ferment the following sugars: amylagdine, cellibiose, esculine, fructose, lactose, maltose, mannitol, melezitose, Na-gluconate, raffinose, rhamnose, salicine, sorbitol, sucrose.

### Identification of the genera *Gluconobacter* isolated from Mnazi

As indicated above, all the strains that were Gram negative (variable), catalase positive, oxidase negative, ellipsoidal to rod-shaped, straight or slightly curved were

**Table 2.** Fermentation of common sugars by *Acetobacter* species isolated from palm wine.

| Bacterial strains | TYC4031 | TP3051 | AYC4031 | GY541 | GY542 | GY553 | GY554 | GY556 | GY557 | GY558 |
|---|---|---|---|---|---|---|---|---|---|---|
| Amylagdine | - | - | - | - | - | - | - | - | - | - |
| Arabinose | - | + | ± | ± | ± | ± | ± | ± | ± | ± |
| Cellibiose | - | - | - | - | - | - | - | - | - | - |
| Esculine | - | - | - | - | - | - | - | - | - | - |
| Fructose | - | ± | - | - | - | - | - | - | - | - |
| Galactose | - | + | ± | + | ± | ± | + | + | - | - |
| Glucose | + | + | + | + | + | + | + | + | + | + |
| Lactose | - | - | - | - | - | - | - | - | - | - |
| Maltose | - | - | - | - | - | - | - | - | - | - |
| Mannitol | - | - | - | - | - | - | - | - | - | - |
| Mannose | - | ± | - | ± | ± | ± | ± | ± | ± | ± |
| Melezitose | - | - | - | - | - | - | - | - | - | - |
| Melibiose | ± | + | - | + | + | ± | ± | ± | + | + |
| Na-gluconate | - | - | - | - | - | - | - | - | - | - |
| Raffinose | - | - | - | - | - | - | - | - | - | - |
| Rhamnose | - | - | - | - | - | - | - | - | - | - |
| Ribose | ± | + | - | ± | ± | ± | - | - | ± | ± |
| Salicine | - | - | - | - | - | - | - | - | - | - |
| Sorbitol | - | - | - | - | - | - | - | - | - | - |
| Sucrose | - | - | - | - | - | - | - | - | - | - |
| Trehalose | - | - | - | ± | + | ± | ± | ± | ± | ± |
| Xylose | + | + | + | + | + | + | ± | ± | ± | ± |

+ = Positive result, - = negative result, ± = variable.

preliminarily identified as acetic acid bacteria according to the biochemical and physiological tests described in materials and methods. All isolated strains of *Gluconobacter* were Gram-variable rods, oxidase negative and catalase positive. They were strictly aerobic and motile on molten agar medium (Table 3). They also showed positive growth at pH 4.5 and 7.0 while that at pH 2.5, 8.0 and 8.5 were negative. All strains showed negative growth at pH 3.0 except TYC4032. Growth at temperatures 15, 25 and 30ºC was positive while that at 45ºC was negative; at 40ºC growth was either positive or negative depending on the strain. Most strains were able to grow in peptone medium. Although most strains showed positive growth on gelatin, liquefaction of gelatin on the other hand was negative. *Gluconobacter* strains registered positive growth on lactate but they were not able to oxidize it to $CO_2$ and $H_2O$ with deposit of $CaCO_3$ around the inoculated zones. Similarly most of them were unable to oxidize acetate. All *Gluconobacter* strains oxidized ethanol to acetic acid in neutral (pH 7.0) and acidic conditions (pH 4.5), but upon further incubation, the acetic acid was not over-oxidized to $CO_2$ and $H_2O$ hence the color of the medium changed from blue to yellow without reverting back to blue. Some of the *Gluconobacter* strains were able to form brown pigmentation on GYP medium, while others were unable to form brown pigmentation on GYP medium.

Fermentation of the most common sugars by *Gluconobacter* strains are shown in Table 4. Like Acetobacter strains, most *Gluconobacter* strains were able to ferment the following sugars: arabinose, xylose, ribose, glucose, galactose, mannose and melibiose. However, unlike *Acetobacter* they were unable to ferment mannose and trehalose. All the strains of *Gluconobacter* were unable to grow in the following sugars: amylagdine, cellibiose, esculine, lactose, maltose, mannitol, melezitose, Na-gluconate, raffinose, rhamnose and salicine; however, unlike *Acetobacter*, some of the *Gluconobacter* strains were able to ferment fructose and sorbitol. All *Gluconobacter* strains were unable to ferment sucrose except TYC3031.

## DISCUSSION

Since their first discovery and reporting as a unique group, the acetic acid bacteria have been labeled with numerous genetic names, which have been the subject of extensive discussion and revision. The eighth edition of Berger's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974) recognized only two genera, *Acetobacter* (motile by petrichous flagella or non-motile)

**Table 3.** Physiological and biochemical characteristics of Gluconobacter strains.

| Bacterial strains | TP3052 | TYC3031 | TYC4032 | TYC3034 | TYC3041 | GY 201 | GY 203 |
|---|---|---|---|---|---|---|---|
| G. Stain | v | v | v | v | v | v | v |
| Catalase | + | + | + | + | + | + | + |
| Oxidase | - | - | - | - | - | - | - |
| Growth in gelatin | + | + | + | + | + | + | + |
| Gelatin liquefaction. | - | - | - | - | - | - | - |
| Growth at pH 2.5 | - | - | - | - | - | - | - |
| Growth at pH 3.0 | - | + | - | - | - | - | - |
| Growth at pH 4.5 | + | + | + | + | + | + | + |
| Growth at pH 7.0 | + | + | + | + | + | + | + |
| Growth at pH 8.0 | - | - | - | - | - | - | - |
| Growth at pH 8.5 | - | - | - | - | - | - | - |
| Growth at 15ºC | + | + | + | + | + | + | + |
| Growth at 25ºC | + | + | + | + | + | + | + |
| Growth at 30ºC | + | + | + | + | + | + | + |
| Growth at 40ºC | + | + | - | - | + | + | + |
| Growth at 45ºC | - | - | - | - | - | - | - |
| Growth in peptone | + | w | + | + | + | - | - |
| [1]Growth in Lactate | + | + | + | + | + | + | + |
| Growth in Acetate | - | - | - | - | - | w | + |
| Oxid. Ethanol at pH 7.0 | + | + | + | + | + | + | + |
| Oxid. Ethanol at pH 4.5 | + | + | + | + | + | + | + |
| Over oxid. Ethanol at pH 7.0 | - | - | - | - | - | - | - |
| Over oxid. Ethanol at pH 4.5 | - | - | - | - | - | - | - |
| Motility | + | + | + | + | + | + | + |
| Brown pigment on GYP | w | w | + | - | - | + | - |

+ = Positive result, - = negative result, v = variable, w = weak.
[1]Strains registered positive growth; however they were unable to oxidize lactate to $CO_2$ with deposit of $CaCO_3$ around the inoculation areas.

and *Gluconobacter* (motile by polar flagella or non-motile), and placed the genus *Gluconobacter* with the family Pseudomonadaceae; however, the genus *Acetobacter* was not assigned to any particular family and was grouped within the genera of uncertain affiliation. The Approved List of Bacterial Names, (Skerman et al., 1980) acknowledged both the genera *Acetobacter* and *Gluconobacter*. The ninth edition of Bergey's Manual of Systematic Bacteriology (Buchanan and Gibbons, 1984) recognized the fact that the genera *Gluconobacter* and *Acetobacter* were closely related; hence they were placed within the family Acetobacteraceae. Members of the family are united by their unique ability to oxidize ethanol to acetic acid. Under this family we have genera Acetobacter, *Gluconobacter* and *Frateuria*. Today, acetic acid bacteria have been classified into 24 different genera. The major genera involved in vinegar production include: *Acetobacter, Gluconobacter, Gluconacetobacter, Asaia, Neoasaia, Saccharibacter, Frateuria* and *Kozakia* (De Vero and Giudici, 2008). From the results (Tables 1 and 2) all the isolated strains were found to be catalase positive, oxidase negative, Gram negative (variable), obligate aerobic and grew at pH 4.5. According to the eighth edition of Bergey's manual of determinative Bacteriology (Buchanan and Gibbons, 1974), these strains should be classified into the genera *Acetobacter* or *Gluconobacter*.

Classification of the isolated strains under the genus *Acetobacter* was based on the ninth edition of Bergey's Manual of Systematic Bacteriology (Buchanan and Gibbons, 1984), as well as the reports of Leifson, (1954) and Gosselé et al. (1983). The strains that were classified under the genus *Acetobacter* were Gram negative (variable), obligatory aerobic rods, motile, growth at pH 4.5, oxidizing lactate and acetate to $CO_2$ and $H_2O$, no or very poor growth on peptone, and able to grow on gelatin but unable to liquefy it. The optimum temperature for these strains as given by De Ley and Swings (1984a and 1984b) was reported to be in the range of 25 to 35$^0$C. According to Holt et al. (1994) both *Acetobacter* and *Gluconobacter* alike were unable to grow at 37$^o$C however, in this study, *Acetobacter* strains showed positive growth not only at 37$^0$C but also at 40$^0$C. Positive growth was also registered at pH 4.5 and 7.0 with negative growth

**Table 4.** Fermentation of common sugars by Gluconobacter species isolated from Mnazi.

| Bacteria strains | TP3052 | TYC3031 | TYC4032 | TYC3034 | DCY3031 | GY 201 | GY 203 |
|---|---|---|---|---|---|---|---|
| Amylagdine | - | - | - | - | - | - | - |
| Arabinose | + | + | + | + | - | ± | - |
| Cellibiose | - | - | - | - | - | - | - |
| Esculine | - | - | - | - | - | - | - |
| Fructose | ± | + | - | + | - | + | - |
| Galactose | + | + | + | + | + | + | - |
| Glucose | + | + | + | + | + | + | - |
| Lactose | - | - | - | - | - | - | - |
| Maltose | - | - | - | - | - | - | - |
| Mannitol | - | + | - | + | - | - | - |
| Mannose | ± | - | - | ± | - | - | - |
| Melezitose | - | - | - | - | - | - | - |
| Melibiose | + | + | ± | + | ± | ± | - |
| Na-gluconate | - | - | - | - | - | ± | - |
| Raffinose | - | - | - | - | - | - | - |
| Rhamnose | - | - | - | - | - | - | - |
| Ribose | + | - | ± | + | ± | ± | - |
| Salicine | - | - | - | - | - | - | - |
| Sorbitol | - | + | - | ± | - | - | - |
| Sucrose | - | + | - | - | - | - | - |
| Trehalose | - | - | - | - | - | - | - |
| Xylose | + | + | + | + | ± | - | - |

± = Variable.

at pH 2.5, 8.5, while only a few were able to grow at pH 8.0 and 3.0. In the acid formation test, all the *Acetobacter* isolates were positive for glucose, xylose, while some were able to produce acids from ribose, trehalose, melibiose, mannose, galactose and glucose. This again is confirmed by the Bergey's Manual of Systematic Bacteriology (Buchanan and Gibbons, 1984). According to this study, strains of *Acetobacter* were unable to form acid from the following sugars: lactose, cellibiose, fructose, mannitol, sorbitol, esculine, maltose and melezitose (Table 1). This again confirms the findings by Minakami et al. (1984) and those provided by the Bergey's Manual of Systematic Bacteriology (Buchanan and Gibbons, 1984). Finally, *Acetobacter* strains as provided in this study, were confirmed and differentiated from *Gluconobacter* strains by the method described by Carr, (1968). Based on this method, *Acetobacter* strains were able to over-oxidize ethanol to acetic acid and finally to $CO_2$ and $H_2O$ through tricarboxylic acid cycle in neutral and acidic conditions (pH 7.0 and 4.5 respectively). Whereas due to non-functional tricarboxylic acid cycle in Gluconobacter, the genera is unable to oxidize most organic acids such as acetic, citric, lactic, malic, pyruvic and succinic (Holt et al., 1994). Upon incubation, all *Acetobacter* strains were able to change the medium from blue to yellow and upon further incubation, it reverted back to blue indicating that the acetic acid was converted into $CO_2$ and $H_2O$. This not only confirms the presence of *Acetobacter* strains, but also differentiates them from the *Gluconobacter* strains.

The classification of the genus *Gluconobacter*, isolated in this study was also based on the ninth edition of Bergey's Manual of Systematic Bacteriology (Buchanan and Gibbons, 1984), as well as the reports of Leifson, (1954) and Gosselé et al. (1983). The physiological properties of *Gluconobacter* strains were similar to those of *Acetobacter*. However, most *Gluconobacter* were able to grow in peptone agar as opposed to *Acetobacter*, which were unable to grow on this medium. *Gluconobacter* strains showed positive growth at $15^0$C while most *Acetobacter* strains were unable to grow at this temperature. While all *Acetobacter* strains were unable to produce brown pigmentation on GYP, some of the *Gluconobacter* were able to produce brown pigment on GYP. Almost all *Gluconobacter* strains were unable to oxidize acetate while some of the *Acetobacter* strains did not oxidize acetate to $CO_2$ and $H_2O$. This however, contradicts the suggestion that all *Gluconobacter* strains were unable to oxidize lactate and acetate while all *Acetobacter* strains were able to oxidize the same (Swings and De Ley, 1981). Most of the sugars that gave positive fermentation with *Acetobacter* strains did the same with *Gluconobacter*. However, trehalose and mannose showed positive fermentation with Acetobacter but

negative with *Gluconobacter* while, fructose and mannitol had negative fermentation with most *Acetobacter* but gave positive results with most *Gluconobacter*. As expected *Gluconobacter* strains oxidize ethanol to acetic acid but were unable to over-oxidize it to $CO_2$ and $H_2O$ in neutral and acidic conditions (pH 7.0 and 4.5 respectively). This test served as one of the major differences between *Gluconobacter* and *Acetobacter* during classification of *Gluconobacter*. This again confirms the findings by Minakami et al. (1984), Swings and De Ley, (1981) and those provided by the Bergey's Manual of Systematic Bacteriology (Buchanan and Gibbons, 1984). The other difference between *Acetobacter* and *Gluconobacter* is inability of the former to oxidize lactate while the latter was unable to do so. This phenomenon also differentiates *Gluconobacter* from *Gluconacetobacter* (Navarro and Komagata, 1999).

Acetic acid bacteria or vinegar bacteria though for a long time have been believed to play little, if any, role during winemaking operations due to their aerobic nature (Drysdale and Fleet, 1988), recent findings have shown that acetic acid bacteria do contribute significantly to volatile acidity in must and wine, hence the spoilage associated with most wines (Joyeux et al., 1984b; Drysdale and Fleet, 1989b). Wine spoilage by vinegar bacteria is through the production of acetaldehyde and acetic acid from ethanol. Acetic acid is one of many chemical components found in wine and is produced in low concentrations by yeasts during alcoholic and by lactic acid bacteria during the malolactic fermentation. However, the concentrations of this acid can be significantly increased through the action of spoilage yeasts, spoilage species of lactic acid bacteria, and, in particular, by acetic acid bacteria. Acetic acid constitutes the major volatile acid in wine (especially mnazi) and is considered objectionable at levels above 1.2 to 1.4 g/L (Margalith, 1981; Ribéreau-Gayon et al., 1975). The inability of acetic acid bacteria to grow under the anaerobic conditions that generally prevail in wine has been recognized by wine-makers for many years, and as such, the control of these bacteria has not been viewed as a major problem to the wine industry. However in recent years, the ability of the acetic acid bacteria to affect wine quality has been the subject of renewed interest and research. For example, there is speculation that these bacteria may survive and grow under the semi-anaerobic to anaerobic conditions that occur in stored wine to affected wine quality by mechanisms other than the production of acetic acid (Drysdale and Fleet, 1985; Joyeux et al., 1984a).

Mnazi when freshly tapped is sweet, oyster white and normally rich in amino acids and vitamins. This alcoholic drink when not preserved turns into vinegar (acetic acid) in 2 - 5 days of continuous fermentation. Vinegar therefore is one of the value added bi-products of mnazi. The fact that acetic acid bacteria of the genera *Acetobacter* and *Gluconobacter* were abundantly in palm wine (mnazi) tapped by traditional methods provide enough explanation as to why mnazi spoils readily 2 - 5 days after tapping. As pointed early, acetic acid bacteria tend to appear after 2 - 3 days after the onset of coconut sap fermentation. This again confirms the fact that palm wine spoilage is mainly cause by the presence of acetic acid bacteria in the wine. The significant populations of acetic acid in mnazi may influence the composition on wine (mnazi), the growth of yeasts during alcoholic fermentation and the growth of lactic acid bacteria during malolactic fermentation (Joyeux et al., 1984a, 1984b; Sponholz and Dittrich, 1984, 1985). This therefore, accelerates the spoilage process through significant increase of acid mainly acetic acid and other volatile components. Some of the volatile substances associated with acetic acid include: gluconic acid and ketogluconic acid (Holst et al., 1982; Izuo et al., 1980; Seiskari et al., 1985; Tramper et al., 1983). The *Acetobacter* and *Gluconobacte*r strains isolated in this study are not only responsible for the spoilage of mnazi but when utilized could serve as useful microorganisms in the production of vinegar. Further research should be conducted with an aim of incorporating the strains isolated in this study into the vinegar manufacturing industry. Success in this line of research will go a long way in improvement of the vinegar industry in Kenya. The fact that substances of industrial importance such as sorbose, dihydroxyacetone, gluconic acid and ketogluconic acid were obtained through oxidation of sugars and sugar alcohols by *Gluconobacter oxydans* qualifies the need for further research on the isolated strains to establish their usefulness in industrial application (Holst et al., 1982; Izuo et al., 1980; Seiskari et al., 1985; Tramper et al., 1983). The authors therefore recommend the use of other procedures such as 16S and/or 23S rRNA gene sequencing, DNA-DNA similarity tests, DNA base composition as well as DNA relatedness and Quinone analysis. These procedures are expected to help in identification of the respective species in the two genera before serious research on industrial application of the identified species is embarked on.

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**REFERENCES**


Asai T (1968). Acetic Acid Bacteria. Classification and Biochemical Activities, University of Tokyo Press. Tokyo and University Park press, Baltimore, p. 103.

Blackwood AC, Guimberteau G, Penaud E (1969). Sur les bactéries acétiques isolées de raisins. C.R. Seances Acad. Sci. Serv. D 269: 802-804.

Buchanan RE, Gibbns NE (1974). Bergey's´s Manual of Determinative Bacteriology (8th ed.). The Williams and Wilkins Co., Baltimore.

Buchanan RE, Gibbns NE (1984). Family VI. Acetobacteraceae. In: Bergey's´s Manual of Systematic Bacteriology, Vol.1 (9th ed.). Holt JG (eds). The Williams and Wilkins Co., Baltimore, pp. 267-78.

Carr JG (1968). Identification of acetic acid bacteria. In Identification methods for Microbiologists, Part B. Gibbs BM, Shapton DA (eds). Academic Press, London, pp 1-8.

De Ley J, Gillis M, Swings J (1984). Family VI. Acetobactaceae. Bergey's Manual of Sytematic Bacteriology, Vol. 1, 19th edn. Williams and Wilkens , MD, USA, pp. 267-274.

De Ley J, Gillis M, Swings J (1984a). Genus 11. *Gluconobacter Asai* 1935, 689 emend. Mut. Char. Asai, Iizuka and Komagata 1964, 100. In: Bergey's Manual of Systematic Bacteriology, Vol. 1 (9th ed.). Holt JG (eds) Williams and Wilkins Co., Baltimore, pp. 275-284.

De Ley J, Swings J, Gossele F (1984b). Genus 1. Acetobacter Beijerinck 1898, 215 In: Bergey's Manual of Systematic Bacteriology, Vol. 1 (9th ed.). Holt JG (eds) The Williams and Wilkins Co., Baltimore, pp 268-74.

De Vero L, Giudici P (2008). Genus-specific profile of acetic acid bacteria by 16S rDNA PCR-DGGE. Int. J. Food Microbial. 125(1): 96-101.

Drysdale GS, Fleet GH (1985). Acetic acid bacteria in some Australian wines. Food Technol. Austr, 37: 17-20.

Drysdale GS, Fleet GH (1988). Acetic acid bacteria in wine making: a review. Am. J. Enol. Vitc 39: 143-154.

Drysdale GS, Fleet GH (1989a). The growth and survival of acetic acid bacteria in wines at differentconcenration of oxygen. Am. J. Enol. Vitic 40: 99-105.

Drysdale GS, Fleet GH (1989b). The effect of acetic acid bacteria upon the growth and metabolism of yeast during the fermentation of grape juice. J. Appl. Bactariol. 67: 471-481.

Du Toit WJ, Lambrechts MG (2002). The enumeration and identification of acetic acid bacteria from South African red wine fermentations. Int. J. Food Microbiol. 74: 57-64.

Faparusi SI (1973). Origin of initial microflora of palm wine from oil palm trees *(Elaeis guineensis).* J. Appl. Bacteriol. 36: 559-565.

Faparusi SI (1974). Microorganisms from oil palm trees *(Elaeis guineensis)* tape holes. J. Food Sci. 39: 755-757.

Faparusi SI, Bassir O (1972). Factors affecting the quality of palm wine. II. Period of storage. West Afr. J. Biol. Appl. Chem. 15: 24-28.

Frateur J (1950). Essai sur la systematique des Acetobacters. Cellule. 53: 287-393.

Gosselé F, Swings J, Kersters K, De Ley J (1983). Numerical analysis of phenotypic features and protein gel electropherograms of Gluconobacter asai 1935 emend. Mut. Char. Asai, Iizuka, and Komagata 1964. Int. J. Syst. Bacteriol., 33: 65-81.

Hehre EJ, Hammilton DM (1953). The biological synthesis of dextran from dextrins. J. Biol. Chem. 192: 161-174.

Hibbert H, Barsha J (1931). Structure of the cellulose synthesized by the action of *Acetobacter xylinus* on glucose. Can. J. Res. 5: 580: ibid., 10: 170.

Holst O, Enfor S, Mattiasson B (1982). Oxygenation of immobilized cells using hydrogen-peroxide: a model study of *Gluconobacter oxydans* converting glycerol to dihydroxyacetone. J. Appl. Microbiol. Biotechnol., 14: 64-68.

Izuo N, Nabe K, Yamada S, Chibata I (1980). Production of dihydroxyacetone by continuous cultivation of *Acetobacter suboxydans*. J. Ferment. Technol. 58: 221-226.

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994). Genus *Acetobacter* and *Gluconobacter*. Bergey's Manual of Determinative Bacteriology, 19th edn. Williams and Wilkens , MD, USA, pp. 71, 84.

Joyeux A, Lafon-Lafourcade S, Ribéreau-Gayon P (1984a). Evolution of acetic acid bacteria during fermentation and storage of wine. Appl. Environ. Microbiol. 48: 153-156.

Joyeux A, Lafon-Lafourcade S, Ribéreau-Gayon P (1984b). Metabolism of acetic acid bacteria in grape must: consequences on alcoholic and malolactic fermentation. Sci. Aliment 4: 247-255.

Kadere TT, Oniang`o RK, Kutima PM, Muhoho SN (2004). Traditional tapping and distillation methods of coconut wine (mnazi) as practiced in the Coastal region of Kenya. Afr. J. Food Agric. Nutr. Dev. 4(1): 1-16.

Kittleman M, Stamm WW, Follman H, Trűper HG (1989). Isolation and classification of acetic acid bacteria for higher percentage vinegar fermentations. Appl. Microbiol. Biotechnol. 30: 47-52.

Kovacs N (1956). Identification of *Pseudomonas pyocyanea* by the Oxidase reaction. In Nature Bd. 178, S. 703.

Leifson E (1954). The flagellation and taxonomy of species of *Acetobacter*. Antonie v. Leeuwenhoek, 20: 102-110.

Loitsyanskaya MS (1965). Tr. Pertergof. Biol. Chem., 192: 161.

Margalith PZ (1981). Flavour Microbiology. Charles Thomas Publishers, Springfield, IL., pp. 167-168.

Minakami H, Entani E, Tayama K, Fujiyama S, Masai H (1984). Isolation and characterization of a new polysaccharide-producing *Acetobacter* species. Agric. Biol. Chem. 48: 2405.

Navarro RR, Komagata K (1999). Differentiation of *Gluconacetobacter liquefaciens* and *Gluconacetobacter xylinus* on the basis of DNA base composition, DNA relatedness, and oxidation products from glucose. J. Gen. Microbiol. 45: 7-15.

Okafar N (1975). Microbiology of Nigerian palm wine with particular reference to bacteria. J. Appl. Bacteriol. 38: 81-88.

Rainbow C (1981). In Brewing Science, Vol. 2 (ed. Pollock JRA), Academic Press, London, p. 491.

Ribéreau-Gayon J, Peynaud E, Ribéreau-Gayon P, Sudraud P (1975). Traite d´oenologie. In: Sciences et Techniques du vin, Tome 2. Dunod, Paris.

Ruiz A, Poblet M, Mas A, Guillamon JM (2000). Identification of acetic acid bacteria by RFLP of PCR-amplified 16S rDNA and 16S-23S rDNA intergenic spacer. Int. J. Syst. Microbiol. 50: 1981-1987.

Seiskari P, Linko Y, Linko P (1985). Continuous production of gluconic acid by immobilized gluconic acid by immobilized *Gluconobacter oxydans* cell bioreactor. Appl. Microbiol. Biotechnol. 21: 356-360.

Sievers M, Sellmer S, Teuber M (1992). Acetobacter europaeus sp. Nov., a main component of industrial vinegar fermenters in central Europe. Syst. Appl. Microbiol. 15: 386-392.

Simonart P, Lauedelout H (1951). Ětude mcrobiologique et biochimique du vin de palme. Bulletin de l'Institut Royal Colonial Belge, 22: 385-401.

Skerman VBD, McGowan V, Sneath PHA (1980). Approved lists of bacterial names. Itn. J. Syst. Bacteriol. 30: 225-420.

Sokollek SJ, Hammes WP (1997). Description of a starter culture preparation for vinegar fermentation. System. Appl. Microbiol. 20: 481-491.

Sponholz WR, Ditrich HH (1985). Uber das Vorkommen von Galacturon und Glucurosaure sowie von 2- und 5-Oxo-Gluconsaure in Weinen, Sherries, obst- und Dessertweinen. Vitis, 23: 214-224.

Sponholz WR, Ditrich HH (1985). Uber die Herkunft von Gluconsaure, 2- und 5-Oxo Gluconsaure sowie Glucuron- und Galacturonsaure in Mosten und Weinen. Vitis, 24: 51-58.

Steel KJ (1961). The Oxidase reaction as a taxonomic tool. J. Gen. Microbiol. 25: 297-306.

Swings J, De Ley J (1977). The biology of *Zymomonas*. Bacteriol. Rev. 41: 1-46.

Swings J, De Ley J (1981). The genera *Gluconobacter* and *Acetobacter*. In: The Prokaryotes, a Hand book on Habitats, Isolation and Identification of Bacteria. Starr MP, Stolp H, Truper HG, Balows B, Schlegel HG (Eds.). springer Verlag, Berlin, pp. 771-778.

Tramper J, Luyben AM, van den Tweel WJJ (1983). Kinetic aspects of glucose oxidation by Gluconobacter oxydans cells immobilized in calcium alginate. Eur. J. Appl. Microbiol. Biotechnol. 17: 13-18.

Valla S, Kjosbakken J (1981). Isolation and characterization of a new extracellular polysaccharide from a cellulose-negative strain of *Acetobacter xylinum*. Can. J. Microbiol. 27: 599.