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# Study of Pineapple Peelings Processing into Vinegar by Biotechnology

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**Abstract:** This study aimed to reduce post-harvest losses of pineapple local variety egbenana by the transformation of juice into vinegar through biotechnological process.Vinegar was produced through two successive fermentations: alcoholic and acetic fermentations. The alcohol fermentation was carried out at 30°C using yeast. Biomass, pH and Brix were evaluated daily during the fermentation. Acetic fermentation was carried out at 30°C using an acetic bacteria strain isolated from pineapple wine previously exposed to ambient temperature (28°C) for 5 days. Biomass, pH and acid levels were monitored each 2 days. The performance of acetic bacteria isolated was also assessed by studying their glucose and ethanol tolerance. The study allowed the isolation of yeast coded *Saccharomyces cerevisiae* (LAS01) and an acetic bacteria coded *Acetobacter* sp. (ASV03) both occurring in the pineapple juice. The monitoring of successive fermentations indicated that the pineapple juice with sugar concentration of 20 Brix, seeded with $10^6$ cells of *Saccharomyces cerevisiae* (LAS01) for alcoholic fermentation for 4 days and afterwards seeded with $10^6$ cells of *Acetobacter* sp. resulted in 4.5 acetic degree vinegar at Brix 5.3% and pH 2.8 for 23 to 25 days. The study of glucose tolerance of the strain of *Acetobacter* sp. showed that the growth of acetic bacteria was important in a juice with high concentration of sugar. However, the concentration of ethanol did not effect on the acetic bacteria growth. These results enabled on one hand to improve the manufacturing technology of vinegar from fruits and on the other hand to produce a starter of yeast and acetic bacteria strains for this production.

**Key words:** Pineapple juice, microbial starter, fermentation, vinegar

## INTRODUCTION

Developing countries, including tropical African countries, produce huge quantities of fruits and vegetables; these are usually consumed fresh and of which a significant fraction is abandoned at production site. As these countries, Togo because processing and/or appropriate conservation experiences during harvest overproduction of some fruits, including pineapple, losses between 30 and 50% of crops, according to the ministry of agriculture raising and peach. Therefore, processing and storage of agro-perishable foods to preserve them over a long period is a necessity for developing countries both economically and socially.

The sugars adhering to the fruit processing waste materials are ideal substrates for fermentations. Several reports suggested the possibilities of alcohol and vinegar production from various fruit processing wastes (Ethiraj and Suresh, 1990). But Gullo *et al.* (2005) reported that the production of vinegar has some sugar in fruit juice and that the concentration of ethanol is not a limiting factor for the growth of acetic bacteria.

Vinegar is commonly used as food ingredient but also for its medicinal properties and for its physiological effects such as invigorating, regulator of blood pressure, diabetes mellitus regulator, appetite stimulator, digestion and absorption of calcium (Ndoye *et al.*, 2007). Consequently, acetic acid bacteria cause an important industrial interest as well as lactic acid bacteria and yeast. Since, the acetic bacteria are involved in the production or spoilage of food, their species identification is lead information for the technologist trying to control a bioprocess in industry (Trcek, 2005). In recent years, there have been major advances in understanding their taxonomy, molecular biology and physiology and in methods for their isolation and identification (Raspor and Goranoviccaron, 2008). However, problems related to environment conditions such as temperature variations and process technology limit the artisanal and industrial applications in tropical regions (Ndoye *et al.*, 2007).The production of traditional balsamic vinegar uses a selection of yeasts and acetic bacteria (De Vero *et al.*, 2006). Ndoye *et al.* (2006) reported that acetic bacteria are gram-negative strictly aerobic bacteria and commonly found in

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nature on vegetable products (fruits, cereals, herbs…). They have the ability to oxidize the different types of alcohol in major food products derived from biotechnology as vinegar. The strains involved in production of high acetic degree vinegar are rarely isolated from the environment with success. Even after the successful isolation, the strains have proven to be less efficient and extremely difficult for handling under laboratory conditions, especially if the idea is to preserve their original high acetic acid resistance (Treck, 2005). A few species of acetic bacteria are able to grow at high sugar concentration, but the sugar tolerance is an important acetic bacteria trait for traditional Balsamic Vinegar production, since this product is special vinegar made from cooked must with a high sugar concentration (Gullo *et al*., 2005).

The present study was undertaken to find of the possibility of using peelings pineapple fruit for vinegar production through biotechnology.

## MATERIALS AND METHODS

**Plant material:** The pineapple *Ananas comosus* (L.) Merr. (Bromeliaceae), local variety egbenana was used in this study. Pineapple peelings were obtained from a local processing factory in Lome town (Togo) during April 2007 to February 2008.

**Preparation of pineapple juice:** The technology of production of vinegar shown in Fig. 1, is based on two successive fermentations of a pineapple juice. There is an alcoholic fermentation in 30°C with a yeast strain followed by an acetic fermentation at 30°C with an acetic bacteria strain.

Lots of pineapple peelings (10 kg) were mixed with 5 L of boiling water (100°C for 15 min) and the saccharine extracts were obtained by manual pressing. These extracts were treated with 50 ppm sulfite. An optimum amount of total dry matter is necessary in the pineapple juice for production of vinegar with high acetic degree; indeed, the obtained juice was concentrated to 20 Brix by evaporation prior to fermentation.

**Alcoholic fermentation of pineapple juice:** The yeast culture that was used for alcoholic fermentation was obtained by inoculating Sabouraud Chloramphenicol agar (Bio Rad, France) with natural wort of pineapple juice. The gender and species were identified by the seeding on gallery API 20AUX (bioMerieux, France). From the culture of 24 to 48 h on Sabouraud Chloramphenicol agar

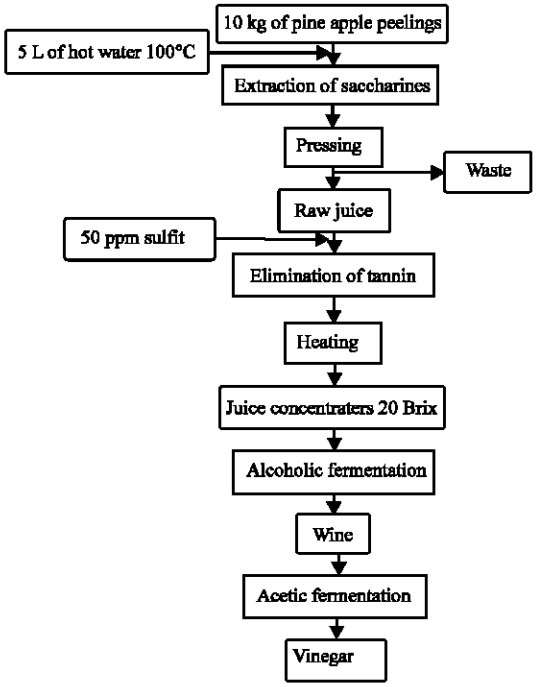


Fig. 1: Flow chart of production of pineapple vinegar

(Bio Rad, France), the yeast strain were seeded extensively on 100 mL broth culture. After 12 to 24 h incubation, 25 mL broths with $10^6$ cfu $mL^{-1}$ were collected for use as inoculum for alcoholic fermentation of pineapple juice, by adding to 450 mL of juice (Brix 20%). The mixture was incubated at 30°C for 72 to 96 h.

**Acetic fermentation of pineapple wine:** The acetic bacteria culture that was used for acetic fermentation was obtained after exposure at ambient temperature (28°C) of 250 mL pineapple wine followed by inoculating on Müller Hinton agar (Bio Rad, France) and of Gram staining. The study of respiration metabolism was performed by growing bacteria on semi-solid beef liver agar (Bio Rad, France) at 30°C for 24 h (Leyral and Vierling, 2007). The production of acetic acid from ethanol was monitored in the mid of yeast extract agar and phenol red (Bio Rad, France) was added to ethanol. The identification of gender was supplemented by oxidase and catalase tests (Guiraud and Galzy, 1980). From the culture of 24 to 48 h on Müller Hinton agar (Bio Rad, France), the acetic bacteria strain, seeded extensively on 100 mL of broth culture. After 12 to 24 h incubation, 25 mL broth with $10^6$ cuf $mL^{-1}$ were collected for use as inoculum for acetic fermentation. This was assayed by adding to 475 mL of previously fermented juice, 25 mL microbial suspension concentration $10^6$ cfu $mL^{-1}$ acetic bacteria. The mixture incubated at 30°C for 23 to 25 days.

**Physico-chemical and microbiological parameters during the fermentation**

**Biomass:** The development of yeast biomass and bacteria biomass was monitored at different stages of fermentation by direct counting with standard plate count and results were expressed in colony forming units (cfu $mL^{-1}$).

**Total sugar:** The total sugar contents of juice was evaluated during the fermentation of pineapple juice with a refractometer (Euromex, HC type 0-32, Holland). The total sugars in the juice was expressed as Brix degree, indicating the mass in gramme of dry matter for 100 g juice.

**The pH:** The pH of the wort fermentation was measured with a digital display pH meter (HANNA, France).

**Acetic acid degree:** The production of acetic acid was determined each 48 h by titration of 1 mL sample with NaOH 0.1 N using phenolphthalein as color indicator. The acidity of vinegar was expressed as degree of acetic acid indicating the mass in gram of pure acid for 100 g of vinegar (Lotong *et al*., 1989).

**Ethanol and glucose tolerance of isolated acetic bacteria:** Acetic bacteria were cultivated on the Muller Hinton agar (Bio Rad, France) at 30°C for 24 h and the broth culture seeded were properly diluted. One milliliter cell suspension containing $10^2$ microbial germs was inoculated Muller Hinton agar Petri dishes with several concentrations of ethanol (2, 4, 6, 8, 10, 12 and 14%) for ethanol tolerance. Glucose tolerance was assayed with the same Muller Hinton agar with different concentration of glucose (16, 18, 20, 22, 24, 26, 28 and 30%) as indicated by Gullo *et al*. (2005).

**Statistical analysis:** The results were processed by the software Excel. Statistical significance was set at $p<0.05$.

## RESULTS

**Assessment of physico-chemical and microbiological parameters during fermentation of acetic pineapple juice**

**Yeast isolated and identified:** The yeast strain isolated was an oval cell, sporulating, apical budding, which fermented and assimilated glucose, galactose, maltose, sucrose, raffinose and bearing hyphae. It was identified as *accharomyces* cerevesiae. It was coded *Saccharomyces cerevisiae* (LAS01).

**Acetic bacteria isolated and identified:** The acetic bacteria strain isolated was a Gram-negative bacillus, an aerobic strict, acid producing from ethanol, catalase positive, oxidase positive. This strain was identified as belonging to *Acetobacter* genus. It was coded *Acetobacter* sp. (ASV03).

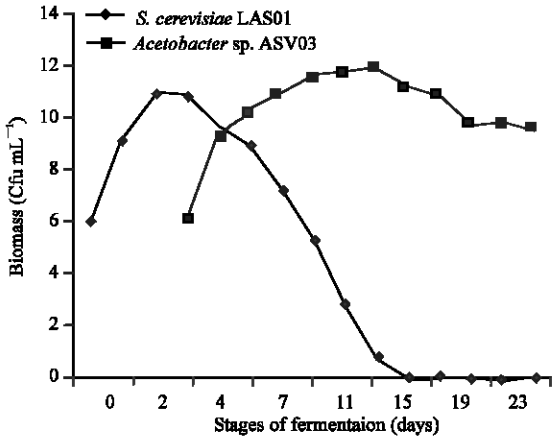


Fig. 2: Yeast and acetobacters biomass during the production of vinegar

**Yeast and bacteria Biomass:** The results of the development of yeast and bacteria biomass during the production of vinegar are shown in Fig. 2.

The growth curve shows three phases: an exponential growth phase from $10^6$ to $1.2\ 10^{11}$ cfu $mL^{-1}$ in the 1st to 3rd day, a stationary phase in time of 3rd to 4th day and a phase of declination to 1.1 cfu $mL^{-1}$ beyond the 4th to 12th day.

The maximum biomass was obtained after 4 days at $1.2\ 10^{11}$ cfu $mL^{-1}$. The growth was maximal after 4 days, this period is the appropriate time start up process in continuous culture: in our case that is acetic fermentation.

The development of bacteria biomass showed that the general growth curve indicated three phases. A phase of rapid growth from $10^6$ to $1.2\ 10^9$ cfu $mL^{-1}$ in the period of 1st to 5th days, a stationary phase in the period of 5th to 11th days and a phase of declination after the 11th day until the 23rd day to $4.4\ 10^5$ cfu $mL^{-1}$.

**Acetic acid production and pH:** Figure 3 shows the evolution of the production of acetic acid and pH changes at different stages of the acetic fermentation.

These results showed an increase in the production of acetic acid from 1.1-4.5 degrees during 1st to 20th day of the acetic fermentation. The changes of pH during acetic fermentation showed that the pH of vinegar decreased from 4.4 to 2.9 during the acetic fermentation. The degree of acidity of vinegar in contrast increased with the length of fermentation. Therefore, increasing the production of acetic acid may the cause of the decrease in pH of vinegar.

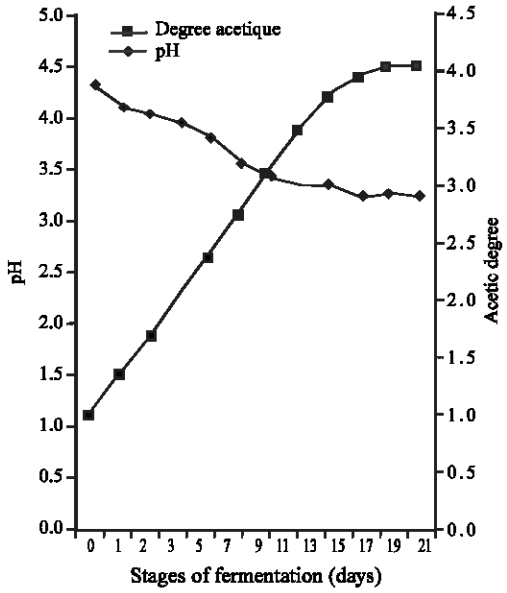


Fig. 3: Evolution of pH and acid levels during acetic fermentation

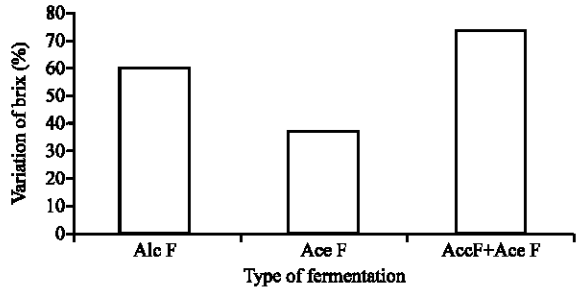


Fig. 4: Variation of Brix during alcohol and acetic fermentation

**Variation of dry content during of alcohol and acetic fermentation:** The results of the evaluation of Brix during acetic fermentation (Fig. 4) showed a variation of dry contents during acetic fermentation due to the use of sugar by yeast and acetic bacteria for growing. It was estimated at 59.5, 36.9 and 73.5%, respectively for the alcoholic fermentation, acid fermentation and alcoholic and acetic fermentation.

**Ethanol and glucose tolerance of acetic bacteria:** The results of ethanol tolerance of acetic bacteria in the pineapple juice are shown in Fig. 5. These results in general showed a slight decrease in the number of strains of acetic bacteria from 86 to 78% between 2 and 6% ethanol and a significant decrease from 78 to 50% between 6 and 14% ethanol. The majority (78%) of acetic bacteria have grown at 6% ethanol and some (58%) at 12% ethanol.

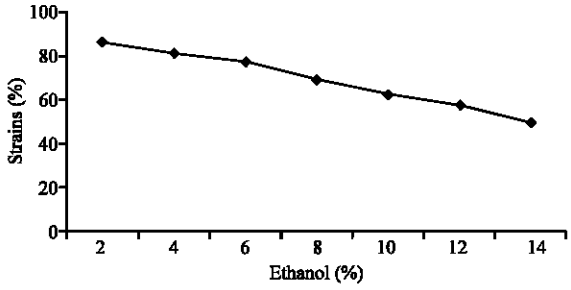


Fig. 5: Strains of acetic bacteria tolerance to ethanol

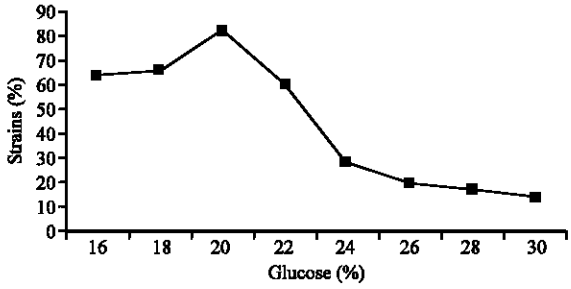


Fig. 6: Strains of acetic bacteria tolerance to glucose

The results of glucose tolerance to acetic bacteria in the pineapple juice are shown in Fig. 6. These results showed a curve of 2 phases: the first increase in the number of strains of acetic bacteria from 64 to 82% between 16 and 20% glucose and a decrease from 82 to 16% between 20 and 30% glucose. The number of strains acetic bacteria able to grow decreases with increasing glucose and the concentration of sugar by 24%, only a small number (28%) strains were able to grow.

## DISCUSSION

The best-known alcohol producing yeast organism is *Saccharomyces cerevisiae* wich is capable of fermenting only hexose sugars to ethanol (Patle and Lal, 2007). According to Gardner *et al.* (1989) the *Saccharomyces* genus is effective yeast in the production of alcohol in pineapple juice.

According to Ndoye *et al.* (2006), acetic bacteria are Gram negative, strictly aerobic and commonly found in nature on different plants (fruits, grains, herbs etc). Among 17 strains of acetic bacteria isolated from tropical fruit and sub-Saharan and used in industrial production of vinegar, more than 60% are identified as belonging to the genus Acetobacter. The remaining strains are identified to be the genus Gluconobacter. Indeed, these two types differ with the following biochemical features: Acetobacter is positive oxidase while Gluconobacter is

negative oxidase (Guiraud and Galzy, 1980). Both species *Acetobacter* frequently isolated from these products are *Acetobacter tropicalis* and *Acetobacter pasteurianus* (Ndoye *et al*., 2006).

Three different phases of yeast growth correspond to the stages of viability, stress and death of yeast during of vinegar. The state of stress is a transitional stage leading to the death of yeast. The study of cytometry monitores of these physiological changes status of yeast during the production of vinegar show that the diacetate carboxy-fluorescein (cFDA), a fluorescent marker, is used by living cells, cells with intact membranes. The yeast live cells expelled during the cytometry cFDA marking so dependent on energy. The dead cells are penetrated by Propidium Iodide (a fluorescent marker) to color their desoxyribonucleic acid (DNA) in red orange (Sekavova *et al*., 2005).

Admittedly, alcohol induced stress in yeast, causing their flocculation, but the stress of yeast is much more related to acetaldehyde which is the first product of ethanol biological oxidation by *Acetobacter*; this acetaldehyde disrupts the enzymatic activity of yeast (Claro *et al*., 2007). Acetic acid content which cause a change in pH extracellular, influencing the intracellular pH of yeast (Valli *et al*., 2005) by significantly reducing its production of ethanol as soon as the acid concentration reached 3% (Graves *et al*., 2007).

The rapid growth phase is the formation of the mother vinegar, the stationary phase is the oxidation of alcohol by bacteria to acetic acid and the phase of declination is the falling of the mother vinegar at the bottom of the bottle indicated the end of acetic fermentation. These results are in accordance with several reports demonstrated that the optimal total biomass is obtained with high concentration of ethanol and acetic acid in the medium (Ethiraj and Suresh, 1990).

According to Valli *et al*. (2005), gender Acetobacter frequently used in industries of vinegar involves a biological oxidation of ethanol in acetic acid by the combination of two types of enzymes: an alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). At beginning of acetic fermentation, which constitutes the second stage of the production of vinegar acid acetic production is quick and important because the oxidation of ethanol by dehydrogenases is spontaneous (Frebortova *et al*., 1997). The stability of acetic acid to 4.5° indicated the end of the acetic fermentation.

The acetic fermentation of pineapple juice requires 23 to 25 days for 4.5 degree vinegar with pineapple juice of 20 Brix content. These results are compared with those of Ethiraj and Suresh (1990) that the acetic fermentation of the juice of mango peelings gives vinegar at 4.65° in 12 days. However, the fermentation of vinegar by the simple batch process is generally slow and requires 4 to 5 weeks for a complete fermentation (Ethiraj and Suresh, 1990). According to Ndoye (2007), depending on the strains, acetic bacteria which produce a final acetic acid concentration of up 1.7° are achieved in modern submerged fermentation processes as so called acetators.

The study of the evolution of acetic acid and the pH in pineapple juice during the production of vinegar shows the existence of a linear relationship between the two settings. This correlation (r = 0.98) indicates a depending between the evolution of acetic acid and the pH in pineapple juice during the acetic fermentation.

The acidity of vinegar (pH = 2.8) is still due to the presence of acetic acid. But all volatile organic acids short chain affects the acidity, flavor and quality of vinegar. These volatile acids, mainly acetic acid and smaller propionic and butyric acids come from raw materials or are generated by the fermentation (Yang and Choong, 2001). Accordmg to Walter (2005), acetic acid and other organic acids (for example: citric acid, tartaric acid, malic acid, succinic acid and lactic acid) determine the acidity of vinegar.

These results show a better utilization of sugar in the pineapple juice in the production of pineapple vinegar than pineapple alcohol. Fermentable sugars adhering to residues in fruit processing substrates are ideal for alcoholic fermentation and acetic fermentation of fruit juice (Ethiraj and Suresh, 1990). These results indicated a significant presence of sugar (5.3%) in pineapple vinegar at the end of its production and are comparable to those found by Ould El Hadj *et al*. (2001), in the study of physical and chemical characteristics of the traditional vinegar with few varieties of dates of the Bowl of Ouargla in Algeria. Indeed dry matter vinegar varieties of dates: harchaya, Deglet-food, hamraya respectively worth 6.59, 10.00 and 11.26%. For Ould El Hadj *et al*. (2001) this rate with significant sugar is the cause of the sweet taste of vinegar. According to Plessi *et al*. (2006), at the end of the processes of acetification, maturation and aging, Traditional Balsamic Vinegar from Mokena (ABTM) generally presents high acidity and high sugar content (up to 70 g 100 $mL^{-1}$).

The preparation and acetic fermentation rarely exceeded 10% alcohol, it would be reasonable not to consider ethanol concentration as a limit to the growth of acetic bacteria. Gullo *et al*. (2005) argued that the increasing of ethanol concentration at acetic fermentation is not significant for the growth of acetic bacteria. High concentrations of ethanol were not tested because according to Lotong *et al*. (1989), high the total concentration of ethanol and acetic acid in the medium is, the less biomass produced.

The concentration of glucose would have a strong effect on bacterial growth. The sugar tolerance trait would be an important technology because vinegar is made with an optimum concentration required by glucose. Gullo *et al*. (2005) argued that tolerance to sugar is not an important factor for technological stress of acetic bacteria in industrial vinegar but this factor is received for the production of traditional vinegar.

## CONCLUSION

A strain of yeast named *Saccharomyces cerevisiae* (LAS01) and an acetic bacteria named *Acetobacter* sp. (ASV03) and acetic bacteria have been isolated in pineapple juice. The production of pineapple vinegar to at least 4.5°, Brix 5.3%, pH 2.8 requires 23 to 25 days for alcohol and acetic complete fermentation. Acidity is stressful and fungicide on yeast during the production of vinegar and is no longer necessary to develop in the process an elimination of yeast before beginning the acetic fermentation. The sugar tolerance is an important technology factor for the growth of acetic bacteria during pineapple juice acetic fermentation unlike that of ethanol which has no limit to the growth of acetic bacteria. All these results can be concluded that post-harvest losses of pineapple fruits may be used to vinegar and have a commercial value.

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