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Banana Vinegars Production Using Thermotolerant *Acetobacter pasteurianus* Isolated From Ivorian Palm Wine

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Abstract

Vinegar or sour wine is a product of alcoholic and subsequent acetous fermentation of sugary precursors. Among acetic acid producing bacteria, only few genera (*Acetobacter and Gluconobacter*) are used in vinegar industry. In this paper, we intended to produce vinegar at 37 °C using two *Acetobacter pasteurianus* strains (S3 and S32). These species were isolated from palm (*Elaeis guineensis*) wine and presented potentialities for industrial vinegar production at 37 °C. Successive fermentations were carried up and semi-continuous acetous fermentation was performed to increase acid production. Concentrated bananas (*Musa ssp.*) juice (11°Brix) was fermented using *Saccharomyces cerevisae* within 7 days, yielding 6.4% alcohol. After fermentation, 60 and 58 g/L acetic acid were produced in vinegars obtained using S3 and S32 stains respectively in 34 days and 5 flow cycles. Malic and acetic acids were the most substantial acids produced in alcoholic juice with 5 631.473 and 2 833.055 mg/L respectively. Among the eight organic acids responsible for vinegars total acidity, acetic acid was major compound with 23 459.416 and 21 268.407 mg/L for S3 and S32 strains respectively. Alcohol and acetic acid fermentation efficiency were 90.9% and 85.39 - 87.63% respectively. All the results above showed that S3 and S32 strains revealed great potentialities for successful industrial vinegar production from overripe banana.

Keywords: acetic acid bacteria, *Acetobacter pasteurianus*, alcoholic and acetous fermentation, vinegar, semi-continuous fermentation

1. Introduction

Food deterioration can be due to a combination of various factors like light, oxygen, heat, humidity and/or contamination of all kind of microorganisms (Yusuf, Jibril, Misau, & Danjuma, 2012). Among these microorganisms, some having beneficial effects are widely used in Biotechnology for production of compound such as vinegar, spirit, wine and antibiotics. This ability allows processing and storage of agro-perishable foods to preserve or to valorize them over a long period of time. That is necessary for developping countries both economically and socially (Seyram, Ameyapoh, Karou, & De Souza, 2009).

Vinegar is produced from ethanol fermentation in a process that yields its key ingredient, acetic acid. It has very good preservative potentialities and is commonly used as food ingredient but also for its medicinal properties. Moreover, it has physiological effects such as invigorating (Johnston, 2005; Johnston, Kim, & Buller, 2004), regulator of blood pressure (Kondo & Tayama, 2001), diabetes mellitus regulator (Ostman, Granfeldt, Persson, & Bjorck, 2005), appetite stimulator, digestion and absorption of calcium (Ndoye, Weekers, Diawara, Guiro, & Thonart, 2007). It is also known to be effective in cancers (Xibib, Meilan, & Moller, 2003), osteosporosis (Kishi & Fukaya, 1999) and neurological diseases (Davalos, Bartolome, & Gomez-Cordoves, 2005). Vinegar can be produced from many kinds of sources like grapes, apples, beetroots, potatoes, honey and some many other tropical fruits like pineapples, dates, oranges, grapefruits, pawpaws or bananas (Ould El Hadj, Sebihi, & Siboukeur, 2001).

Vinegar production through biotechnological means has acquired considerable interest due to possible utilisation of acetic acid bacteria for acetic acid production. According to Beheshti, and Shafiee (2009), 50 types of volatile and aromatic compounds are produced into biotechnological vinegar increasing its final quality.

This study allows promoting the use of the two thermotolerant *Acetobacter pasteurianus* strains (UFHB*LB*003 (S3) and UFHB*LB*032 (S32)) in vinegar production from overripe banana. They were isolated from Ivorian palm wine and showed good potentialities for vinegar production at 37 °C (Konate et al., 2014).

An investigation was first conducted to check out the best alcohol and acetous fermentations combinations models. The best model giving the highest acid rate was held up and acid production was increased by using semi-continuous acetous fermentation instead of continuous fermentation in models.

2. Materials and Methods

2.1 Biological Materials

Overripe bananas were obtained from banana markets in Yopougon and Abobo (District of Abidjan) where overproduction and lack of store systems were responsible of products losses (Figure 1).

Figure1. Bananas in senescence phase used for vinegar production

Note: Bananas were obtained from markets where adequate store system is missing. They are in senescence phase and exposed to sun and wind all along the selling time. The leftovers that are not sold become practically out of use and are nearly going towards dustbin. They're picked up for vinegar production.

2.2 Characteristics of Strains

Yeasts (*Saccharomyces cerevisiae*) (Lesaffre 59703 Marcq, FRANCE) were provided from market on freeze-dried form.

Acetic acid bacteria were from Laboratory of biotechnology in University of Felix Houphouët-Boigny, Côte d'Ivoire. They were isolated from Ivorian palm (*Elaeis guineensis*) wine. Previous studies showed that these strains had great abilities for vinegar processing (Konate et al., 2014). They were characterized by genotypic and phenotypic methods and were strictly aerobic and exhibited optimal growth on HS medium at 37 °C. Cells were Gram-negative, motile and identified as *Acetobacter pasteurianus* strains on the basis of 16S rRNA gene sequence analysis. They produced catalase, utilized glycerol and ethanol, and growth with tolerated 9% ethanol and 6% acetic acid. They were also able to tolerate 5% glucose and their acetifying capacity was found to be higher than 30g/L at 37 °C after one week.

2.3 Juice Production

Bananas were carefully peeled, sliced and boiled in distilled water for about 15 minutes. A paste was obtained by grinding with a Moulinex type A98026F, France. Five hundred grams (500 g) of paste were mixed to 1L of water followed by mechanic pressure to prepare the juice. This juice was submitted to heating at 80 °C in order to prevent microbial contaminations and to concentrate sugar until 20° Brix was reached.

2.4 Inoculums Preparation

Yeast inoculum was prepared by revitalizing 5 g of yeast powder in 20 mL of 10% sucrose solution for 1 hour maximum at 30 °C. This suspension was then used to inoculate 1L of juice.

Bacteria inoculum (0.7 unit of OD_{600}) was obtained by growing cells in YGM medium containing yeast extract 2.5 g, glucose 5 g, mannitol 5 g, $MgSO_4$ 0.25 g, NH_4HPO_4 0.25 g, KH_2PO_4 0.25 g and sodium citrate 0.25 g in a final volume 250 mL. Ethanol 2.5% and acetic acid 0.5% are added after sterilization at 121 °C for 20 min (Ndoye et al., 2006).

2.5 Modelisation for Simultaneous Vinegar Fermentations by Yeasts and Acetic Acid Bacteria

Alcoholic fermentation was monitored into flasks loosely plugged with cotton wool for one week until total soluble solids dropped to constant lowest Brix. Banana wine so obtained was clarified by centrifugation at 4 500 rpm, 25 °C for 15 min. This alcoholic juice was used for both investigations and final vinegar production.

Three models were tested. In the first model, both organisms (yeasts and acetic acid bacteria) were simultaneously inoculated into the fermenting banana juice. No aeration was first applied during the first 48 h before creating aeration to promote acetification. Fermentations were performed at 37 °C. In the second model, bacteria were inoculated into the fermenting medium, 2 days after yeasts fermentation started at 30°C. Temperature was then changed into 37 °C and aeration was provided for acetous fermentation. The last model was to lead alcoholic fermentation within one week at 30 °C before inoculating bacteria for acetous fermentation at 37 °C after removing yeasts by centrifugation.

2.6 Acetous Fermentation

Successive alcohol and acetous fermentation was carried up after a previous investigation study and semi-continuous acetous fermentation method was used to increase vinegar production. Five hundred milliliters (500 mL) vials were used and 150 mL of alcoholic juice were seeded with 1 mL of bacterial suspensions (OD_{600} = 0.7) and incubated in shaking baths at 37 °C. Acid production was determined by titration with NaOH 0.5 N using phenolphthalein as pH indicator. Acetification cycles (Figure 2) were started-up with medium containing 5 % alcohol. It was performed using the first decreasing or constant acid value as the end of a cycle and by renewing the medium with alcoholic juice until 150 mL to start a new one. Fermentation was stopped when constant acid amounts were noticed. A centrifugation at 10 000 rpm, 25 °C for 10 min was effected to clarify the final vinegar (Macias, Caro, & Cantero, 1997; De Ory, Romero, & Cantero, 2002; 2004).

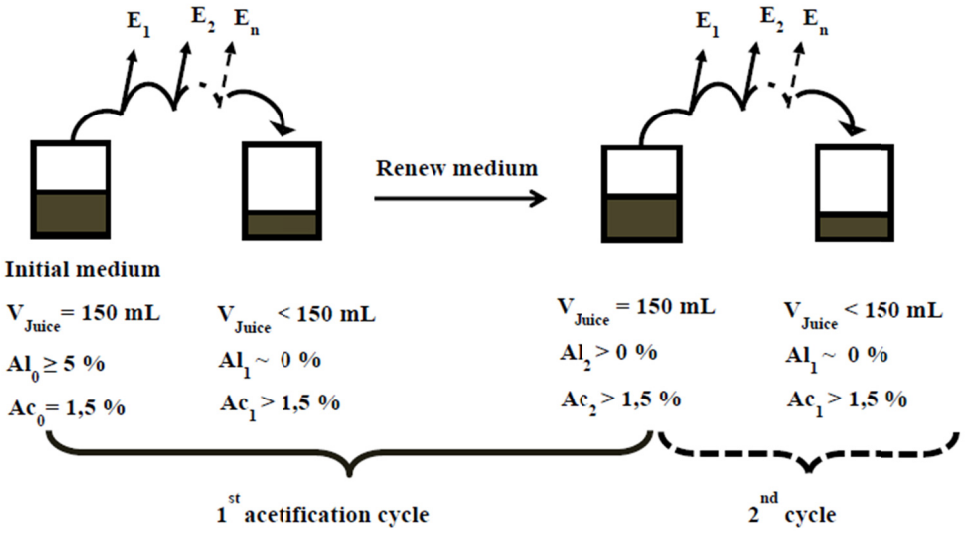

Figure 2. Protocol for the semi-continuous operation procedure

E_1, E_2, E_n = Essay 1, Essay 2, Essay n; Al = Alcohol rate; Ac = Acidity of juice

Note: One hundred fifty milliliters (150 mL) of banana juice was inoculated with 1 mL of acetic acid bacteria seed culture, 0.7 OD_{600} unit. Acetic acid strains were isolated from *Elaeis guineensis* wine of Côte d'Ivoire (Ivorian palm wine). Acid production was checked every day until first constant or increasing acid rate was

obtained. The medium in renewed by adding alcohol juice. The medium is readjusted to 150 mL for a new cycle. Final vinegar is obtained when acid yield is high and when no acid is more produced.

The process of banana vinegar production is depicted in Figure 3.

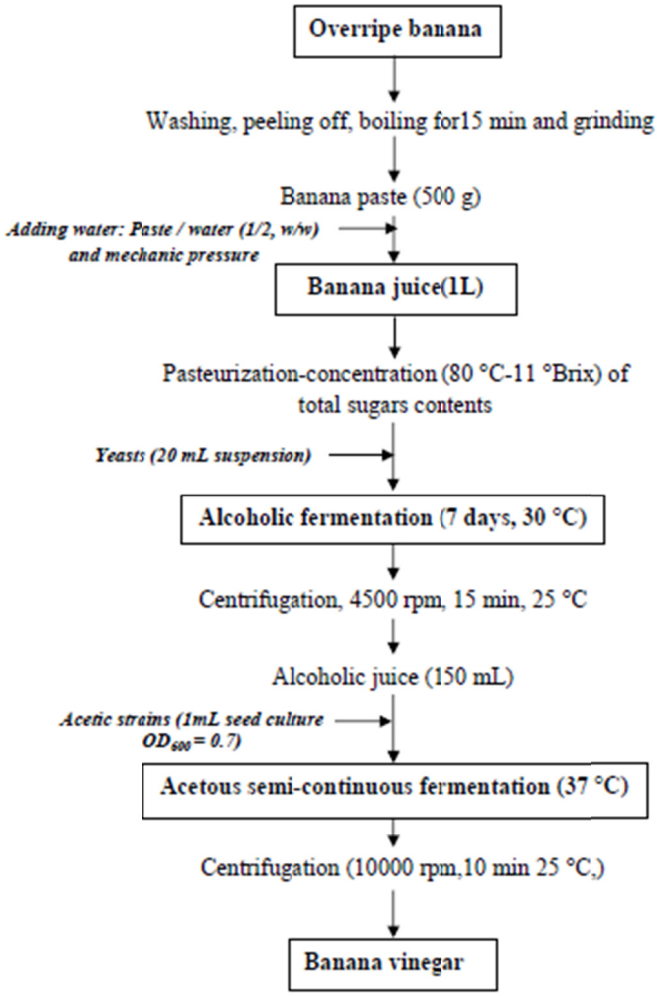

Figure 3. Process of banana vinegar (Ameyapoh et al., 2010, modified)

Note: Overripe bananas were obtained on markets where overproduction and lack of store systems are causing wastes. They are first washed properly and peeled before obtaining banana juice, alcohol juice and finally acidified juice (vinegar). Vinegar was obtained from alcoholic juice using acetic acid strains isolated from *Elaeis guineensis* wine of Côte d'Ivoire (Ivorian palm wine), selected after thorough screening and presenting high potentialities for vinegar production.

Modifications were the use of commercial yeasts, alcoholic fermentation within one week to obtain the maximum alcohol amount and centrifugations to clarify wine and vinegars.

2.7 Samples Analysis

Dry matter contents were determined by desiccation in a drying oven at 105 °C until a constant weight is obtained (AOAC, 2000). Total and reducing sugars contents were determined using the 3.5-DNS acid method (Bernfeld, 1955; Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Brix degree was determinated using a refractometer (C-2 COMECA SA). pH was recorded on the pH-meter HANNA Instruments pH and titratable acidity (TA) was obtained by the volumetric method with phenolphthalein using the following formula: TA (g/l) = N_{NaOH} x V_{NaOH} x 1000 x $M_{acetic\ acid}$ / V_{assay}, where: TA: Titrable acidity (g/L), N_{NaOH}: NaOH normality (0.5 N),

V_{NaOH}: NaOH volume used for titration, $M_{acetic\ acid}$: Molar mass of acetic acid (60 g/mol), V_{assay}: volume of the assay (mL), Multiplied by 1000 to obtain acetic acid yield in g/L.

Alcoholic contents were analyzed by gas chromatography system (Shimadzu GC 14 A, Kyoto, JAPAN). We used Porapak Q column (Length : 1.80 cm ; Diameters : 100-120 mm), Helium as gas vector at 2.0 kg/cm^2 pressure and 2.0 µL of both standard ethanol sample (48%). The flame ionization detector (FID) was also used and sample solutions were injected and in accordance with areas obtained, alcohol degree is calculated (Ogbonna, Mashima, & Tanaka, 2001)

Total acid contents were quantified by a HPLC system (CECIL, Multiwave length Detector) (Reddy & Reddy, 2005). Analytical HPLC grade solvents and standards were used to perform analysis. Solvent (25 mM KH_2PO_4) was from MERCK (Darmstadt, GERMANY). External standard acids (Tartric acid, Malic acid, Ascorbic acid, Citric acid, Oxalic acid, Formica acid, Lactic acid and Acetic acid), were from CHARLAU (Sentmenat, SPAIN). Two analytical methods were used: Method 1were conducted as follow: column C18 (5µm; 150 × 4.6 mm) that was eluted with 25 mM KH_2PO_4 , pH 2.5 as the mobile phase at a flow rate of 1.5 mL/min and a sample injection volume of 10 µL. Wave length was set at 210 nm and retention times of detected acids were inferior to 3 min. In the Method 2 the same characteristics were used excepted that wave length was set at 215 nm and retention times were superior to 3 min with flow rate of 0.6 mL/min. Based on standard acids concentrations, areas obtained from peaks on chromatograms and retention times, acid contents of samples were calculated.

3. Results

Banana juice was obtained with 80 ± 2.1% extraction rate. This juice sugar contents were concentrated until 11 °Brix was reached.

Alcohol fermentation was successfully carried out at 80 °C, giving 6.4 ± 0.36% alcohol after gas chromatography analysis (Figure 4). Alcoholic fermentation efficiency (FE) was 90.9%. It was calculated as follows : % FE (Ethanol) = % Ethanol (v/v) × 100 / % Sugar (°Brix) × 0.64 (Pardeep, Kocher, & Phutela, 2011).

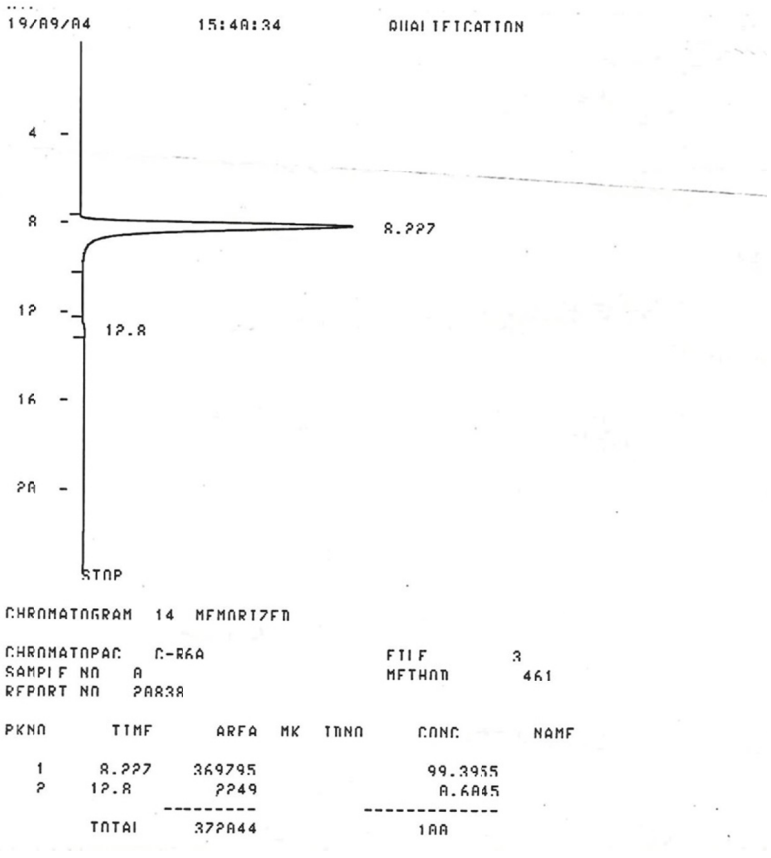

Figure 4. Chromatogram showing alcohol analysis in alcoholic juice

Note: Banana juice obtained after alcoholic fermentation was analysed by gas chromatophy means. According to areas given by chromatograms, alcohol rate was calculated. Experiments were performed in triplicate. Investigation studies showed that model 3 was better than the two others. The highest acid productions obtained with this model were 23.6 g/L (for S3 after 7 days) et 20.5 g/L (for S32 after 11 days).

Semi-continuous acetous fermentation performed after investigations yielded 58 and 60 g/L (from 1.5 g/L initially in alcoholic juice) for S3 and S32 respectively. Acetic acid fermentation efficiency calculated was 87.63% and 85.39% (for S3 and S32) as follows: % FE (Acetic acid) = % volatile Acidity (w/v) × 100 / % Ethanol (v/v) × 1,043 (Pardeep et al., 2011). These acids yields were obtained after 34 days of fermentation at 37 °C, in 5 acetification cycles (Figure 5).

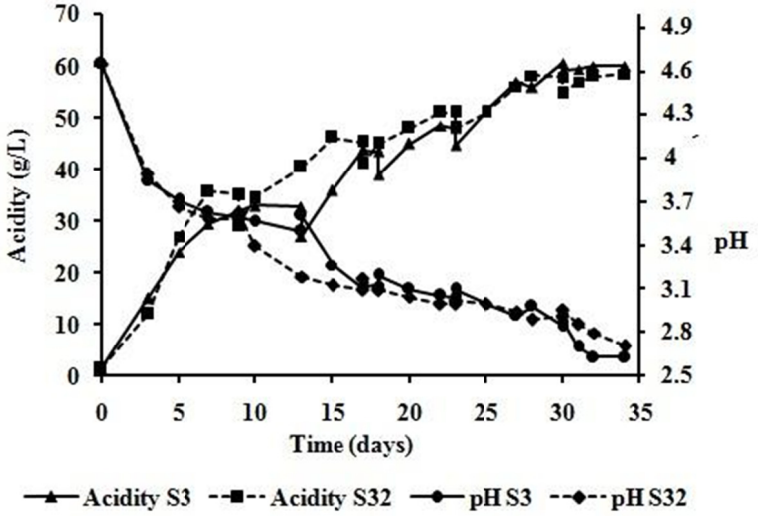

Figure 5. Banana vinegar production by semi-continuous method from two thermotolerant *Acetobacter pasteurianus*

Titratable acidity was obtained by the volumetric method with phenolphthalein as pH indicator and pH was directly read on the pH-meter. Acid production was checked every day until the first constant or decreasing acid rate was reached. The medium is then renewed by adding alcohol juice and readjusted to 150 mL for a new cycle. Fermentation continues until bacteria become unable to produce acid due to the high acid yield.

Results of succinct analyses of banana juice, alcoholic juice and vinegars are showed in Table 1.

Table 1. Analysis of banana, banana juice and fermented juice contents

	DMC (%) [1,2]	***pH*** [1]	***RS*** [1]	***TS*** [1]	***Brix***	***Acidity*** [1]
BB	39.25± 0.17	4.92± 0.05	11.95 ± 0.62	40.23 ±5.65	3	1.4° ± 0.65
CJ	12.16± 0.35	5.11± 0.05	13.15 ± 2.15	78.18 ±0.05	11	0.05° ± 0.01
AJ	3.87± 0.41	4.66± 0.06	0.85 ± 0.19	6.17 ± 1.9	2	0.15° ± 0.90
S3 Vinegar	2.27± 0.33	2.63± 0.03	0.79 ± 0.16	7.25 ± 0.72	1.5	6° ± 0.10
S32 Vinegar	2.08± 0.27	2.71± 0.02	0.57 ± 0.18	8.36 ± 4.26	1.5	5.8° ± 0.20

[1]Values expressed as mean ± standard deviation of samples analyzed in triplicate.

[2]Percentage of dry matter content (DMC).

BB = Boiled banana; CJ = Concentrated Banana; AJ = Alcoholic juice; Vinegar S3 = Vinegar obtained from S3; Vinegar S32 = Vinegar obtained from S32; RS = Reducting sugars; TS= Total sugars.

Dry matter analysis of banana paste showed 39.25%.

The mean value of pH observed in overripe bananas was 4.92. It turned into 5.11 in banana juice before decreasing until 2.63 and 2.71 respectively in vinegars obtained from S3 and S32.

Total and reducing sugar analyses showed 40.23% total sugar (TS) and 11.95 % reducing sugar (RS) (Table 2).

Concentrated juice with 78.18% TS and 13.15% RS decreased in vinegars (7.25-8.36% and 0.79-0.57%, for vinegar with S3 and S32). The results of the evaluation of Brix during alcohol and acetic fermentations showed decreasing rates of dry contents. Banana juice (11°Brix) was finally 2 °Brix at the end of acetic fermentation

HPLC analysis of acid contents in alcoholic and produced vinegars, eight major organic acids (Table 2): Tartaric, Malic, Ascorbic, Citric, Oxalic, Formica, Lactic and Acetic acids. In alcoholic juice, Malic and Acetic acids were the most important acids produced with 5 631.5 and 2 833.1 mg/L respectively. Acetic acid was the major acid produced in vinegars obtained from alcoholic juice with 23 459.4 mg/L and 21 268.4 mg/L for S3 and S32 strains respectively. Figure 6 shows chromatograms of 4 organic acids analyses by HPLC means in vinegars with method 2. It presents acetic acid as the major organic acid produced among detected acids (all detected acids concentrations are provided by Table 1 above).

Table 2. Organic acids contents of banana vinegars by HPLC analysis

	Organic acids contents (mg/L)							
	[1]Tartaric	**[1]Malic**	**[1]Ascorbic**	**[1]Citric**	**[2]Oxalic**	**[2]Formic**	**[2]Lactic**	**[2]Acetic**
Alcoholic Juice	1885.8	5631.5	36.6	0	667.4	1679.1	354.6	2833.1
S3 Vinegar	37.4	5632.6	59.1	328.5	516.6	277.4	205.9	**23459.4**
S32 Vinegar	3543.9	5691.5	623.3	180.4	500.4	233.9	243.3	**21268.4**

[1]Acids detected by method 1;[2]Acids detected by method 2.

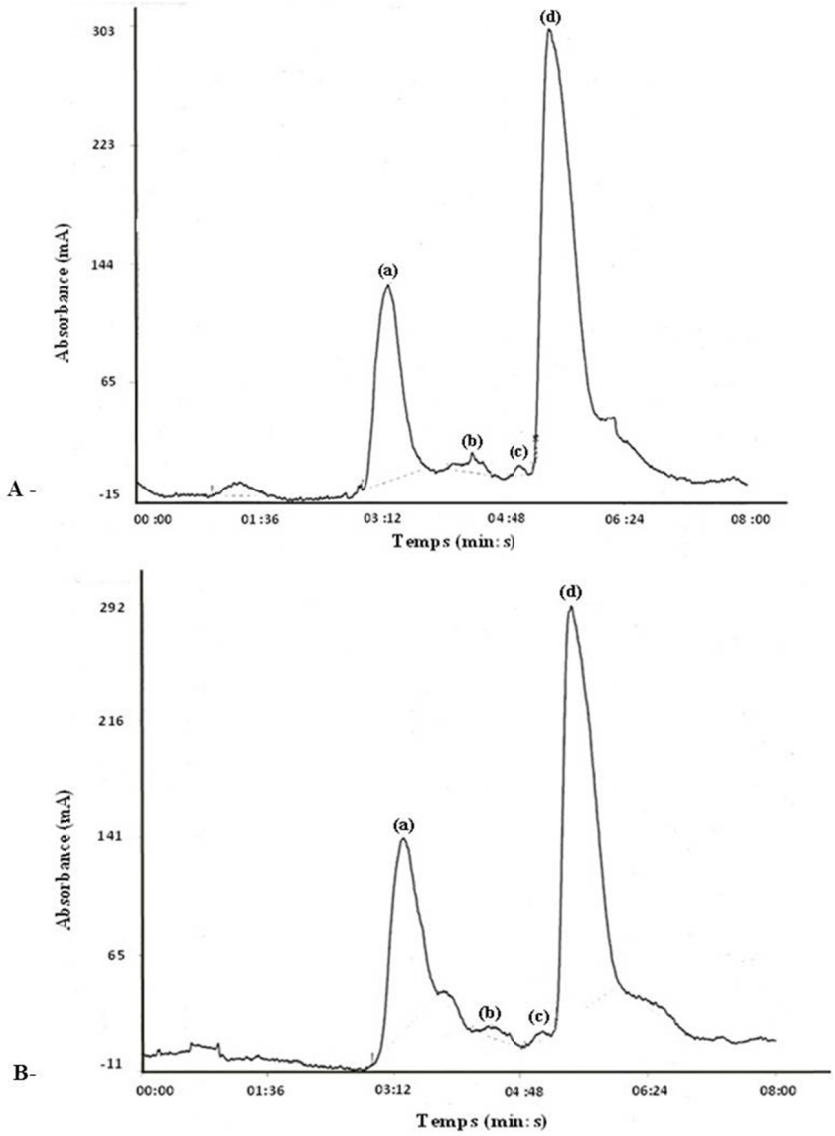

Figure 6. Chromatogram showing acetic acid analysis by HPLC with method 2

Note: (A) was from S3 vinegar and (B) was from S32 vinegar. (a): Oxalic acid, (b): Formic acid, (c): Lactic acid, (d): Acetic acid. Analysis used column C18 (5µm; 150 × 4.6 mm). Acids were eluted with 25 mM KH_2PO_4 (pH 2.5) as the mobile phase at a flow rate of 0.6 mL/min and 10 µL for sample injection volume. Wave length was 215 nm and retention time was superior to 3 min. Based on standard acids concentrations, areas obtained from peaks on chromatograms and retention times, acid contents of samples were calculated.

4. Discussion

Alcoholic fermentation after one week showed 6.4% alcohol. This alcohol rate produced from 11° Brix was sufficient and higher than that obtained by Ameyapoh et al. (2.24% from 20 °Brix juice) and lower than 8.9 % reported by Kaur et al. (2011). These authors used more concentrated juice for alcoholic fermentation (More than 15 °Brix). Consequently, we suggest that obtained 6.4% alcohol rate could be increased if the juice was more concentrated. Moreover, Muhammad (2012) reported 12 % alcohol when adding substances (NPK and 5% bread yeast) in palm juice and Siler and Morris (1996) obtained 13.9 to 16.7% alcohol with 35 °Brix juice fermentation after 35 days. Our banana juice was not enriched, so it couldn't enable enough yeast to higher alcohol productions. The 6.4% alcohol rate can also be justified by ethanol fermentation efficiency (FE) that was higher and found 90.9%. This efficiency could be due to fermentation duration (one week instead of 2-4 days most of times), suitable fermentation conditions and yeasts performance. It was higher than FE (84.8%) reported by Kaur

et al. (2011). These authors suggested that variability in alcohol production could be due to metabolic behaviour and adaptability of yeast to different sugar concentrations and fermentation conditions.

The semi-continuous acetification yielded 6% and 5.8% Acetic acid respectively with S3 and S32 vinegars. FE was 87.63% and 85.39% for S3 and S32. Ndoye et al. (2007) produced experimental vinegar in bioreactor by semi-continuous method at 35°C and obtained 8-9 % of acetic acid within 35 days. Their acetic acid yield was greater-than our productions because of the use of bioreactor where acetification parameters are carefully under control. Ameyapoh et al. (2010) and Yusuf et al. (2012) produced both 47 g/L from mango and banana at 30 °C within respectively 15 and 11days in continuous fermentation. 45 g/L Banana vinegar was also produced in continuous fermentation by Seyram et al. (2009) within 23-25 days at 30 °C while Kaur et al. (2011) used immobilized *Acetobacter aceti* cells to produce 47 g/L and 44 g/L total and volatile acidity by semi-continuous fermentation at 30 °C in 36 h. Comparatively to these productions, vinegars produced by the two thermotolerant *Acetobacter pasteurianus* were interesting considering their capacity of acetification at high temperature (37 °C). Gullo, De Vero and Giudici (2009) reported that *A. pasteurianus* strains have positive effects to start acetification processes from musts that not contain acetic acid initially. They are also able to promote the growth of other naturally occurring bacteria. According to FDA (Food and Drug Administration of the United States), vinegar is defined as a liquid containing 4% acetic acid corresponding to 4 g acetic acid per 100 mL of alcoholic fermented solution obtained from sugar solutions (Beheshti & Shafiee, 2009). Furthermore, Ndoye et al. (2006) stated that *Acetobacter* strains capable of producing 1.7 g/L acetic acid can be used as acetators in fermenting processes. From then on, these strains present potentialities to produce industrial vinegar. However, some experiments in bioreactors should be carried out to confirm this predisposition.

The highest acid concentrations in both vinegars were obtained with acetic acid (23459.4 mg/L for S3 and 21268.4 mg/L for S32). These results are confirmed by Walter (2005) who showed that acetic acid is the major acid produced in vinegars and constitute with organic acids like Citric acid, Tartric acid, Malic acid and Succinic acid, the total acid contents in vinegars. Yang and Choong (2001) also agree that all volatile short chains acids affect total acidity in acetic fermentation but acetic acid remains the main acid produced with Propionic acid and butyric acid in very low yields.

The low rate of dry matter (39.25%) is banana paste is in accordance with 23.96 to 42.63% dry matter in pulps obtained by Coulibaly, Nemlin and Kamenan (2007) from some banana varieties. Olivier et al. (2009) mentioned also 41.1% and Assemand, Camara, Kouamé, Konan and Kouamé (2012) reported 37.87% to 42.8% when characterizing some «Agnrin» banana varieties. These variations of dry matter contents in bananas can be explained by differences between ripening states of bananas. According to Assemand et al. (2012), the reduction of dry matter contents of bananas in ripening state is due to the increasing water rate that was helped by water osmotic migration. Otherwise, the decreasing rates observed all along vinegars making until vinegars (2.27% and 2.08 %) are attributable to transformation steps (pressure and centrifugation).in order to clarify vinegars.

Concerning pH variations, Olivier et al. (2009) reported pH 5.6 as the highest value observable in bananas in ripening phase. Hailu, Workneh, and Belew (2012) observed also the same variations in ripening bananas .This was due to acidification brought by amylolytic enzymes and fruits water content that increased after degradation of polymeric carbon hydrates making their conversion easier into acids (Hsiao & Siebert, 1999). The decreasing pH values until 2.63 and 2.71 in vinegars were attributable to volatile acids brought in juices through alcoholic and acetic fermentations.

Total sugar (TS) and reducing sugar (RS) rates obtained were more or less comparable with those obtained by Limpaiboon, Kankaew and Wongwicharn (2011) and Lii, Chang, and Young (1982). These authors obtained respectively 22.16% and 33.6% TS. Moreover, Assemand et al. (2012) was reporting 29.97% TS and 8.66% RS. These various rates of sugar contents in bananas were justified by Kouame, Camara, and Dick (2010) by the difference between banana varieties compositions and the ripening system used. Otherwise, Forster, Rodríguez, and Romero (2002) affirmed that changes into bananas composition are linked to farming methods and to soils structures of regions. The decreasing sugar contents from concentrated juice to vinegars, proportional to Brix variations was due to the use of sugar by yeast and acetic bacteria for growing (Ameyapoh et al., 2010).

5. Conclusion

The present study successfully achieved vinegar production from banana. Alcoholic fermentation within one week with *Saccharomyces cerevisae* and acetous fermentation during 34 days using two *Acetobacter pasteurianus* strains isolated from Ivorian palm wine were performed. 6.4% alcohol and 5.8-6 acetic acid degree vinegars were yielded with 90.9% and 85.39%-87.63% fermentation efficiency for alcoholic and acetous

fermentations respectively. The semi-continuous fermentation method of banana wine was well experimented and consequently, the two acetic strains are really empowered for industrial vinegar production.

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