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REGULAR ARTICLE

Isolation of thermotolerant and high acetic acid-producing *Acetobacter pasteurianus* from Ivorian palm wine

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Abstract

The aim of this work was to identify acetic acid bacteria expressing technological characteristics for further use in vinegar production in tropical countries. It was focused on isolation and identification of thermotolerant acetic acid bacteria strains from *Elaeis guineensis* wine of Côte d'Ivoire (Ivorian palm wine). A screening was performed to find out strains with high vinegar production, tolerant against high ethanol, acid and sugar concentrations as well as tolerating high production temperatures. Among 104 isolated strains, 5 were selected for their growth ability, acetification capacity (acetic acid production higher than 30 g/l) and suroxydation at 37°C. Tolerance against 6% acetic acid and 9% ethanol was observed. Osmotolerance study showed tolerance against 5% and 10% glucose giving 100% and 50% relative growth, respectively. Resistance against desiccation showed survival rate of 50% at 37°C after 5 h treatment and 10% after 20 h treatment. The best aeration rate in flasks for acetification was 70%. Polyphasic identification study based on biochemical, physiological and molecular characterization showed that the 5 isolates were all Acetobacter pasteurianus. Owing to their potentialities, these strains may be used as starters in vinegar production after conducting preservation studies.

Key words: Acetic acid bacteria, Acetic fermentation, *Acetobacter pasteurianus*, Gram staining

Introduction

Acetic acid bacteria (AAB) are a large group of obligate aerobic gram negative bacteria with the ability to oxidize ethanol to acetic acid (Matsushita et al., 1994).They are widely distributed in natural habitats and classified in the family of *Acetobacteriaceae*. Members of this family are useful in industrial production of vinegar (Adachi et al., 2003). AAB can use substrates such as glucose, ethanol, lactate or glycerol as energy sources. However, most of these compounds are not completely oxidized into CO_2 and H_2O but several metabolites, especially acetic acid, are accumulated in the growth medium. AAB are commonly found in nature because of their high resistance to acidity and the variety of substrates that they can use (De Ley et al., 1984). These bacteria can be isolated from vinegar, fruits, flowers, bees honey, sugar cane juices, soil, water and alcoholic beverages (Yamada et al., 1999). Vinegar can be produced from several sources such as grapes, apples, beetroots, potatoes, honey and some many other tropical fruits like pineapples, dates, oranges, grapefruits, pawpaws and bananas (Ould el Hadj et al., 2001).

Vinegar production through biotechnological means has acquired considerable interest due to the possible use of acetic acid bacteria to produce acetic acid. The recommendations of European Union Norm 2-3-1 AFNOR / CEN (NF EN 13188, 2000) had even allowed the use of foods arising from natural materials processing. In addition, secondary metabolites produced into biotechnological vinegar can increase its final quality. According to Beheshti and Shafiee (2009), around 50 types of volatile and aromatic

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compounds are produced when vinegar is obtained by biotechnological methods.

In African sub-Saharan countries temperatures are generally above 30°C while common A A B are mesophilic microorganisms and their optimum growth temperature is between 25°C and 30°C (Gullo et al., 2008). In such conditions, the production of vinegar with AAB can be disadvantageous for factories because of the cooling expenses of bioreactors (Adachi et al., 2003). There by vinegar producing technology using thermotolerant strains that are able to grow and produce acetic acid at higher temperatures would be advantageous in these tropical countries (Ndoye et al., 2006)**.** Therefore, there is a real need to find out strains that are tolerant to uttermost growth conditions (temperature, desiccation, alcohol and acid), and also capable to produce high level of acetic acid (Lu et al., 2000).

Thermotolerance, and tolerance against alcohol and acetic acid were previously studied (Ndoye et al., 2006; Amornrut et al., 2008; Kanlaya et al., 2009). Illeghems et al. (2013) have isolated an acetic acid bacterium (*Acetobacter pasteurianus* 386B) originating from a spontaneous cocoa bean heap fermentation that proved to be an ideal functional starter. It was able to dominate the fermentation process, thereby resisting high acetic acid concentrations and temperatures. Generally, strains were able to grow at temperatures up to 37-40°C. Alcohol tolerance was found until 10% (v/v) and acetic acid tolerance was more than 4% (v/v). Those authors checking thermotolerant acetic acid bacteria mentioned that using these strains can be economic by reducing cooling water expenses of bioreactors until 30°C (optimum growth temperature of common acetic acid bacteria).

The potential sources of bacteria strains tolerant to such drastic conditions are found in tropical fruits and beverages such *Elaeis guineensis* wine of Côte d'Ivoire (Ivorian palm wine), a popular local alcoholic beverage. This beverage is known to naturally and frequently contain acetic acid bacteria (Okafor, 1975). In this study, AAB strains were first isolated from Ivorian palm wine and then a screening was performed to select strains with high potentialities for vinegar production. The selected strains were subjected to polyphasic characterization.

Materials and Methods

Isolation and screening

The biological material used was a three days old palm wine that was expected to contain acetic acid strains (Okafor, 1975). Owing to the acidity increasing of the beverage a personal communication allowed us to obtain palm wine for analyses. The juice obtained from cut down palm trees was conserved until it becomes wine within three days. The strains isolations were performed following Duthathai and Wasu (2007) method which was modified by adding Nystatine to the medium after sterilization. This medium was GYC (Glucose Yeast Carbonate) (Duthathai and Wasu, 2007), pH 6, containing 0.5% glucose, 2% glycerol, 1% yeast extracts, 1% peptone, 1.5% potatoe extract, 1% $CaCO_3$, 4% ethanol, 0.0016% bromocresol green and 1.5% agar. Dilutions from the sample (the 3 days old palm wine) were prepared in peptone water and 100 µl of various dilutions were then spread in Petri dishes and incubated at 30°C for 3-4 days under aerobic conditions. Isolated colonies with yellow zones around were streaked on Hestrin-Schramm $CaCO_3$-Agar medium to confirm acid production by formation of clear zones around colonies (Andelib and Nuran, 2009). This medium was composed of 0.05% glucose, 0.3% peptone, 0.5% Yeast extract, 1.5% $CaCO_3$, 1.2% agar, and 1.5% ethanol was had after sterilization. Culture was performed at 30ºC for 2-7 days. Confirmed acid forming colonies with the biggest clear zones were subjected to further biochemical tests.

They were allowed to a secondary screening in liquid medium. Modified Hestrin-Schramm (HS) $CaCO_3$ broth composed of 2.5% ethanol, 0.5% Yeast extract, 0.5% peptone, 0.27% Na_2HPO_4 and 0.01% $MgSO_4$ was used as basal medium for that purpose (Andelib & Nuran, 2009). YGM/Mg^{2+} medium (Yeast Glucose Mannitol with $MgSO_4$) (Ndoye et al., 2006) containing yeast extract 1%, glucose 2%, mannitol 2%, $MgSO_4$ 0.01%, ethanol 2.5% and acetic acid 0.5% (Ndoye et al., 2006) was used for seed culture until optic density at 600 nm (OD_{600}) of 0.5 was achieved. Culture experiments were performed in 250 ml-Erlenmeyer flasks containing 150 ml of basal medium. The media were inoculated with 1ml of seed culture. Cultures were carried out in a shaking bath at 30°C, 35°C, 37°C, 39°C and 40°C.

Bacterial growth was followed within 10 days and evaluated at OD_{600}. Acetic acid production was measured by titration with NaOH 0.5 N with phenolphthalein as pH indicator. This titration was performed as follow: 10 ml of the broth medium were mixed thoroughly with 3-5 drops of phenolphthalein [phenolphthalein, 0.1 g; ethanol, 60 ml; distilled water, 40 ml] and then 0.5 N sodium hydroxide (NaOH, 20 g; distilled water, 1000 ml) were added until appearance of pale pink

color in the flask. The amount (g) of acetic acid (Aaa) produced in 1000 ml (1 l) of medium was calculated using the following formula:

Aaa (g/l) = N_{NaOH} x V_{NaOH} x 1000 x $M_{acetic\ acid}$ / V_{assay}, where:
Aaa: acetic acid yield (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

Phenotypical and biochemical characterization

Selected strains were submitted to characterization. Gram staining was performed and cells motility was observed by hanging drop method (Webley, 1953). Catalase test was performed with hydrogen peroxide and oxidase test with oxidase disks.

Overoxidation was investigated with HS broth containing bromocresol green. It was based on the capacity of strains to oxidate acetate to CO_2 and H_2O giving yellow color to the medium that turns finally into green (Holt et al., 1994).

Biochemical studies were performed by checking acid production in modified Hestrin-Schramm (HS) broth containing 1% sugar as the only source of carbon (Andelib and Nuran, 2009) and 0.0016% bromocresol green. The following sugars were used: galactose, glucose, mannitol, acetic acid, lactic acid, glycerol, sucrose, mannose, sorbitol, ethanol, fructose, and xylose. One ml of each strain suspension (OD_{600} = 0.5) was used to inoculate tubes containing 10 ml of each sugar. Acid production is indicated by changing color of the initial blue-green media into yellowish one.

Bacterial tolerance against acetic acid and alcohol were analyzed in HS broth containing respectively acetic acid 1-10% and alcohol 1-15% as sole carbon sources in the media. Ten (10) ml of each acid or alcohol solution were inoculated with 1 ml of a 0.5 OD_{600} seed culture. Cells viability was estimated after incubation at 37°C for 48 h by counting colonies with and without treatment. Strains were considered tolerant to the treatment if $\Delta Log N_{10}$ ($LogN_0$ - $LogN_1$) ≤ 5 (Legrand-Sow, 2004) where, $\Delta Log\ N_{10}$ = Decimal Logarithm variation, N_0 = Number of viable cells before treatment, N_1 = number of viable cells after treatment.

The influence of aeration on acetic acid production was also studied in flasks. For that purpose, volumes of aeration (ratio of medium volume / flask volume) ranging from 10 to 90% were prepared with HS broth. Culture media were inoculated with 0.5 OD_{600} seed cultures using 1 ml for every 150 ml of broth. Incubation was achieved in a shaking bath at 130 rpm at 37°C within 7 days. Acetic acid production in each flask was determined by titration with NaOH 0.5 N using phenolphthalein as indicator.

Osmotolerance of strains was studied with glucose concentrations ranging from 2 to 30% in modified HS medium and measurement of absorbance at 600 nm. Incubation in a shaking bath at 130 rpm at 37°C within 7 days was observed. Cells relative growth (RG) was calculated with considering as 100% RG, the highest OD_{600} observed (Jojima et al., 2004).

Resistance test against desiccation was carried out by preparing suspensions from selected strains, into YGM/Mg^{2+} medium at 30°C with 130 rpm shaking. At 0.5 OD_{600}, each suspension was centrifuged at 9600 g for 10 min at 4°C. After washing twice with potassium phosphate buffer (KPB) 50mM (pH 5.5-6), cell pellets were resuspended in the same buffer. One milliliter of that solution was submitted to cell counting while one other milliliter was filtered onto sterile polycarbonate membranes (0.45 µm). Thereafter, the filters were exposed to drying at 37°C during 72 h in half-opened Petri dishes. At time intervals, filters containing dried cells were put into 10 ml of peptone water and incubated for 10 min at 30°C on YGM/Mg^{2+} medium (Laura et al., 2001, modified). Viable cells were then submitted to plate counting in triplicate. The ratio of viable cells in the rehydrate to initial culture gives the desiccation effect ton strains (Lewis et al., 2010). According to modifications sterile polycarbonate membranes (0.45 µm) were used instead of pieces of sterile gauze (1 cm^2) that were imbibed separately with 1 ml of a 48-h culture (10^6 cfu/ml) of each strain in tryptose broth. YGM/Mg^{2+} medium were also used instead of blood agar medium. In addition, inoculums were washed and centrifuged before using.

Molecular characterization

The genomic DNA preparation was performed by the heat shock methods. Bacteria were grown in YGM/Mg^{2+} agar medium for 24 h at 30°C. Cell suspensions were made using some colonies in 400 µl of milli-Q water into Eppendorf tubes. The tubes were then frozen at -20°C for 20 min before heating them in a heat block at 100°C for 20 min. Afterwards, tubes were centrifuged at +4°C for 10 min at 14000 rpm. The supernatants containing total genomic DNA were poured in new sterile

tubes and conserved at +4°C or -20°C if not used immediately.

The following Polymerase Chain Reaction (PCR) amplification was based on the analysis of the 16S rDNA. It was monitored with an universal primer consisting of a forward primer 16F27: 5′-AGAGTTTGATCCTGGCTCAG-3′ and a reverse primer 16R1522: 5'-AAGGAGGTGATCCAGCCGCA-3', localized respectively on positions 8-27 and 1541-1522 according to the nomenclature system in force with *Escherichia coli* (Brosius et al., 1978). The PCR kit, Hot Master Mix 5 PRIME (Dominique Deutcher, France) was used. It was composed of 20 µl of Mix, 0.2 µl of each primer, 1 µl ADN (50 ng) and supplemented by sterile milli-Q water to achieve a final volume of 50 µl. PCR amplification was carried out in a thermocycler (Mastercycler Personal, Eppendorf, Pecq, France) with the following procedure: a first denaturation step of the DNA at 95°C for 2 min, then denaturation at 95°C for 1 min, primers annealing at 58°C for 30 sec, primers extension at 65°C for 2min and a final extension cycle at 65°C for 7 min. A total of 35 cycles were done.

Isolates were then identified by sequencing the PCR products in a sequencer ABI 3730xl 96-capillary DNA Analyzers (Eurofins genomics, Paris, FRANCE). Genomic DNA of strains and their PCR products were sent to Eurofins genomics, Paris, FRANCE for sequencing and the sequencer ABI 3730xl 96-capillary DNA Analyzers was used. The analyses were performed by Eurofins platform by SANGER methods.

Sequences analyses were performed with National Center for Biotechnology Information (NCBI) software using nucleotide Basic Local Alignment Search Tool (BLAST) method (http://www.blast.ncbi.nlm.nih.gov). BLAST finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families.

Analysis of samples

Experiments were performed in triplicate for samples where standard deviations were calculated and in duplicate for those presented as the mean standard values of two replicates. All these analysis were done with Microsoft Office Excel 2007.

Results and Discussion

We aimed in this study to look for bacterial strains capable of growing well at high temperatures and producing high level of acetic acid. Physiological, biochemical and molecular studies were performed to characterize selected strains.

Acetic acid bacteria were obtained from palm wine. First day palm wine was sweet. No acetic acid bacteria were detected in it (personal communication). Third day palm wine were sour meaning the presence of microorganisms responsible for this transformation. This three days palm wine (pH 3.4 - 4.1) was used to obtain a total of 104 acetic acid strains.

All isolates showed yellow zone on GYC medium (Figure 1 a) and clear zones on HS CaCO3-Agar plates (Figure 1 b). They were all Gram-negative (Figure 1 c) and motile by means of peritrichous flagella, and the colonies were circular and non-pigmented. They were all fermenting alcohol into acetic acid (Figure 1 d), negative for oxidase, and positive for catalase. Oxidation of acetate to CO_2 and H_2O was found positive in all strains and this ability to oxidize acetate was used to confirm their membership to the genus *Acetobacter spp.*

From primary screening based on diameter's length of clear zones, twenty (20) strains with diameter up to 1.2 cm were selected. They were then allowed for a secondary screening with checking good bacterial growth and high acetic acid production at increasing temperature. As results, 20 strains grew and produced acid at 30°C, 16 among the 20 strains grew and produced acid at 35°C, 13 among the 16 strains grew and produced acid at 37°C and only 2 among the 13 strains grew and produced acid at 39°C. Neither growth nor acid production was observed beyond 39°C. At least, five (5) strains were selected. They were composed of the two acid producing strains at 39°C (UFHB-LBAAB*003* (S3) and UFHB-LBAAB*032* (S32)) and three other strains that were among the highest acid producers at 37°C (UFHB-LBAAB*004* (S4), UFHB-LBAAB*011* (S11) and UFHB-LBAAB*013* (S13)). All five strains showed good growth and acid production up to 30 g/L at 37°C (optimum temperature). The following results concern all the studies allowed to characterize these five strains.

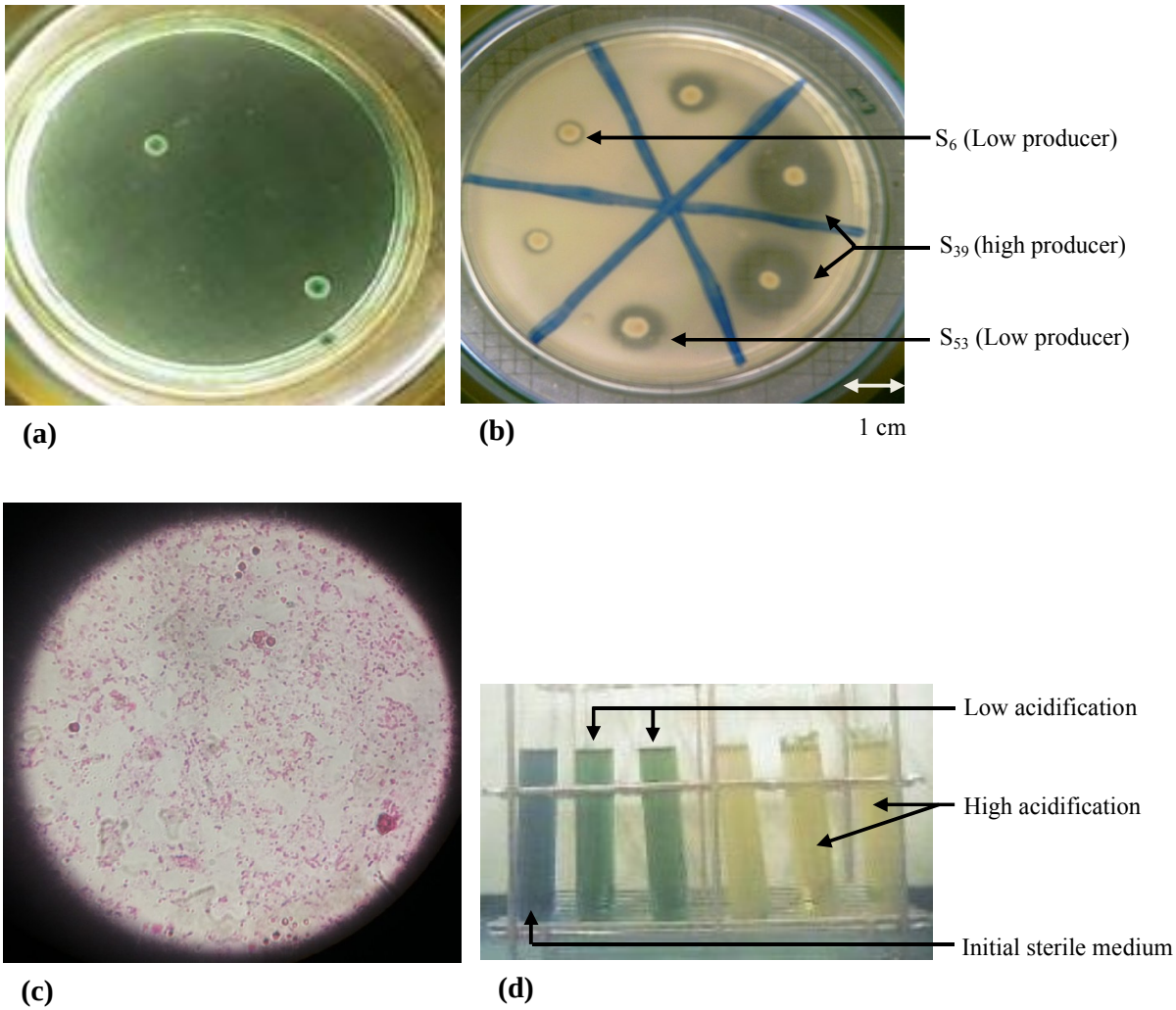

Figure 1. Acetic acid bacteria isolation from Ivorian palm (*Elaeis guineensis*) wine and acetification checking. (a) Isolation of acetic acid bacteria using GYC medium. Incubation was performed at 30° C within five days. Colonies showing yellow zone around were purified for further studies. (b) : Screening of purified acetic acid bacteria isolated on GYC medium, based on acetic acid production. Purified strains were repiquated onto modified Hestrin-Schramm (HS) $CaCO_3$ medium within 4-5 days at 30°C. Strains giving biggest clear zone sizes were considered as high producers. (c): Gram staining (gram negative) of an isolated *Acetobacter pasteurianus* strain. Culture was obtained after 24 hours incubation at 30° C in YGC culture media. (d) : Acetification checking with *Acetobacter pasteurianus* strains. Modified Hestrin-Schramm (HS) broth with ethanol as the only carbon source was used. Cultures were incubated at 30° C within 72 hours. Two strains showed low acetification and the three other strains showed high acetification.

Other biochemical and confirmatory tests of selected strains were shown in Table 1. The isolates were able to oxidize xylose, glycerol, glucose, ethanol, acetic acid, lactic acid and unable to oxidize Galactose, mannose, sucrose, mannitol, sorbitol and fructose. From then on, they seemed to be *Acetobacter* or *Gluconacetobacter* by referring on previous work made by Urakami et al. (1989) and Lisdiyanti et al. (2002).

Following PCR amplification (Figure 2) and analysis of the 16SrDNA of the studied strains allowed their accurate identification. The 16SrDNA sequences showed high levels of similarity of the five strains to *Acetobacter pasteurianus*. We obtained 98% of similarity for UFHB-LBAAB*004* (S4) and UFHB-LBAAB*011* (S11) and 99% for UFHB-LBAAB*003* (S3), UFHB-LBAAB*013* (S13) and UFHB-LBAAB*032* (S32).

Table 1. Biochemical analysis of the 5 selected *Acetobacter* strains isolated from Ivorian palm wine.

Carbon sources	Isolates				
	S3	S4	S11	S13	S32
Galactose	-	-	-	-	-
Xylose	+	-	-	-	+
Mannose	-	-	-	-	-
Glycerol	+	+	+	+	+
Sucrose	-	-	-	-	-
Mannitol	-	-	-	-	-
Glucose	+	+	+	+	+
Sorbitol	-	-	-	-	-
Ethanol	+	+	+	+	+
Fructose	-	-	-	-	-
Acetic acid	+	+	+	+	+
Lactic acid	+	+	+	+	+

Incubation was performed at 30°C for 48 h with 130 rpm shaking in a Modified HS culture medium. S3: strain UFHB-LBAAB*003*, S4: strain UFHB-LBAAB*004*, S11: strain UFHB-LBAAB*011*, S13: strain UFHB-LBAAB*013,* S32: strain UFHB-LBAAB*032*. (-) no growth; (+) good growth and acidification

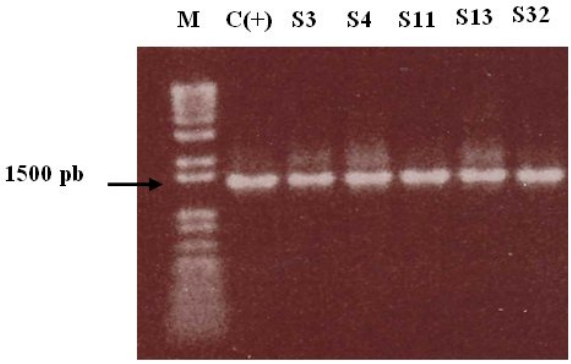

Figure 2. Agarose gel electrophoresis of five strains of acetic acid bacteria. M was Mark, C(+) was positif control (*Lactobacillus pentosaceus*), S3, S4, S11, S13 and S32 were the five selected strains. It is known that the length of acetic acid bacteria 16S rDNA was about 1500bp with the PCR kit, Hot Master Mix 5 PRIME (Dominique Deutcher, France).

The bacterial growth (Figure 3) presented three phases characteristic of diauxic growth. A first growth phase during the first five days of incubation at 30°C (with 0.34-0.52 OD_{600}) and during the first three days at 35°C, 37°C and 39°C (with respectively 0.13-0.33, 0.14-0.29 and 0.10-0.15 OD_{600} for cultures at 35°C, 37°C and 39°C). Then, a lag phase followed during two days. Thereafter, a second growth phase occurred with 0.52-1.09 OD_{600} at 30°C and respectively 0.54-0.89, 0.60-1.18, 0.65 OD_{600} for cultures at 35°C, 37°C and 39°C.

The diauxic curves observed can be explained by total consumption of the first carbon source (alcohol) and the utilization of acetic acid obtained by alcohol oxidation as a second carbon source. Chinnawirotpisan et al. (2003) and Saeki et al. (1997, 1999) justified this biochemical phenomenon as follow: *Acetobacter* strains always show three growth characteristics in alcohol media. The first one is the alcohol oxidation into acetic acid, the second is a no growth step and the last one is the overoxidation of accumulated acetic acid.

In Figure 4 shows that the highest acetic acid productions were obtained between the seventh and the ninth day of incubation at 30°C and between the fifth and the seventh day of culture at 35°C, 37°C and 39°C. These concentrations were respectively 15,0-26.1, 20.4-27.6, 31.5-33.9 and 11.4-12.3 g/l at 30°C, 35°C, 37°C and 39°C. It can be noticed that 37°C was the temperature that gave the highest acid concentrations (31.5-33.9 g/l) after seven days of incubation.

Acid production up to 30 g/l at 37°C in 7 days is very interesting for vinegar production. It's one of the highest acetic acid productions at this temperature. In fact, Amornrut and Wasu (2008) obtained 8.97 g/l à 37°C with acetic strains isolated from fruits. An *Acetobacter* strain isolated by Ndoye et al. (2006) produced 25 g/l in two days at 37°C, but the medium used was enriched. It contained 4 carbon sources which may rapidly increase biomass, final acid production and diversify the acid content.

Besides, the 5 strains mentioned above can be used as acetators; indeed Ndoye et al. (2007) affirmed that strains able to produce 1.7° of final acetic acid can be used in fermentations processes as acetators.

The higher productions at this temperature can be explained by a possible tolerance to temperature acquired by the strains from their natural or transferred areas (Legrand-Sow, 2004). However, Gullo and Giudici (2008) affirmed that minimum and maximum growth temperatures are difficult to define because they depend on the variability among the species and the composition of culture medium.

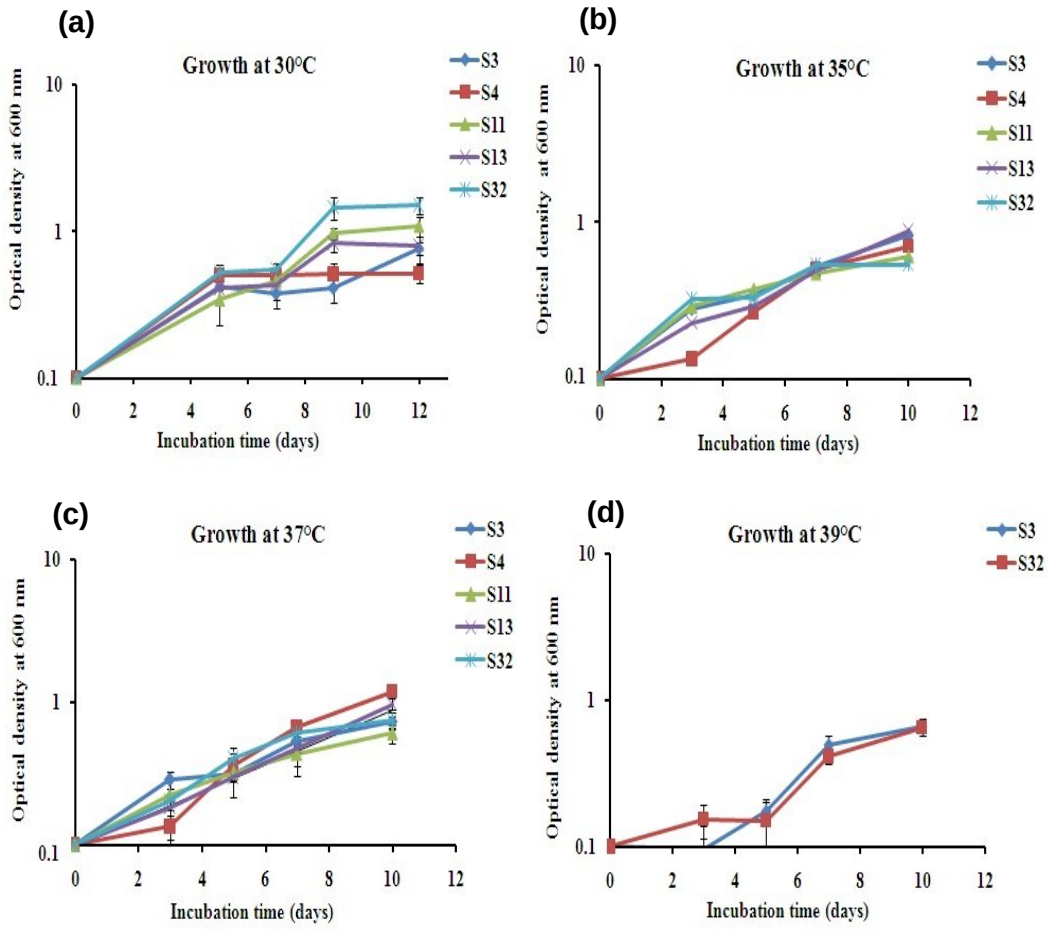

Figure 3. Growth of 5 selected A. *pasteurianus* strains isolated from Ivorian palm wine. Cultures were incubated at 30°C (a), 35°C (b), 37°C (c) and 39°C (d). The HS culture medium used was supplemented with ethanol 1.5% as sole carbon source. S3: strain UFHB-LBAAB*003*, S4: strain UFHB-LBAAB*004*, S11: strain UFHB-LBAAB*011*, S13: strain UFHB-LBAAB*013,* S32: strain UFHB-LBAAB*032*.

Figures 5a and 5b showed respectively the alcohol and acid tolerance tests. Both presented two main growth phases. The first phase was a progressive decrease phase corresponding to 0-4% alcohol (Figure 5a) and 0-3% acetic acid (Figure 5b) in the medium and the second phase was an increase one with 4-9% alcohol and 3-8% acetic acid in the medium. A third phase (lag phase) appeared up to 9% alcohol in the medium. The values of $\Delta LogN_{10}$ ($LogN_0$ - $LogN_1$) that indicate tolerance were inferior to five when alcohol and acid rates were respectively inferior to 9% and 7%.

These results indicated that the strains were tolerant to 8% alcohol and 6% acetic acid according to Legrand-Sow (2004). It was also noticed that approximately 4% alcohol and 3% acetic acid were the optimal rates for bacterial growth.

The 4% alcohol rate for the best growth (Figure 5 a) was also observed by Kanlaya and Wasu (2009) with *Gluconobacter* strains isolated from honey. Some strains of Sharafi et al. (2010) were able to tolerate 7% alcohol and other strains until 11%, while 78% of the strains isolated by Seyram et al. (2009) grew well with 6% alcohol and tolerated until 12%. Du Toit and Pretorius, (2002) noticed that AAB cells remain viable until 10 to 14% (v/v) of ethanol in wine but several studies performed on different AAB sources showed that high ethanol concentration (10% - 15%) could alter their amino acid requirement (Gosselé et al., 1981; Drysdale and Fleet, 1988). Generally, ethanol tolerance is a species and strain-dependent

trait that is conditioned by temperature, pH and oxygen (Drysdale and Fleet, 1988; Du Toit and Pretorius, 2002). For instance, Gullo and Giudici (2006) noticed that among 25 *Gluconacetobacter europaeus* strains 11 were able to grow at 5% ethanol while the other *G. europaeus* strains grew up to 10% ethanol.

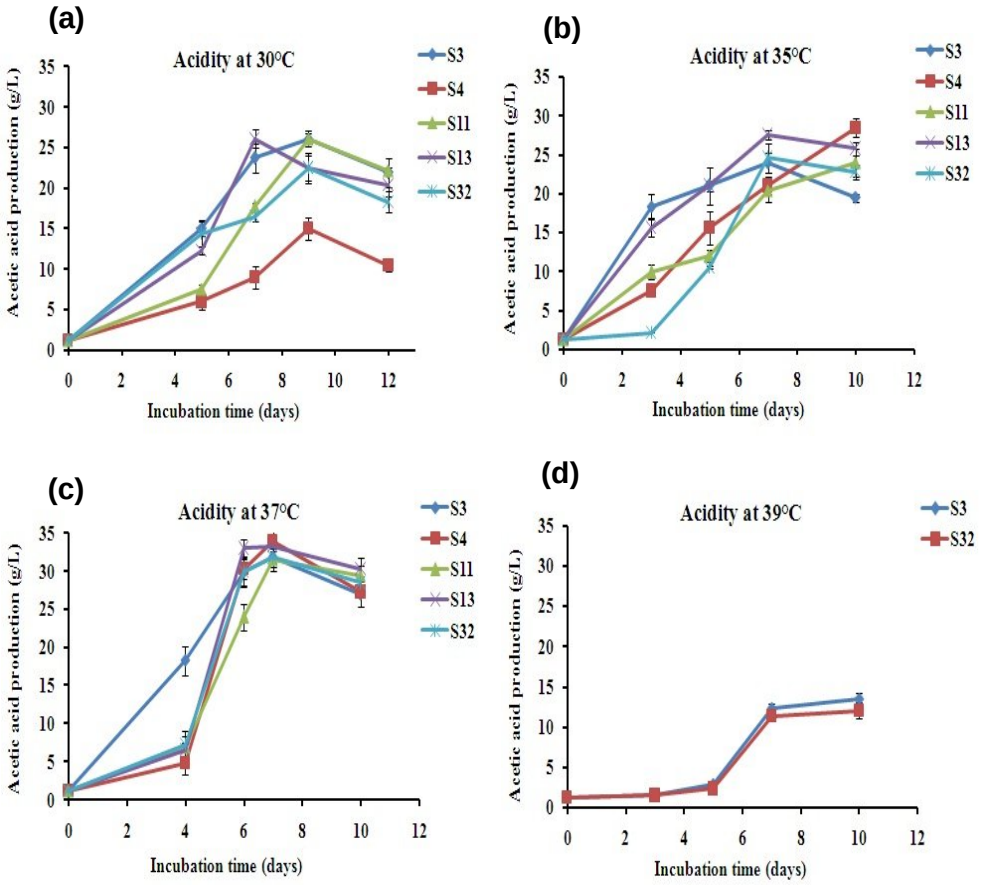

Figure 4. Acetic acid production by 5 selected *A. pasteurianus* strains isolated from Ivorian palm wine. Cultures were incubated at 30°C (a), 35°C (b), 37°C (c) and 39°C (d). The HS culture medium used was supplemented with ethanol 1.5% as sole carbon source. S3: strain UFHB-LBAAB*003*, S4: strain UFHB-LBAAB*004*, S11: strain UFHB-LBAAB*011*, S13: strain UFHB-LBAAB*013,* S32: strain UFHB-LBAAB*032*.

The five selected strains were not tolerant enough to acid (Figure 5 b) comparatively to *Acetobacter polyoxygenes* and *Acetobacter europaeus* which are acidophilic strains isolated by Saeki et al. (1997). Those strains tolerated 15 to 21% acetic acid and needed 4-8% acetic acid for optimal growth. Overwise, they have similar characteristics with some strains isolated by Sharafi et al. (2010) that gave better growth ability at 3% acid.

Concerning the influence of aeration (Figure 6 a), it was noticed that under 40% of air in flasks, acetic acid production was low (inferior to 10 g/l). From 40% to 60% air in flasks, it increased rapidly and achieved 16.9-18.9 g/l. Acid production continued increasing slowly until 70% air in flasks, reaching 17.9-19.7 g/l. No significant production occurred after that rate.

This high aeration rate needed by the strains to produce acid depends probably on the aerobic growth characters known in bacteria from *Acetobacteriaceae* family. Matsushita et al. (1994) affirmed that ADH and ALDH are amoung respiratory components of acetic acid bacteria, located in the cytoplasmic membrane. Furthermore, these enzymes are responsible of acetic acid production in extracellular side of bacteria (Ndoye et al., 2006). Macias et al. (1997) have also showed that an optimum dissolved oxygen concentration in the medium is necessary to obtain a maximal growth and acetification. For instance, Ndoye et al. (2006) used 80% aeration rate to obtain the highest growing characteristics of their *Acetobacter* strains.

This importance of aeration would be due to oxygen. In fact, Adams (1998) affirms that oxygen is a limiting factor in acetification processes because it is only sparingly soluble in aqueous media and both temperature and solute content condition its solubility. In industrial wine vinegar produced by submerged culture in semi-continuous processes, the concentration of dissolved oxygen is the most important parameter that allows bacterial growth. However, it has been established that a high dissolved oxygen value can inhibit AAB growth and that the optimum concentration in semi-continuous processes is 1-3 mg/kg (Rubio-Fernández et al., 2004).

The osmotic stress assay showed that 5% glucose was the optimal concentration giving the highest relative growth: 10-15% glucose gave about 50% relative growth and 20% glucose was lethal for the isolates (Figure 6 b). Therefore, it's better not to go above 8-9% glucose for further uses of the selected bacteria in technological processes. This agrees with previous studies made by Gullo and Giudici (2006) which indicated that even if sugars are excellent carbon sources for acetic acid bacteria, it is better not to reach high concentrations that can inhibits their growth. However, Jojima et al. (2004) showed that some extremophilic strains are able to grow up to 40% glucose.

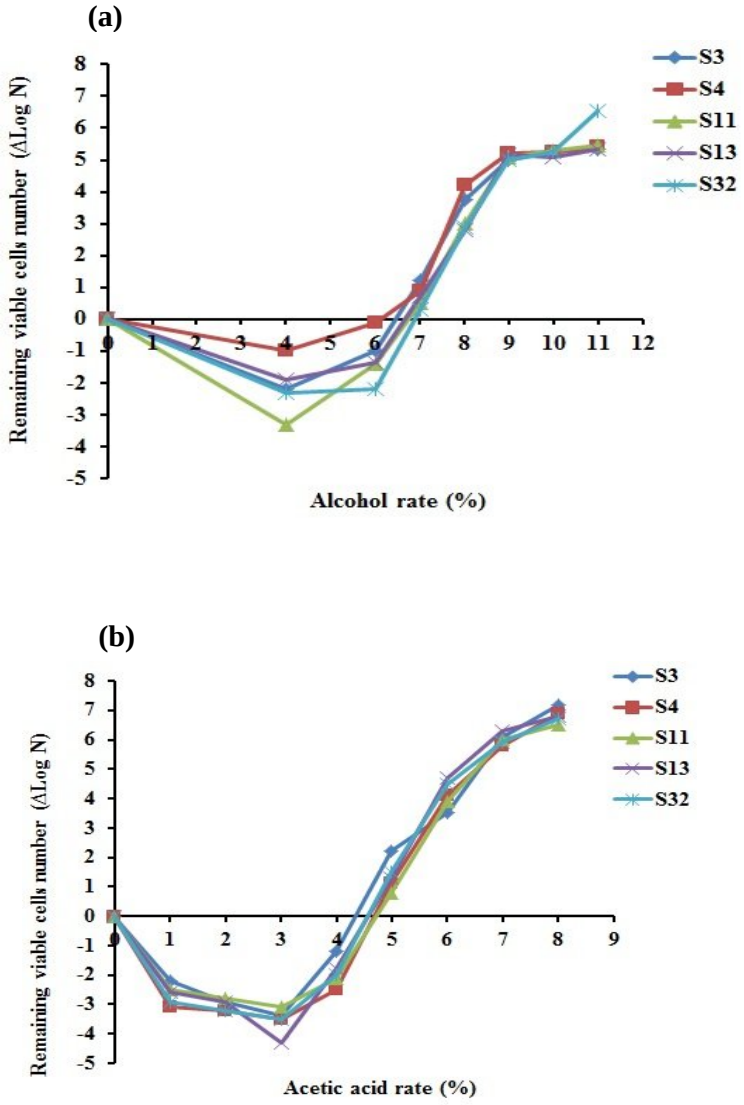

Figure 5. Tolerance to alcohol (a) and acetic acid (b) with estimation of the remaining viable biomass of 5 *Acetobacter pasteurianus* isolated from Ivorian palm wine. Modified HS medium was used with ethanol concentration ranging from 4 to 11% and acetic acid from 1 to 8%. Cultures were incubated at 37° C for 48 h with 130 rpm shaking. S3: strain UFHB-LBAAB*003*, S4: strain UFHB-LBAAB*004*, S11: strain UFHB-LBAAB*011*, S13: strain UFHB-LBAAB*013,* S32: strain UFHB-LBAAB*032*.

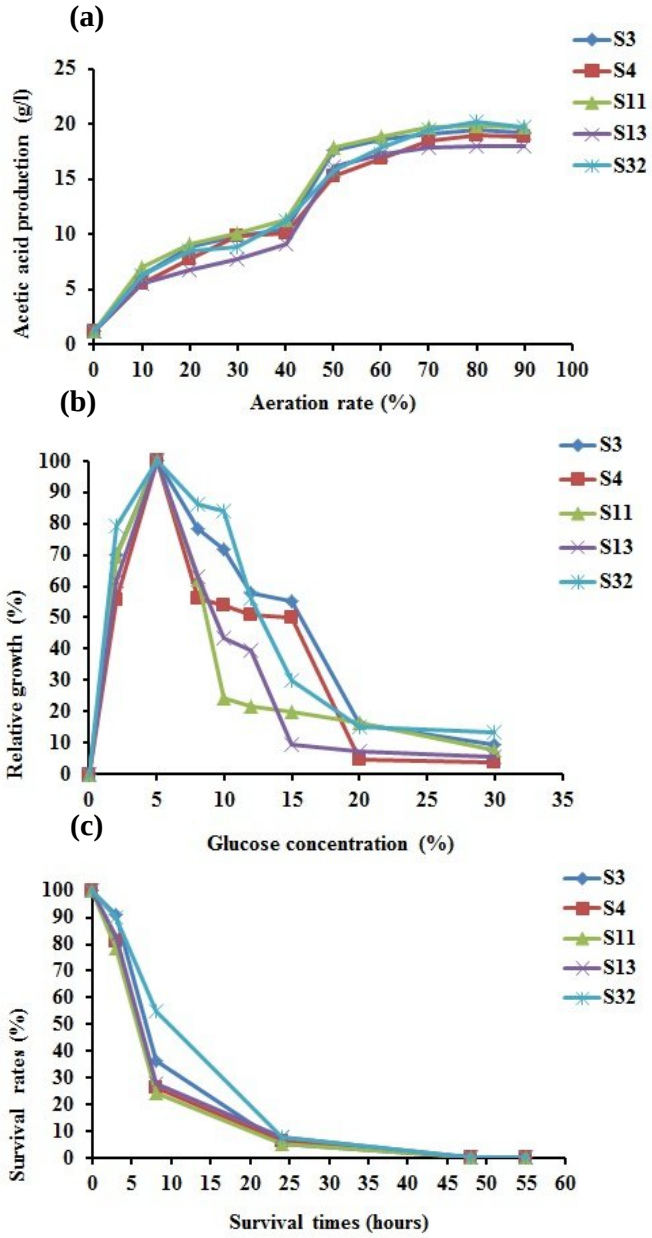

Figure 6. Physiological characterization of 5 *Acetobacter pasteurianus* strains isolated from Ivorian palm (*Elaeis guineensis*) wine. (a) Airing effect on acetic acid production. Modified HS medium was used and supplemented with 1.5 % ethanol as sole carbon source. Cultures were incubated at 37°C with 130 rpm shaking for 48 h. (b): Relative growth of strains with glucose concentrations ranging from 2 to 30% in modified HS medium. Bacterial growth was studied after 7 days at 37 °C with 130 rpm shaking. (c): Resistance against desiccation. YGM/Mg^{2+} medium was used for cells growth at 30°C with 130 rpm shaking during 72 h, then cells were exposed to drying at 37°C during 72 h. S3: strain UFHB-LBAAB*003*, S4: strain UFHB-LBAAB*004*, S11: strain UFHB-LBAAB*011*, S13: strain UFHB-LBAAB*013,* S32: strain UFHB-LBAAB*032*

Resistance against desiccation of the 5 strains (Figure 6 c) showed that their resistance went decreasing considerably during the ten first hours. Nevertheless there still remaining more than 50% survival strains after 5 h, more than 10% survival strains after 15 h and less than 0.3% after 48 h. No survival strain was observed after 50 h.

This result showed that the selected five *A. pasteurianus* strains were not resistant enough to desiccation. This can be due to their gram negative membranes that are naturally less resistant to desiccation than positive ones. Strain S32 is the

only one that remains having about 30% survival rate after 15h treatment. This suggests that resistance to desiccation could be strain dependant, as it was observed in other gram (-) bacteria like *Campylobacter jejuni* and *C. coli* (Fernández et al., 1985). We did not use any interference substance in this study to attenuate the temperature effect while Birgit et al. (2005) showed that bacterial resistance to desiccation is increased when using interference substance. They suggested that the higher the concentration of the interfering substance the higher was the survival rate of gram negative bacteria. However, there still remaining viable cells after 48 h incubation with the 5 strains even if their rates are considerably low (0.03 to 0.25 %). Comparing to those of Laura et al. (2001) where among 11 *Arcobacter butzleri* only 2 were able to resist to desiccation at 37°C after 48h. Lewis et al. (2010) have shown that proportion of surviving cells is linked to desiccation time. They suggested that there is a correlation between microhabitat and desiccation survival, according to sampling location. Their strains isolated from a more barren and exposed location exhibit much greater desiccation resistance than those adapted to more sheltered microenvironment, with resistance beyond one week. The studied 5 strains were isolated from palm wine where growth conditions are not so hard, hence they could not be naturally as resistant. This indicates that a particular attention should be brought on the preservation of these strains as starters. Indeed, atomization and other desiccation methods like freeze-drying can be critical and must be preceded by accurate studies. Whatever, the use of starters in lyophilizated form is advantageous by avoiding risks linked to industrial manipulation with liquid inoculum. It reduces also considerably the starting time of starters activities, facilitates their carrying and their stability through the conservation time (De Vuyst, 2000).

Conclusion

In this work, 104 strains were isolated from Ivorian palm wine. After thorough screening, five strains presenting the best potentialities were characterized. Morphological, biochemical, physiological and molecular examination indicated that all isolates were all *Acetobacter pasteurianus* strains and that they can be successfully used for high vinegar production. They showed acceptable acid and alcohol tolerance. Their acetic acid production above 30 g/l is one of the best rates obtained by acetic bacteria and their thermotolerant properties at 37°C are useful in sub-African countries where temperature is generally above 30°C. Nethertheless, accurate studies must be performed on these strains according to their preservation as starters for industrial vinegar production.

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