Isolation and Characterization of Indigenous Acetobacter Strains from Cashew Apple and Their Potential Use as Vinegar Producers in Côte d'Ivoire

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Article

Isolation and Characterization of Indigenous *Acetobacter* Strains from Cashew Apple and Their Potential Use as Vinegar Producers in Côte d'Ivoire

Yaya Anianhou Ouattara [1], Doudjo Soro [1], Kouakou Romain Fossou [2,*], Ahou Cinthia Ines Yebouet [2], Ismael Abraham Cissé [3], Dogbo Marius Akissi [4], Adolphe Zézé [2], Emmanuel Assidjo [1] and Kouassi Benjamin Yao [5]

[1] Laboratoire de Nutrition et de Technologie Alimentaire (LANTA), Unité Mixte de Recherche et d'Innovation en Sciences Agronomiques et des Procédés de Transformation, Institut National Polytechnique Félix HOUPHOUËT-BOIGNY, Yamoussoukro, Côte d'Ivoire
[2] Laboratoire de Microbiologie, Biotechnologie et Bio-informatique (LaMBB), Unité Mixte de Recherche et d'Innovation en Sciences Agronomiques et des Procédés de Transformation, Institut National Polytechnique Félix HOUPHOUËT-BOIGNY, BP 1093 Yamoussoukro, Côte d'Ivoire
[3] Laboratoire central des Biotechnologies, Centre National de Recherche Agronomique (CNRA) Km 17, Unité de phyto/physiopathologie, 01 BP 1740 Abidjan, Côte d'Ivoire
[4] Laboratoire de Biotechnologie et microbiologie (LBMA), UFR des Sciences et technologie des Aliments , Université Nangui Abrogoua, BP 1740 Abidjan, Côte d'Ivoire
[5] Laboratoire des Procédés Industriels de Synthèse de l'Environnement et des Énergies Nouvelles (LAPISEN), Unité Mixte de Recherche et d'Innovation en Sciences Agronomiques et des Procédés de Tansformation, Institut National Polytechnique Félix HOUPHOUËT-BOIGNY, Yamoussoukro, Côte d'Ivoire
* Correspondence: romain.fossou@inphb.ci; Tel.: (+225 0708274742)

Abstract: Côte d'Ivoire has the main cashew tree culture area worldwide, but the cashew apple produced is still underutilized although its potential for industry. In the present study, the aim was to isolate and identify acetic acid bacterial strains, and to assess their potential use for cashew apple-based vinegar production. Vinegar mother from fermented juice was used to isolate acetic acid bacteria on a standard glucose-based medium. Physiological and biochemical tests followed by 16S rRNA gene analysis and phylogeny were used for isolate characterization. Moreover, their acetic acid production capacity was assessed. As results, five (05) strains of the *Acetobacter* genus were isolated. Phenotypic and phylogenetic analysis revealed that four of them, namely OYA2, OYA6, OYA9, and OYA10, belong to *A. tropicalis* / *A. senegalensis* species complex with 99,7% or 100% similarity. The last strain, OYA7, match to *A. syzygii* (99,7%). All the isolates were resistant to 15° alcohol (v/v) and grew well between pH 5.0 and 6.5. Their optimal growth temperatures varied between 27°C and 37°C, and only isolate OYA6 grows up to 40°C. They produced vinegar with yield ($Y_{p/s}$, g/g) varying from 0.82 to 0.92, and acetic acid content (g/L) of 80.67 ± 2.1, 70.26 ± 0.9, 70.11 ± 1.7, 68.70 ± 1.5 and 67.22 ± 0.40 were obtained with OYA6, OYA7, OYA2, OYA10, and OYA9, respectively. Thus, the isolate OYA6 appeared as the best candidate for vinegar production, owing to its superior yield and thermotolerance ability that need to be further explored for industry use purpose.

Keywords: *Acetobacter*; indigenous microbiome; vinegar ; acetic acid; Cashew apple valorization

1. Introduction

Acetic acid bacteria (AAB) are generally known as vinegar-producing micro-organisms. They occur naturally on fruits and vegetables at the fermentable stage. They belong to the *Acetobacteraceae* family of the *Alphaproteobacteria* class [1]. The genus type of the family, namely *Acetobacter*, encompasses 34 species validly published to date, (https://lpsn.dsmz.de/genus/acetobacter); accessed on 17 August 2024) (**Figure A1**). AAB are a group of Gram-negative or Gram-variable bacteria, obligatorily aerobic, ellipsoidal or rod-shaped, non-spore-forming, and peritrich or polar flagellates.

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In general, they have a rapid oxidative fermentation capacity to oxidize ethanol to acetic acid and to oxidize a wide range of sugars and sugar alcohols to the corresponding organic acids in the presence of oxygen, although some are very low producers [2,3].

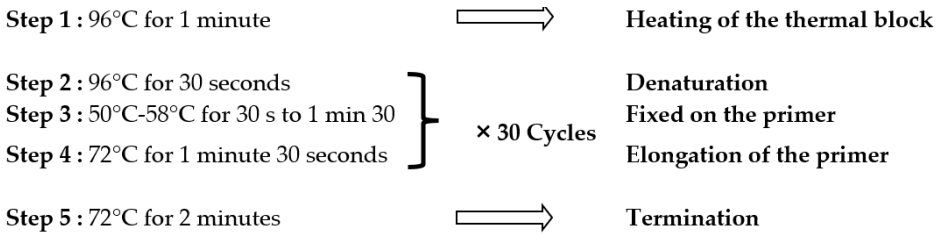

Figure 1. PCR programming phase.

Vinegar is a precious additive and food supplement that has been used around the world for thousands of years [4]. According to the FAO [5] and the European Union standard (NF EN 13188-October 2000)[6], vinegar is produced from agricultural materials containing starch and/or sugar by the process of double fermentation, alcoholic and acetic. Acetic acid is the principal aromatic compound in vinegar, and techniques have been developed to improve vinegar production. In general, these improvements increase the rate at which ethanol is converted to acetic acid by bacteria of the genus *Acetobacter*. The temperature at which they grow and metabolize varies between 25°C and 30°C [7]. Temperature control is of particular concern, as ethanol oxidation is an exothermic reaction. An increase of 2 to 3°C causes deterioration in the rate of acetification and even inefficiency in vinegar production [8]. In tropical countries, an important selection parameter is the strain's thermotolerance, which, combined with the interest in vinegar quality, has led to a growing search for potential strains. The thermotolerant strains are used to avoid a low rate of acetification or fermentation stoppage [9]. Moreover, the use of strains also capable of high acid production is a desirable asset. To date, several acetic acid bacteria have been selected from tropical countries in African. For example in Senegal (West Africa), Ndoye et al. [10] selected two strains of *Acetobacter tropicalis* and *A. pasteurianus* for their ability to grow at 40°C and 45°C, respectively, and proposed them for the production of an artisanal spirit vinegar. These thermotolerant isolates, especially those from *A. tropicalis* species, are being used for industrial vinegar production in the sub-Saharan Africa [11]. A similar experiment was carried out in Ghana for isolating indigenous *Acetobacter* using traditional heap fermentations of cocoa beans, leading to the characterization and the taxonomic description of *Acetobacter ghanensis* sp. nov [12]. Thus, the bioprospection and selection of indigenous *Acetobacter* strains constitute a promising way for fostering the production of acetic acid and vinegar using domestic biomass [13,14].

Acetic acid bacteria could be difficult to isolate, due to the "viable but unculturable state" phenomenon [15]. It is also very difficult to correctly identify acetic acid bacteria down to the species level based on their physiological and biochemical characteristics [3]. Thus, for proper identification of AAB, the use of molecular methods and phylogenetic reconstruction is recommended [16]. As for their cultivation, the culture medium for acetic acid bacteria is generally composed of glucose, ethanol, peptone, yeast extract, calcium carbonate, and agar [17]. When the composition of carbon sources is altered, selective isolation of acetic acid bacteria can be expected [18]. For example, strains of *Asaia bogorensis* and *Asaia siamensis* were first isolated using sorbitol or dulcitol instead of glucose [19]. Thus, several carbon sources are used as enrichment cultures to efficiently isolate acetic acid bacteria to date [20], using diverse "non-conventional" and/or underutilized products such as mangoes [10], cocoa beans [12], papaya and sugarcane [21], in addition to standard fermented products like beer, vinegar and cider [16]. The cashew apple fruit is classified among the underutilized products in low-technological nations in Africa despite having high nutritional content [22]. In Côte d'Ivoire, the country which is the largest nut producer worldwide (1.300.000 tons of raw cashew nuts in 2024), the majority of apple fruit (an estimated of 7.776.870 tons) was left to rot in the field after removal of the raw nut [23–26]. This situation leads to important greenhouse gas (CH_4 and CO_2) emissions being released as a result of the non-controlled decomposition process [26]. Thus,

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there is a need of prospects for sustainable development of the cashew industry [23], and in the last decade, the Ivorian government has elaborated ambitious objectives to increase the percentage of products processed domestically [26]. In this context, this study aims to isolate and identify acetic acid bacteria from cashew apples, and to assess their potential to produce cashew apple-based vinegar.

2. Materials and Methods

2.1. Bacterial Strains and Isolation Technics

Ripe cashew apples were randomly collected from cashew tree in Yamoussoukro (6.74 N 5.38 W), the political capital of Côte d'Ivoire . The apples were washed and stored in a vat for 10 days. The apples were then pressed and 2L of the liquid collected was fermented at 30°C for 4 weeks. A vinegar mother solution was formed on the fermenting liquid. From this mother solution, 100 mL were collected and a decimal dilution was made up to 10^{-9}. About 200 µl of the diluted solution were used to inoculate each agar plate (Ø 100 mm Petri dish) and all agar plates inoculated with solution from 10^{-3} to 10^{-5} dillutions were incubated at 30°C for 5 days. After the cells growth, single strain purity was verified by several replicates on nutrient agar [27].

2.2. Culture Medium

A medium based on glucose, yeast extract, calcium carbonate, penicillin and mycostatin (GYC) was used for isolation and culture. GYC culture medium consisted of Yeast extract 1%; Glucose 7.5%, Calcium carbonate 3%; Agar-agar 2% and Penicillin 0.5%. It was sterilized at 121°C for 15 min in an autoclave (HV 50 Hirayama, Japan), cooled to 45°C, and then 5% Mycostatin was added. Each Petri dish was filled with ca. 15 mL of prepared GYC medium for subsequent utilization.

2.3. Biochemical and Cultural Identification of Isolated Strains

Biochemical and cultural identification tests were carried-out to confirm that the strains isolated belonged to the *Acetobacter* genus. For cultural identification, we noted the shape, size, and color of the colonies. Conventional biochemical tests, including Gram stain, catalase, and oxidase tests, were performed according to the guidelines of Bergey's Manual of Systematic Bacteriology [28].

2.4. Molecular Identification

2.4.1. PCR Amplification of 16S rDNA and PCR Programming

Presumptive *Acetobacter* strains were subjected to genomic DNA amplification sequence analysis with primers 16Sd (5'-GCTGGCATGCTTAACACAACAT-3') and 16Sr (5'-GGAGGTGATCCAGCCGCAGGT-3') [29]. DNA was extracted from fresh cells following standard methods [30]. Briefly, for each isolate, a single colony was picked from a fresh culture on GYC medium and resuspended in 50 µl sterile water. After a brief vortex, the mixture was incubated at 95 °C for 15 min, centrifugate and the supernatant containing the release DNA was used as a template for the analysis. As for the PCR, the total reaction volume per strain was 50 µl **(Table 1)**. For each amplification reaction, a microtube containing only the DNA-free reaction medium was used as a negative control.

Table 1. PCR reaction medium composition.

	Concentration	Sample volume
2X oneTaq standard buffer	2 X	25 µl
Amorce 1 (avant)	10 µM	5 µl
Amorce 2 (inverse)	10 µM	5 µl
ADN	Ca. 10 ng/µl	3 µl
Distilled sterile water	-	12 µl

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Total reaction volume for each amplification	50 µl

PCR reaction conditions, carried-out in a thermocycler (Eppendorf, France) using a standard PCR program **(Figure 1)**. PCR products were visualized by electrophoresis at 100V for 45 min on a 1% agarose gel in 1X TBE (Tris Borate EDTA) buffer. The molecular weight of reaction products was estimated with reference to a 1Kb molecular weight marker (100 bp to 12,000 bp) (Thermo Scientific™ SM0313, Thermofischer, France) to verify the expected size of amplified 16S rDNA which is around 1450 bp with the primers used [30].

2.4.2. 16S rDNA Sequencing

The PCR product was purified and sequenced using the Sanger method in the Eurofins laboratory (France). The sequences obtained were then assembled into double stranded DNA using the Bio Edit version 7.25 program. The sequences obtained were compared with those deposited in GenBank, using the BLAST algorithm (NCBI). BlastN with NCBI/GenBank online standard databases [nucleotide collection (nr/nt) and Whole-genome shotgun contigs (wgs)] was used for the identification of the isolates. The presumptive taxonomic affiliations obtained were further confirmed using (i) phylogenetic reconstructions and (ii) similarity level calculations between the novel sequences and the sequences of the selected reference strains [31]. All 16S rDNA gene sequences generated for the 05 isolates, and used in this study were deposited in GenBank under the accession numbers PP359644 to PP359648.

2.4.3. Phylogenetic Analysis and Similarity Distance Calculations

The phylogenetic analysis was inferred with the MEGA software version 7.0.26 [32]. Prior to the phylogenetic reconstruction, we carried-out a multiple sequence alignment by aligning the 16S rDNA gene sequences from the five isolates with those of the reference type strains collected in GenBank (**Table 2**). Briefly, 16S edited sequences were aligned with MUSCLE as implemented in MEGA software v.7, and a subsequent phylogenetic tree was reconstructed using the neighbor-joining (NJ) and maximum likelihood (ML) methods [33,34], with a Bootstrap value of 1000 [35]. The best-fit nucleotide substitution model of the aligned sequences was selected according to the Bayesian information criterion (BIC) [36] in MEGA software v.7. Finally, a pairwise similarity distance was calculated to infer the genetic similarity level between the sequences of isolated bacteria from Côte d'Ivoire and the selected reference sequences [37,38].

Table 2. List of the 15 reference strains of Acetobacter and corresponding GenBank accessions used in this study. Their genome sequences were used as data for species of the Acetobacter found to be genetically related to the acetic acid bacteria isolated from the Ivorian cashew apples. They were mainly used for the phylogeny reconstruction and for the genetic similarity value calculations.

	Reference type strain	Isolation source & Country	16S data[1]	Genome data	Reference
1	*A. cerevisiae* LMG 1625^T	Beer (ale) in storage; Canada	AJ419843	LHZA01000074	Cleenwerck et al.[39]
2	*A. cibinongensis* NRIC 0482^T	montana (fruit); Indonesia	AB681085	BAOZ01000003	Lisdiyanti et al.[40]
3	*A. conturbans* LMG 1627^T	Laboratory-scale cider fermentations, UK	MN744433	WOSY01000049	Sombolestani et al. [16]
4	*A. ghanensis* 18895^T	Traditional heap fermentations of cocoa beans; Ghana	EF030713	BAPC01000052	Cleenwerck et al. [12]

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5	*A. indonesiensis* 16471^T	Fruit of *Annona muricata*; Indonesia	AB032356	BJXQ01000038	Lisdiyant et al.[41]
6	*A. lambici* LMG 27439^T	fermenting lambic beer ; Belgium	HF969863	WOTD01000082	Spitaels et al.[42]
7	*A. malorum* LMG 1746^T	Rotting apple ; Belgium	AJ419844	LHZC01000018	Cleenwerck et al.[39]
8	*A. nitrogenifigens* DSM 23921^T	Kombucha tea ; India	AY669513	BAPG01000028	Dutta & Gachhui.[43]
9	*A. okinawensis* JCM 25146^T	Sugarcane (stem); Japon	AB665068	BAJU01000075	Lino et al.[21]
10	*A. orientalis* NRIC 0481^T	Canna (flower) ; Indonesia	AB681086	BAPI01000013	Lisdiyanti et al.[40]
11	*A. sacchari* VTH-Ai14^T	stems of sugarcane; Thailand	LC103252	LC103252	Vu et al.[44]
12	*A. senegalensis* LMG 23690^T	mango fruit ; Senegal	AY883036	LHZU01000060	Ndoye et al.[8]
13	*A. syzygii* NBRC 16604^T	Malay rose apple (fruit); Indonesia	AB681084	BJVT01000030	Lisdiyanti et al.[40]
14	*A. thailandicus* BCC 15839^T	flower of the blue trumpet vine; Thailand	AB937775	*Not available*	Pitiwittayakul et al.[45]
15	*A. tropicalis* LMG 19825^T	Coconut juice; Indonesia	AB032354	LHZQ01000125	Lisdiyanti et al.[40]

[1] For the sequences analyses, we preferentially used the 16S sequence retrieved from the whole-genome shotgun contigs (wgs) of each strain (when possible), for quality concerns. Some type strains include more than one ambiguous positions (N, W…) per 100 bp count in their 16S rDNA data archived in GenBank, under Nucleotide collection (nr/nt) and appeared inapropriate for the analysis. For example, the 16S (nr/nt) sequence of *A. senegalensis* LMG 23690^T (AY883036), which is underlined in Table 2, diverged too much (> 2%; ca. 30 divergent nucleotides, including 5 gaps) from the 16S sequences retrieved from the genome of the same type strain obtained from two independent sources (cf. GenBank 16S locus_tag LHZU01000060 & 16S locus_tag BAPN01000029).

2.5. Selection of the Strains According to Physiological and Biochemical Characteristics

The selection of high-performance acetic acid bacteria strains comes down to their ability to withstand high levels of temperature, pH, and ethanol [46]. Strain kinetics are performed to determine growth optima. *Acetobacter* isolates harvested from fresh colonies grown on plates were used for the experiment. For each isolate, selected colonies were used to inoculate 10 mL of GYP broth (Glucose, Yeast Extract, and Peptone) and incubate for 7 days. Cell growth was estimated by measuring optical density (OD) at 600 nm using a spectrophotometer [47]. Bacterial growth rate was represented by μ_{max}, the number of cells produced per unit of time. The details of each test are provided below:

- **Thermo-tolerance test**: Cultures were incubated at temperatures of 30 °C, 35 °C, 37 °C, and 40 °C.
- **Effect of pH:** Bacteria were grown at pH 3, 5, 6, 6.5 and 7
- **Ethanol tolerance test:** Bacteria were grown in GYP medium supplemented with at 5°, 10° and 15° alcohol (v/v).

2.6. Acetic Acid Production

The *Acetobacter* strains isolated and identified were used as a ferment to produce cashew apple vinegar. *Acetobacter* strains were grown on YG broth (yeast extract, glucose) to an OD_{600} of 0.7. Then 10 g/L were used to inoculate cashew apple alcohol to carry-out acetic fermentation. Batch

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fermentation (semi-continuous) was carried out in 500 mL Erlenmeyer flasks with 150mL alcoholic solution, at room temperature of 30°C ± 3 and pH 6 **(Figure 2)**. Samples were taken at defined time intervals and analyzed.

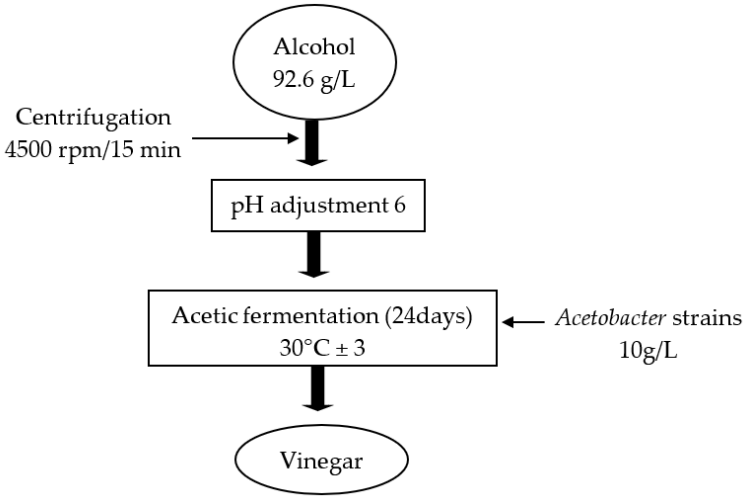

Figure 2. Acetic fermentation process.

2.7. Estimation of Acetic Acid

Acetic acid concentration was determined using the titratable acidity method described by AOAC (1990). A sample (1 mL) was pipetted into the 250 mL Erlenmeyer flask, and 20 mL distilled water and 5 drops of phenolphthalein were added to the flask. The sample solution was titrated with 0.1 N sodium hydroxide until a pink color was obtained. The acetic acid content was calculated using the following formula:

$$\text{Acetic acid content (\%)} = \frac{V\ (mL)\ \times\ N\ \times 60.05}{10\ \times\ W\ (g)} \qquad (1)$$

Where V = volume of final 0.1 N NaOH solution,
N = normality of NaOH solution,
60.05 = molar mass of acetic acid (g/mol)
W = weight of vinegar sample.

2.8. Fermentation Parameters

Yields were calculated at the end of fermentation. Volumetric **acetic acid productivity** (Q_P , g/L/h) was calculated as the ratio between acetic acid concentration at the end of the run (P_f , g/L) and fermentation time (t,h).

$$Qp = \frac{Pf}{t} \qquad (2)$$

P_f : (g/L) Acetic acid concentration at end of fermentation; t (h): Fermentation time.

Yield of acetic acid on ethanol consumed ($Y_{p/s}$, g/g):

$$Yp/s = \frac{Pf}{(E0\ -\ Ef\ )} \qquad (3)$$

Where E0 and Ef g/L Initial and final ethanol concentration.

Ethanol conversion was calculated using this equation,

$$Conversion\ (\%) = \frac{(E0\ -\ Ef)}{Ef} \qquad (4)$$

The efficiency of conversion of ethanol to acetic acid was estimated by the following relationship:

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$$\eta = \frac{Yp/s}{Yth} \times 100 \quad (5)$$

Where Yth is the theoretical value of $Y_{p/s}$ (1.36 g/g).

2.9. Statistical Analysis

The data collected were analyzed using STATISTICA 7.1 software. Mean ± SD were calculated and analysis of variance was considered significant when the p-value is inferior to α=5%. Tests for significant difference in mean values were performed using Duncan's multiple range test ($p < 0.05$).

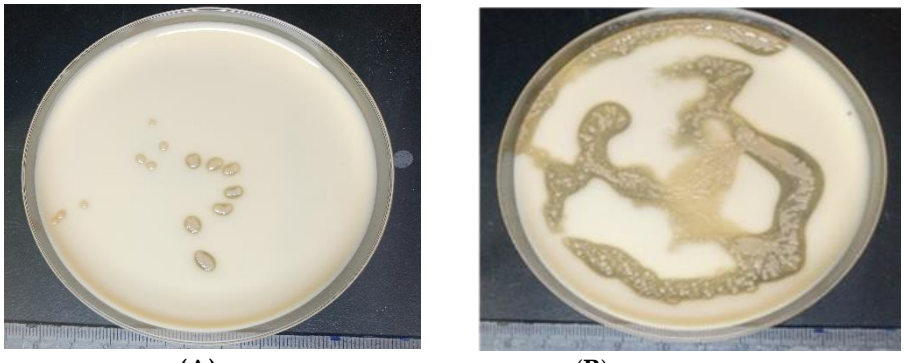
3. Results and Discussion

Acetic acid bacteria (AAB) with good acid-producing potential can be found in cashew apple juices in different tropical countries [27]. The isolates characterized so far are intensively tested for acetic acid production and tolerance against different stresses, including ethanol, but their identification rely often on physiological and biochemical technics [27]. The genetic identity of the acetic acid bacteria isolated from the microbiome of cashew apple in tropical areas is still unknown. In Côte d'Ivoire, vinegar production trial with cashew apple have been explored, but the *Acetobacter* strains used have been isolated from various sources such as palm wine and fermented cocoa beans [48]. Thus, the present work which aims to isolate and assess the functional features of acetic acid bacteria using native fermented cashew apple juices provides the first comprehensive analysis of cashew apple microbiome in Côte d'Ivoire. The study used a multi-approach to analyse five (05) indigenous acetic acid bacteria from Ivorian cashew apples, including a biochemical and microscopic traits assessment, a molecular identification using 16S rRNA gene sequences, the test of tolerance of isolates against ethanol, temperature, and pH stresses as well as the assessment of their potential use as vinegar producers in laboratory conditons.

3.1. Isolation, Screening, and Identification of the Strains for Production of Acetic

3.1.1. Biochemical and Cultural Identification of Isolated Strains

After 5 days of incubation at 30°C, colonies showing a halo due to acidification of the GYC medium were selected [14,49] (**Figure 3).** In consequence, isolation results showed the presumptive presence of 05 acetic acid bacteria on GYC medium. When visualized, OYA2, OYA6, OYA10, OYA7, and OYA9 have the same brown color, but they have two types of shape, which consist of regular (e.g. OYA2) and irregular (e.g. OYA9) shapes (**Figure 3**).



(A) **(B)**

Figure 3. Appearance of bacteria isolated with regular (A) and irregular (B) shape.

Microscopic examination revealed that the bacteria are motile, the cells are long, filamentous and have pink fusiform swellings, making them Gram-negative **(Figure 4)**. For Cire et al. [50],

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microscopic examination of acetic acid bacterial colonies isolated in mango alcohol in Senegal, in west-Africa, showed the same shape and Gram staining.

Colonies are colorless after a few seconds in the presence of N-dimethyl paraphenylene diamine oxalate. This means that isolated strains are oxidase-negative. Also, the catalase test performed on the bacteria enabled us to distinguish catalase-positive bacteria. All the morphological and biochemical characteristics of strains isolated from the Ivorian fermented cashew apple juice are presented in **Table 3**.

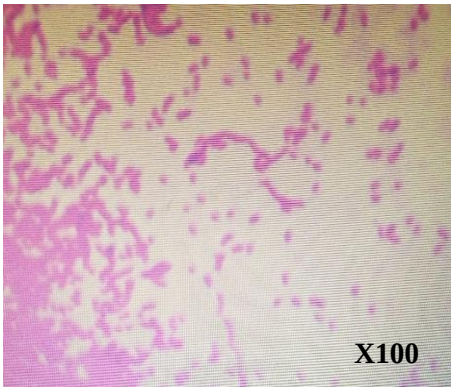

Figure 4. Gram-negative bacilli observed after gram staining.

Table 3. Biochemical and cultural characteristics of isolated strains.

Test	Morphology	Color	Motility	Gram staining	Catalase	Oxidase
OYA10	Irregular	Brown	+	-	+	-
OYA2	Irregular	Brown	+	-	+	-
OYA9	regular	Brown	+	-	+	-
OYA6	Irregular	Brown	+	-	+	-
OYA7	Irregular	Brown	+	-	+	-

Acetic acid bacteria are difficult to identify down to species level on the basis of physiological and biochemical characteristics [3]. There are several reasons for this difficulty, including the occurrence of spontaneous mutations and related phenotypic changes [51]. Mutation causes a loss of several biochemical traits, such as ethanol oxidation, non-production of cellulose, non-production of brown pigment. For these reasons, an accurate identification of acetic acid bacteria required a sequencing of molecular markers such as the 16S rRNA coding gene.

3.1.2. Molecular Identification

The BlastN analysis allowed us to identify our isolates as *Acetobacter* strains. These taxonomic affiliations were further confirmed using a combination of a phylogenetic analysis and a similarity level calculation between the sequences of our isolates and those of the selected reference type strains (**Table A1**). As preliminary analyses showed the Ivorian isolates to belong to *Acetobacter syzygii* species, as well as to *Acetobacter tropicalis* and *Acetobacter senegalensis* species (**Figure A1**), subsequent phylogenetic analysis included the 16S sequence of all the 12 types strains closely related to these two lineages and available at the time of writing (August 13th, 2024). An additional three type strains of *Acetobacter* genus representative of the remaining lineages were also selected, making a total number of 15 *Acetobacter* reference strains for the final phylogenetic analysis (**Figure 5**). The analyses showed that strain OYA7 match to *Acetobacter syzygii* and shares 99.7% of similarity value with its type strain NBRC 16604^{T}, while all the remaining isolates (i.e. OYA2, OYA6, OYA9 and OYA10) are genetically related to both the *A. tropicalis* and *A. senegalensis* species **(Figure 5).**

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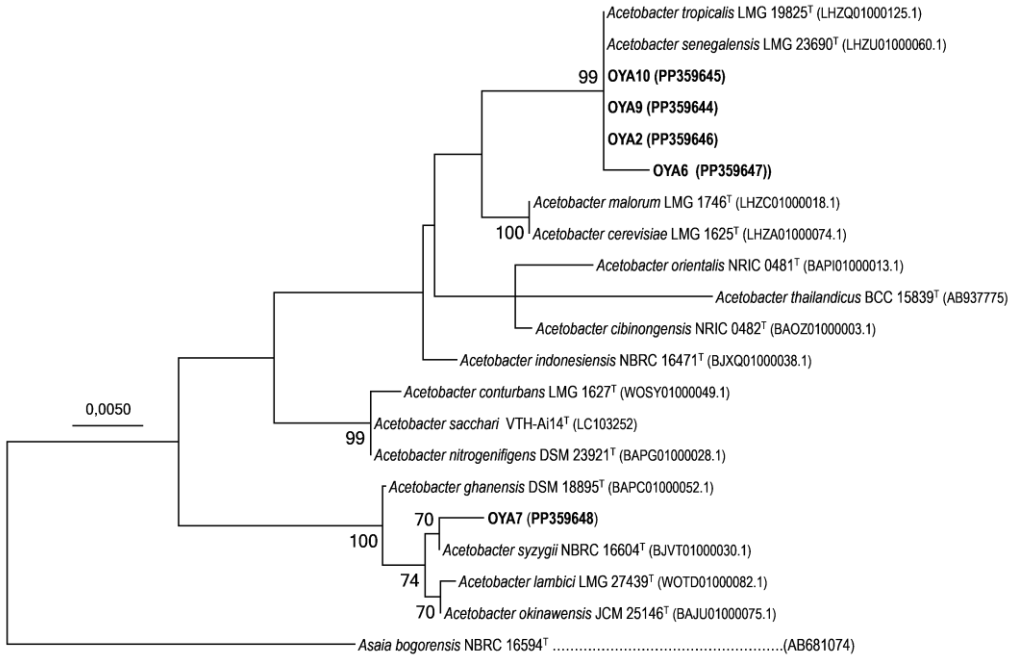

Figure 5. Maximum Likelihood (ML) phylogenetic tree based on 16 S rRNA gene sequences of Acetobacter strains isolated from Cash apple in Côte d'Ivoire and related species of the genus. All the sequences were aligned with MUSCLE and analysis used the T92 +G model, a total of 967 positions, 1000 replicates with bootstrap values higher than 70% shown at branch nodes. The sequence accession numbers are shown within parentheses next to the species name. Scale bar indicates the number of substitutions per site. Asaia bogorensis NBRC 16594^T was used as an outgroup.

The analysis showed that the 16S sequences of isolates OYA10, OYA9 and OYA2 are 100% identical to *Acetobacter tropicalis* strain LMG 19825^T & *A. senegalensis* strain LMG 23690^T, (hereinafter referred to as *A. tropicalis* / *A. senegalensis* species complex) while the isolate OYA6 is 99.7% similar to the type species of these two species **(Figure 5; Table A1)**. It is worth mentioning that the 16S rRNA gene sequence retrieved from the whole genome sequence (wgs) of *A. senegalensis* LMG 23690^T (Accession number LHZU01000060.1) was used in this analysis instead of the 1386 bp-sequence archived under GenBank accession AY883036 (NR_043252) (deposited by Ndoye et al. in 2007 [8], which contained too much degenerate nucleotides (e.g. K, W, N) and may have quality concerns. With the data retrieved from the wgs, *A. tropicalis* type strain LMG 19825^T & *A. senegalensis* type strain LMG 23690^T shared 100% identical 16S rRNA gene sequence. Similar findings have been reported in previous studies for the 16S rRNA gene sequence of these two type species (e.g. [42,45]). The same trend of results was also obtained for two other couples of *Acetobacter* species, namely *Acetobacter malorum* LMG 1746^T (LHZC01000018.1) and *Acetobacter cerevisiae* LMG 1625^T (LHZA01000074.1), as well as *Acetobacter sacchari* VTH-Ai14^T (LC103252) and *Acetobacter nitrogenifigens* DSM 23921^T (BAPG01000028.1) (**Figure 5**), confirming the limited taxonomic resolution of the 16S rRNA gene to resolve the phylogeny of AAB species [16,52]. Although it is still recognized as universal molecular marker for identifying bacterial isolates, the 16S rRNA gene sequences (partial and full-length) were sometimes found to be too conserved for accurately differentiating many bacteria species, including soil and plant-associated bacteria [44,52–54]. As for the AAB species, the use of protein-encoding genes, such as *dnaK*, *groEL* and *hsp60*, has been shown to provide reliable delineations & phylogenies, to reflect overall genome similarities, and to match (digital) DNA–DNA hybridization (DDH) values [16,44,52]. These metrics could be applied to the 05 isolates for further taxonomic analysis. These analyses could help to determine whether these bacteria isolated from fermented cashew apple juice in Côte d'Ivoire, mainly OYA6 and OYA7, may represent novel lineages of acetic acid bacteria.

It is interesting to note that, by Blast analysis, the Ivorian isolates from the *A. tropicalis* / *A. senegalensis* species complex (OYA2, OYA6, OYA9 and OYA10) were found to be closely related to

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many thermotolerant AAB isolated from fermented plant beverages such as *Acetobacter* strain FF82 (Accession number LC462264.1) and *Acetobacter* strain FF35 (Accession number LC462263.1). The Ivorian isolates are also genetically similar to many other strains isolated from different substrates such as palm wine in Nigeria (e.g. *Acetobacter* strain AFF6; Accession number MT158803.1 for OYA6), sugarcane juice in India (e.g. *Acetobacter* strain MA; Accession number MG279707.1 for OYA2, OYA9 and OYA10) or cocoa beans in fermentation (e.g. *Acetobacter* strain At1 ; Accession number MN909151.1 for OYA6). The isolate OYA7, which is genetically related to *A. syzgii* species, appeared also highly similar (99,7%) to diverse *Acetobacter* strains isolated from food-derived sources such as a kefir grain (e.g. *Acetobacter* strain GM1 ; Accession number KX987248). Moreover, *Acetobacter senegalensis* type strain LMG 23690^T which was also isolated from the same geographical region in Africa, i.e. the tropical sub-Saharan Africa, as the isolates OYA2, OYA6 OYA7, OYA9, and OYA10, was shown to be a thermotolerant acetic acid bacterium [8]. This strain was isolated in Senegal more than a decade and used nowadays for industrial vinegar production [11]. All these functional characteristics of the published strains that were found to be closely related to OYA2, OYA6, OYA7, OYA9 and OYA10 prompt us to perform various phenotypic analyses of our *Acetobacter* isolates to assess their ability to serve as good thermotolerant candidates for vinegar production. The data obtained are reported in the next section.

3.1.3. Physiological Characterization of Isolated Acetic Acid Bacteria Strains

The 05 strains isolated and identified as acetic acid bacteria were physiologically characterized to determine the effect of temperature, pH, and alcohol during their growth.

- Effect of temperature

Figure 6 below shows the effect of temperature on the acetic acid bacteria strains growth. It shows that the optimum growth temperature for the 05 strains ranges from 27°C to 37°C. Only strain OYA6 grows up to 40°C. Acetic acid bacteria are mesophilic, with optimum growth temperatures between 25 and 30°C. Above this optimum temperature, essential enzymes such as alcohol dehydrogenase and aldehyde dehydrogenase can be denatured, causing deterioration in the rate of acetification and even inefficiency in vinegar production [55]. However, the isolation of acetic acid bacteria able to grow at high temperatures will be important for the vinegar industry, in order to limit losses linked to bioreactor cooling, particularly in tropical countries [56].

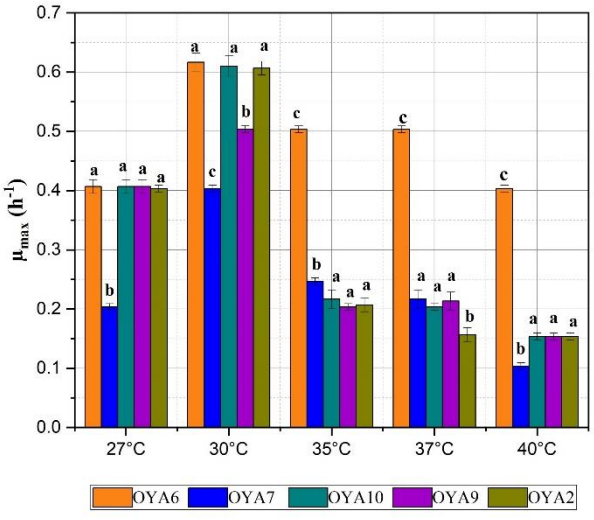

Figure 6. Effect of temperature on isolates growth.

- Effect of pH

The optimum pH for growth was between 5 and 7 (**Figure 7**). These results are comparable to those of Cire et al. [50], who reported a pH of 5-6.5 as the optimum condition for acetic acid bacteria growth. It should be noted that the optimum pH for growth of AAB lies between 5-6.5 [57,58]. In our work conditions, no growth was observed at pH 3 for any of the five (05) strains. On the other hand,

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strong growth was observed at pH 6.5 for all the isolates, but the *Acetobacter* OYA6 strain showed the strongest growth. These results are congruent with the growth partern expected from *Acetobacter* strains in general, although growths at lower or higher pH values have been also reported [59,60].

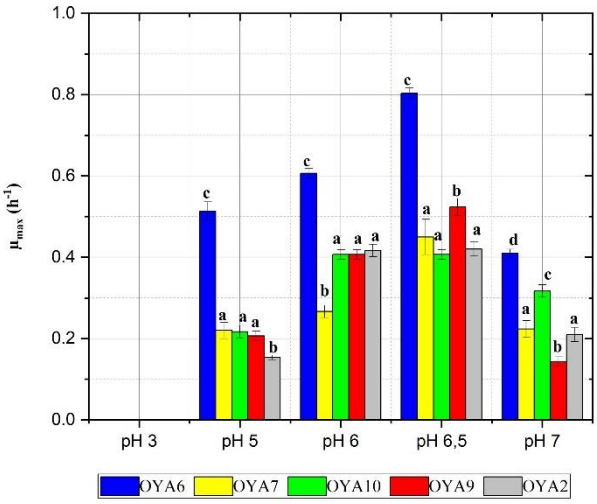

Figure 7. Effect of pH on isolates growth.

- Effect of alcohol

The 05 strains were grown at 5°, 10° and 15° alcohol (v/v) **(Figure 8)**. These results are comparable to those of Sharafi et al. [49], who reported the isolation of bacterial strains able to grow at 5-11% v/v ethanol. This result confirmed the growth of AAB in the presence of low ethanol concentration [61,62] and showed that these strains are resistant to alcohol. This could be of interest to the vinegar industry in the search for ethanol-resistant acetic acid bacteria. Ethanol is the main substrate for acetic acid production. When there is a high concentration of ethanol, it can cause stress for acetic acid bacteria, inhibiting their growth and reducing acetic productivity [63]. However, obtaining high acetic acid concentrations requires the presence of acetic acid bacteria capable of tolerating high ethanol concentrations. In addition, an acetification process using high ethanol concentrations could improve the quality of the vinegar produced [64]

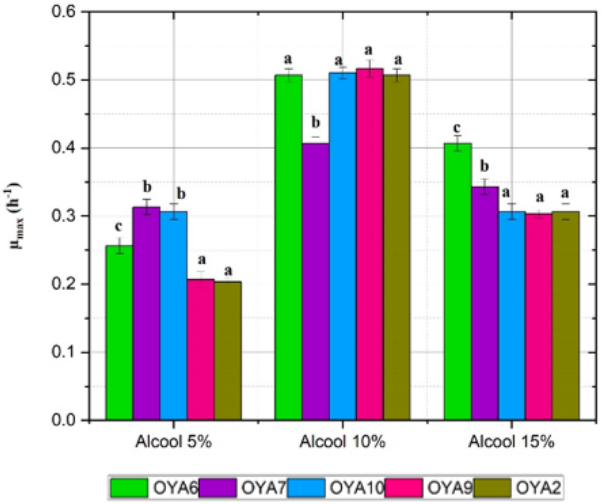

Figure 8. Effect of ethanol on isolates growth.

3.2. Acetic acid Fermentation

Acetic fermentation parameters were monitored and recorded in **Table 4**. All strains of acetic acid bacteria isolated from were fermented cashew juice from Côte d'Ivoire were found to be capable of producing acetic acid [65]. This could be explained by the fact that ethanol was oxidized by acetic

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acid bacteria to produce acetic acid [66]. Acetic acid production evolved progressively over time until day 24 (or 576 hours) of fermentation, with maximum values of 80.67 g.L^{-1} with strain OYA6, and values of 70.26 ± 0.9 g/L; 70.11 ± 1.69 g/L; 68.70 ± 1.51 g/L and 67.22 ± 0.37 g/L, respectively for strains OYA7, OYA2, OYA10, and OYA9 (**Table 4**). Statistical analysis reveals a significant difference in acetic acid production by *Acetobacter* OYA6 compared to strains OYA2, OYA7, OYA9, and OYA10. This is because *Acetobacter* OYA6 also grew well in the fermentation medium. In the present study, *Acetobacter* OYA6 had good physiological properties regarding the temperature, pH, and ethanol stresses. The experiments showed that the type of aeration are important factors in acetic acid production by *Acetobacter* strains, as reported before [49]. We showed that the use of semi-continuous fermentation could contribute to a high final acidity. It should be noted that in both continuous and semi-continuous modes, acetification cycles are achieved by renewal of the initially alcoholic medium inoculated with acetic acid bacteria. This hypothesis is supported by different authors [67] who claim that semi-continuous or continuous fermentation is this fermentation mode used in industry to improve yield. Moreover, Acourene et al. [68] have shown that every 36 hours of continuous acetic fermentation can produce date vinegar grading between 7.2 and 8.5 degrees acetic with an hourly productivity of between 0.458 - 0.488 g/L/hour. Successive analysis by acidity determination in our study reveals the moment of alcohol exhaustion in the medium. Furthermore, the lowest residual alcohol content is attributed to OYA6 (5.18 ± 0.13 g/L). All fermentations obtained had a residual alcohol content of less than 15 g/L standard recommended by current regulations [5]. The kinetics of acetic fermentation revealed the trend in acetic acid concentration. It can be seen that all curves show the same trend, reflecting the oxidation of ethanol by *Acetobacter* isolates as a function of time. These results corroborate those of authors who have carried-out acetic fermentation kinetics [47,49]. The five strains used in this study show better growth and ethanol oxidation capacity. With 92.6 g/L initial ethanol, no overoxidation was observed during acetic fermentation, as overoxidation is unfavorable to vinegar production. According to Saeki et al. [69], overoxidation does not occur when oxidizable ethanol and other carbon sources remain in the culture medium, or when the acetic acid concentration exceeds 4.5%. Thus, overoxidation can be avoided with a high initial ethanol concentration, and with ethanol-tolerant *Acetobacter* strains [56]. Taken togther, we can conclude that our strains have different features suitable for fruit vinegar production.

Table 4. Acetic fermentation parameters with isolated acetic acid bacteria strains.

Strain of acetic acid bacteria	Fermentation time (h)	Initial alcohol (g/L)	Final alcohol (g/L)	Acetic acid produced (g/L)	Q_P (g/L/h)	$Y_{P/S}$ (g/g)	Conversion (%)	η (%)
OYA6	576	92.6 ± 3.43 ^{a}	5.18 ± 0.13 ^{a}	80.67 ± 2.1 ^{c}	0.14	0.92	94.41	67.85
OYA7	576	92.6 ± 3.43 ^{a}	10.34 ± 0.26 ^{b}	70.26 ± 0.9 ^{a}	0.12	0.85	88.83	62.8
OYA10	576	92.6 ± 3.43 ^{a}	10.49 ± 0.55 ^{b}	68.7 ± 1.51 ^{ab}	0.12	0.84	88.67	61.52
OYA9	576	92.6 ± 3.43 ^{a}	10.61 ± 1.69 ^{b}	67.22 ± 0.37 ^{b}	0.12	0.82	88.54	60.28
OYA2	576	92.6 ± 3.43 ^{a}	11.2 ± 1.15 ^{b}	70.11 ± 1.69 ^{a}	0.12	0.86	88.54	62.88

All values are mean ± SD (n = 3); Means with different superscript letters in the same row are significantly different at $p < 0.05$ by Duncan's multiple range test. a > b > c >d.

4. Conclusions

The search for acetic acid bacteria (AAB) in cashew apple juice in this study aimed at isolating and characterizing indigenous microorganisms capable of vinegar production in Côte d'Ivoire. The five (05) *Acetobacter* isolates are affiliated to *A. syzgii* (OYA7) and *A. tropicalis* / *A. senegalensis* species complex (isolates OYA2, OYA6, OYA9 & OYA10), and proved to be promising, as they can produce

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vinegar with, at least, acetic acid content of 67 g/L. These indigenous isolates are resistant to alcohol up to 15° alcohol (v/v) and their optimal growth occurs in acidic conditions, between pH ranging from 5 to 6.5. The results obtained also indicate that the five isolates can grow at moderate temperatures in the range of 27°C to 37°C. Interestingly, only strain OYA6 is capable to grow at 40°C and is also able to produce vinegar with 80 g/L of acetic acid; thus sharing similar features with the reference strain *A. senegalensis* LMG 23690^T (**Table 5**). This thermotolerant strain of AAB was isolated from mango fruits in Senegal more than a decade and used for industrial vinegar production in developing countries, mainly in West Africa [11]. Taken together, the isolate OYA6 appeared as the best candidate that could be used for producing a cashew apple-based vinegar in the Ivorian industrial context. To make this objective effective, additional tests needs to be carried-out in future experiments, including the viability test of these indigenous *Acetobacter* isolates under different stress conditions as well as a fermentation test in an industrial controlled environment (e.g. bioreactor) as required [11,70].

Table 5. Main futures of the 05 *Acetobacter* strains isolated from cashew apple in Yamoussoukro, Côte d'Ivoire, together with the reference strain *A. senegalensis* LMG 23690$^{T.}$

	Isolate / Strain	16S rDNA-based Identification	16S Genbank accession	Stress at 40°C (Temperature)	Stress at 10° & 15° (v/v) of alcohol	Acetic acid produced (g/L)	$Y_{p/s}$ (g/g)
1	OYA2	*A. tropicalis & A. senegalensis* (100%)	PP359646	doesn't grow	resistant	70.11 ± 1.69	0.86
2	OYA6	*A. tropicalis* (99.7%)	PP359647	still grow	resistant	80.67 ± 2.1	0.92
3	OYA7	*A. syzygii* (99.7%)	PP359648	doesn't grow	resistant	70.26 ± 0.9	0.85
4	OYA9	*A. tropicalis & A. senegalensis* (100%)	PP359644	doesn't grow	resistant	67.22 ± 0.37	0.82
5	OYA10	*A. tropicalis & A. senegalensis* (100%)	PP359645	doesn't grow	resistant	68.7 ± 1.51	0.84
6	CWBI-B418[1] (=LMG 23690^T =DSM 18889^T)	*A. senegalensis*	AY883036	still grow	resistant	-	Up to 0.88

[1]Data for the reference strain were obtained from Ndoye et al.[8] and Shafiei et al.[70].

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