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Screening of Thermotolerant Acetic Acid Bacteria Involved in Cocoa Fermentation in Six Major Cocoa Producing Regions in Côte d'Ivoire

Pelepo M. Coulibaly[1*], Bernadette G. Goualié[1], Lamine Samagaci[1], Honoré G. Ouattara[1] and Sébastien L. Niamké[1]

[1]Biotechnology Laboratory, Department of Biosciences, University Félix Houphouët-Boigny, 22 BP 582, Abidjan 22, Côte d'Ivoire.

Authors' contributions

This work was carried out in collaboration between all authors. Author PMC designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors BGG and LS managed the analyses of the study. Authors SLN and HGO managed the literature searches. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: This work intends to screen and to identify thermotolerant acetic acid bacteria with acetic acid production capacity at high temperature in cocoa beans fermentation from six cocoa producing regions of Côte d'Ivoire.
Study Design: Thermotolerant acetic acid bacteria were isolated from cocoa fermentation. These thermotolerant strains were biochemically characterized and tested for the production of acetic acid in culture medium.
Place and Duration of Study: This study was performed in Biotechnology Laboratory, University Félix Houphouët-Boigny (Côte d'Ivoire) from January to November 2017.
Methodology: Several strains of acetic acid bacteria were isolated from the traditional cocoa beans

**Corresponding author: E-mail: pelepomichou@yahoo.fr;*

heap fermentation process occurred in six major cocoa producing regions of Côte d'Ivoire. These isolates were screened to select thermotolerant strains that were able to produce a good amount of acetic acid. Biochemical identification of thermotolerant acetic acid bacteria was carried out on the basis of biochemical characteristics analysis as acid production from ethanol, oxidation of acetate and lactate, ketogenesis from glycerol or mannitol, formation of water-soluble brown pigment, growth on different carbon sources and acid production from sugars and sugar alcohols.

Results: A total of 821 acetic acid bacteria strains were isolated from the cocoa beans heap fermentation of these six regions. Among them, 26 (31.15%) showed growth capacity at 45°C and six (6) grown at 50°C. These 26 strains displayed also acid production capacity at 35°C and at 45°C with acid amount ranged from 1.2 to 24.63 and 0.80 to 1.70 respectively. Biochemical analyses of these thermotolerant strains revealed that the isolates belong to three genera notably *Acetobacter*, *Gluconacetobacter* or *Gluconobacter*. Moreover, all strains were able to grow in medium containing 10% ethanol and to produce acid from various carbohydrates sources. In addition, strain T6HS14 displayed acetoin production capacity while 8 strains were able to produce brown pigment on Yeast extract-Ethanol-Peptone-Glucose medium.

Conclusion: This study highlighted the presence of thermotolerant acetic acid bacteria strains involved in Ivorian cocoa fermentation. Furthermore, some isolates displayed a diversity of technological properties which could be used for the improvement of cocoa fermentation process. These predictors, however, need further work to validate reliability.

Keywords: Acetic acid bacteria; thermotolerance; identification; cocoa fermentation.

ABBREVIATIONS

AA : Acetic acid
AAB : Acetic Acid Bacteria
DHA : Dihydroxyacetone
GYC : Glucose-Yeast extract-Carbonate
LAB : Lactic Acid Bacteria
YEPG:Yeast extract-Ethanol-Peptone-Carbonate
YPM: Yeast extract-Peptone-Mannitol

1. INTRODUCTION

Cocoa beans (*Theobroma cacao L.*) are the basis raw products for chocolate and cocoa powder production [1]. In cocoa post-harvested processing, fermentation is an important step to obtain good cocoa quality because of its great impact on flavor, color and aroma of cocoa products [2]. During cocoa fermentation, microorganisms initiate complex biochemical reactions that allow the production of cocoa and chocolate with desirable organoleptic characteristic. In this process, yeasts oxidize the sugars contained in the pulp into alcohol and breakdown the pulp by pectinolytic enzymes production. Lactic acid bacteria (LAB) are well-known for their ability to breakdown citric acid and to acidify the cocoa beans heap by lactic acid production. *Bacillus sp.* is reported to participate in the degradation of cocoa pulp by enzymes production [3]. Concerning acetic acid bacteria (AAB), they play an important role during cocoa fermentation [4]. Indeed, they oxidize ethanol, initially produced by yeasts into acetic acid which is one of the key metabolite for production of good quality chocolate. Other roles are also assigned to the acetic acid bacteria including the production of flavor molecules (aldehydes, acetoin and esters) involved in chocolate aroma characteristic [5]. Moreover, the genus *Acetobacter* is characterized by the ability to metabolize lactic acid [6]. All these biochemical reactions resulting from the activity of microorganisms involved in the process of cocoa pulp fermentation lead to variation of fermentation conditions such as temperature and pH. Indeed, the conversion of sugars into ethanol by yeast and the conversion of ethanol into acetic acid by acetic bacteria are exothermic reactions which lead to increase of temperature ranging from 45 to 50°C [7]. The acetic acid diffuses into cotyledons and acidifies the beans. This acidification coupled with temperature increase, allow the death of the cocoa seed embryo.

As a reminder, temperature increase and acetic acid production during cocoa fermentation seemed to be crucial to obtain cocoa of good quality [8]. The temperature increase in fermenting heap could also inhibit the growth capacity of microorganisms during cocoa beans fermentation particularly AAB. The optimum temperature of acetic bacteria ranges between 25 and 30°C [9], therefore, strains which could resist to this stress may be considered as valuable microbial starter to obtain fermented cocoa beans of quality.

However, only few studies concerning AAB characterized by this technological property have been investigated for the improvement of cocoa fermentation process [10]. Particularly in Côte d'Ivoire, AAB strains have been selected for their high acetic acid production capacity [11] but the thermotolerance aspect has not been fully investigated.

The aim of this study was to select and biochemically identify thermotolerant AAB with high acetic acid production capacity for their use as microbial starter in order to control cocoa fermentation and solve the issue of variability in cocoa beans quality in Côte d'Ivoire.

2. MATERIALS AND METHODS

2.1 Cocoa Fermentation and Sampling

Cocoa pods, mainly *forastero* variety, were harvested from six regions of high cocoa producing areas (Cavally, Gbôklé, Gôh, Haut-Sassandra, San-Pedro and Tonpki) of Côte d'Ivoire (Fig. 1). Beans were spontaneous fermented during 6 days in "National Floristic Center" of Felix Houphouet-Boigny University in traditionally conditions by heap fermentation using banana leaves as previously described [12]. Fifteen (15) kilograms of cocoa beans in heaps were covered and left to ferment. Beans fermentation was conducted during 6 days and samples were taken every 12 hours since the start of the fermentation. Each 12 hours of fermentation, pH and temperature were recorded and 200 g of beans were collected for biochemical and microbiological analysis. The temperature was measured by inserting a thermometer (Alla, France) in the middle of the fermenting cocoa beans mass. The pH of the pulp was also recorded by using a pH meter (Eutech instrument, Singapore). Standard buffer solution of pH 4.0, 7.0 and 10.0 was used as reference for calibration.

2.2 Biochemical Analysis

To determine the acid amount in cocoa beans pulp, 10 g of the collected sample were placed in a flask containing 100 mL of distilled water. The extract was filtered through a Whatman filter paper and acid yield was quantified by titration with NaOH (0.1 N) [13]. Acid production was calculated using the following formula:

$$\text{Acidity (meq/100 g of beans)} = (N \times V_1 \times 10^4) / (m \times V_0)$$

2.3 Isolation and Enumeration of AAB

The isolation of AAB was performed immediately after sampling as previously described [11]. Briefly, 225 mL of peptone solution were added to 25 g of beans in a sterile Stomacher bag and then homogenized for 5 minutes. After this, 1 mL of the homogenate samples was 10-fold diluted in trypton salt buffer, from which 0.1 mL of aliquots was plated on Duthathai medium [glucose (0.5%), yeast extract (1%), casein peptone (1%,) $CaCO_3$ (1%) and agar (1,5%)]. Bromocresol green (0.0016%) and nystatin (50 µg/ mL) were also added to monitor pH variation and inhibit fungal growth respectively. The plates were incubated at 30°C for 5 days. After incubation, acetic acid bacteria isolates were selected based on the presence of a clear halo around colonies and identified based on following tests: Gram staining, catalase and oxidase. After enumeration, colony count was expressed as cfu per gram of cocoa pulp-bean mass. The strains isolated were kept at -20°C in Luria Bertani medium supplemented with glycerol 20% for further studies.

2.4 Screening of Thermotolerant AAB

The screening of thermotolerant AAB isolates was carried out according to [14] method with some modifications. Briefly, 10 mL of Yeast extract-Ethanol-Peptone-Glucose (YEPG) broth [glucose (1%), yeast extract (1%), casein peptone (2%), supplemented with ethanol (4%)] were inoculated with colonies of AAB and incubated at 30°C for 24 hours. After incubation, 100 µL of this pre-culture [with OD (optical density) = 0.5 at 600 nm] were used to inoculate 10 mL of YEPG broth. The cultures were then incubated at different temperatures (35; 40; 45, 50; 55 and 60°C) during 6 days. After incubation, AAB strains with growth capacity at different temperatures were selected on plates containing YEPG agar supplemented with bromocresol green and incubated at 30°C for 3 days.

2.5 Acetic acid Production by Thermotolerant AAB

Acetic acid production was carried out according to the method described by [15]. The thermotolerant isolates were grown in liquid medium supplemented with ethanol in order to assess their ability to produce acetic acid from ethanol. From pure colonies obtained on agar

plates, a pre-incubated culture of 24 hours was done in the carbonate broth (0.05% glucose, 0.3% peptone, 0.5% yeast extract and 4% ethanol). The pH of the medium was adjusted to 6 before sterilization at 121°C for 15 minutes. Then, 1 mL of this pre-culture (OD $_{600\ nm}$ = 0.5) was used to inoculate the same carbonate broth contained in 150 mL vials. This broth was

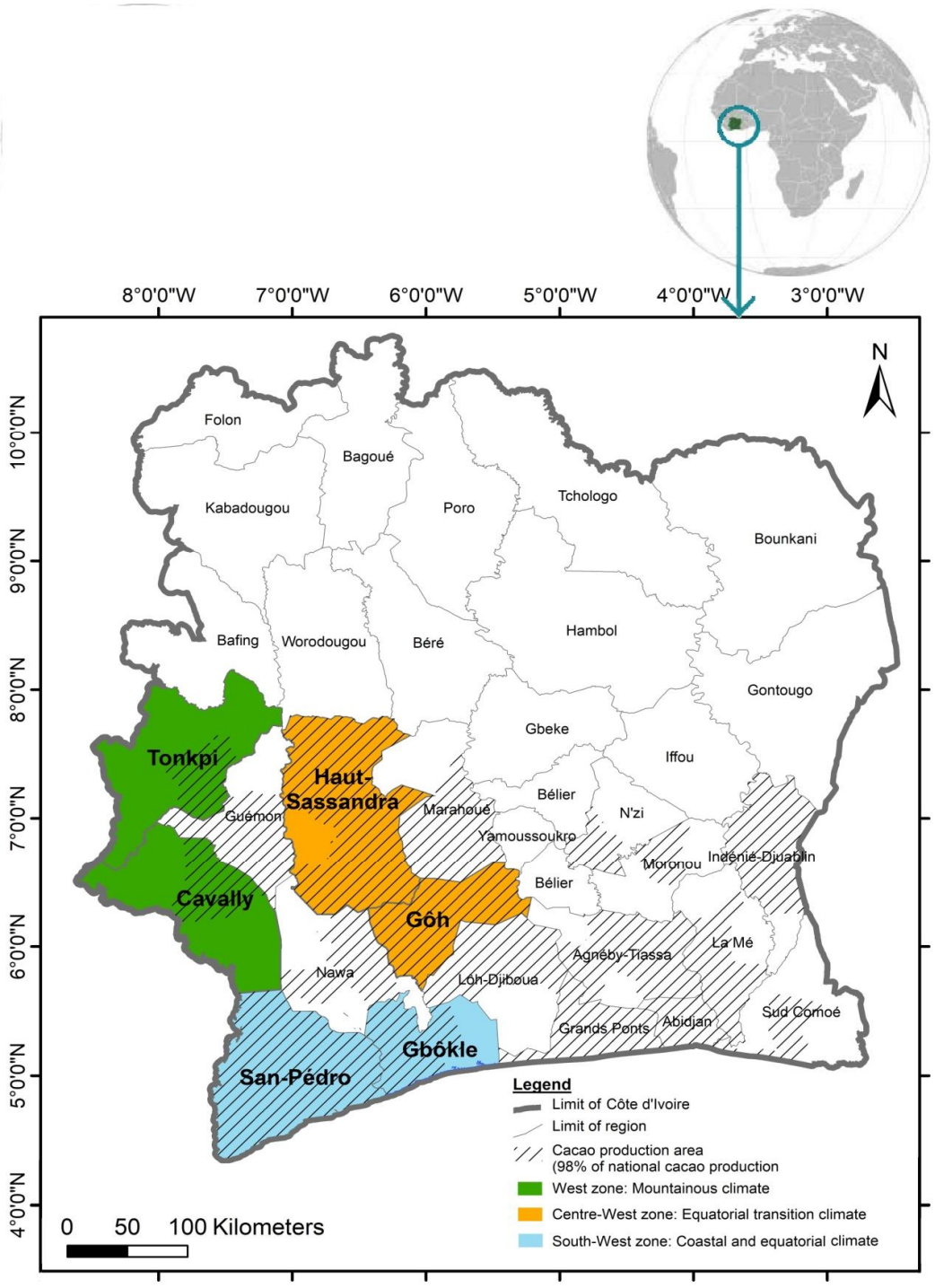

Fig. 1. Areas of cocoa pods sampling

subsequently incubated at 30°C for 10 days under agitation at 130 rpm. At the beginning of incubation and each 24 hours, 10 mL of culture medium were sampled for the measurement of pH, absorbance and amount of acid produced. The absorbance was measured at 600 nm using a spectrophotometer (Pioway UV 752, China). The pH of culture medium was determined by using pH meter and acid amount (expressed in grams of acetic acid per liter) was determined as described previously. Moreover, acid production capacity of each tested strains was evaluated at different temperatures (35, 40, and 45°C) under agitation and acid amount in the medium was determinate as previously described.

2.6 Phenotypic Characteristics of Thermotolerant Acetic Acid Bacteria

AAB showing good growth and high acid production under high temperature (45°C) were selected for biochemical characterization. The first step of this characterization was the ability to oxidize both acetic and lactic acids. Briefly, after sterilization of culture broth at 121°C during 15 minutes and cooled to 45°C, acetic or lactic acid was added (with final concentration in the culture medium at 1% and 0.1% respectively) as sole carbon source. Then, 3 mL of this medium were inoculated with 100 µL of pre-culture (OD 600nm = 0.5) and incubated at 30°C for 10 days in aerobic conditions. The capacity of strains to metabolize the carbon source was assessed by the change of medium colour from yellow to green, comparatively to the negative control. Purified cultures were streaked onto carbonate agar (0.05% glucose, 0.3% peptone, 0.5% yeast extract, 1% $CaCO_3$, 1.2% agar supplemented with 4% ethanol) plates to confirm acetic acid production by formation of transparent zones around colonies. Next, other biochemical tests including acid production from sugars, growth capacity in medium containing 10% ethanol, nitrate reduction, ketogenesis from glycerol and mannitol, acetoin production and formation of brown pigment were performed. Acid production from sugars particularly glucose, fructose, sucrose, galactose, xylose, trehalose, mannose and glycerol was investigated at 30°C during 7 days in broth consisting of 0.5% yeast extract and 0.002% of bromocresol green supplemented with 1% of tested sugar [16]. Growth capacity in presence of high concentration of ethanol was evaluated in liquid medium containing 0.05% glucose, 0.5% yeast extract, 0.3% casein peptone supplemented with 10% ethanol [17]. The nitrate reduction to nitrite capacity was evaluated during 10 days at 30°C in liquid medium containing peptone (1%) and potassium nitrate (0.2%). After incubation, nitrate reduction was revealed by addition of Griess (Sigma-Aldrich, USA) reagent [18]. The ability to assimilate mannitol was investigated by inoculating the Yeast extract-Peptone-Mannilol (YPM) medium (2.5% mannitol; 0.5% yeast extract and 0.3% peptone) with 100 µL of pre-culture (OD 600 nm = 0.5) of tested strains. Incubation was done at 30°C for 10 days [16]. Ketogenesis from glycerol or mannitol was performed by the methods previously described by [19]. Pigment production capacity was detected in Glucose-Yeast extract-Peptone (GYP) or YEPG agar medium by change of medium color from beige to brown after 10 days of incubation [20]. The acetoin production test was carried out according to Voges-Proskauer reaction previously described [21].

2.7 Statistical Analysis

All experiments were performed in triplicate, and the mean and standard deviation were calculated. Significant differences were detected using the test of Tukey with a 0.05 threshold, using the IBM SPSS Statistica to determine any significant difference between the experiments.

3. RESULTS

3.1 Cocoa Fermentation Parameters

The temperature inside the fermenting cocoa mass varied between 28 to 29°C at the start and reached a peak at 40 to 47°C after 24 to 72 hours. Then, a gradual decrease of temperature was observed, dropping at 28 to 32°C at the end of fermentation (Fig. 1). However, among all studied regions, the high peak of temperature was obtained in Cavally fermenting cocoa heap after 48 h of the process while the lower peak of temperature was recorded in San-Pedro after 24 h of fermentation. Moreover, maximal temperature was reached at different time for the six study areas. Indeed, Gbôklè and San Pedro reached this value after 24 h, Haut-Sassandra and Cavally after 48 h, and Gôh and Tonkpi reached a peak of temperature after 72 h of fermentation (Fig. 2).

Concerning the pH values, the same evolution was observed in all cocoa fermentations. Indeed, at the beginning of the fermentation, the pH values ranged from 3.4 to 4.0 and decreased to approximately pH 2.9 to 3.6 during the first 24 hours. Then, an increase of pH values was

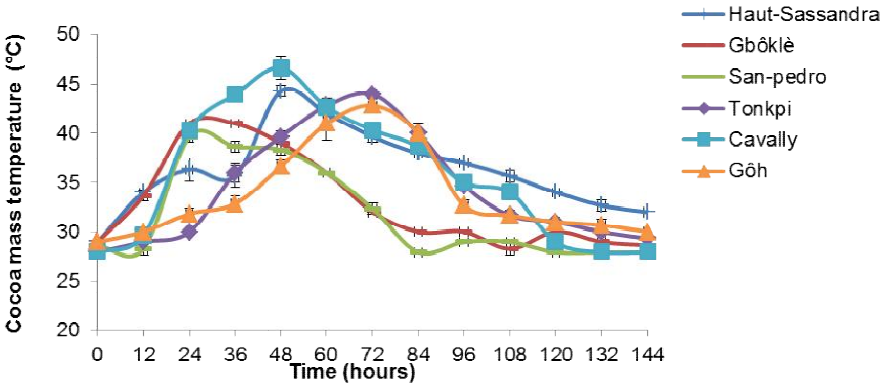

Fig. 2. Evolution of cocoa mass temperature during cocoa heap fermentation

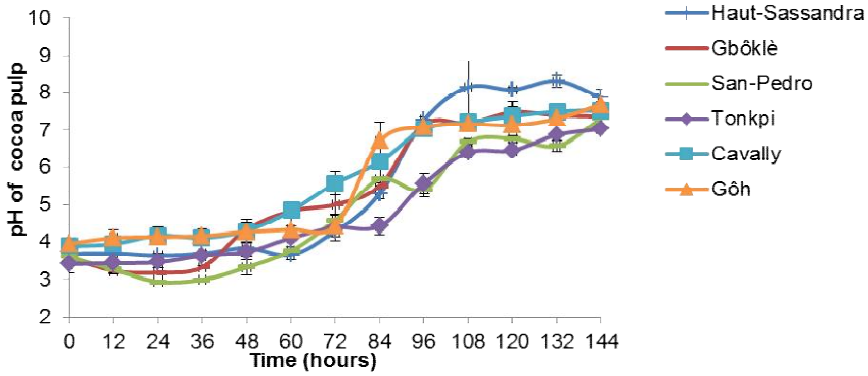

Fig. 3. Evolution of cocoa pulp pH during cocoa heap fermentation

observed until the end of the process with values ranged from pH 7.0 to 7.9 after 144 h. However, Haut-Sassandra region displayed a pH value of 8.1 after 108 hours before decreasing to pH value 7.9 at the end of the process (Fig. 3).

The titratable acidity of each fermenting cocoa heap was also monitored during the fermentation process. At the start, the results indicated that pulp titratable acidity ranged from 0.60 to 1.87 meq/100 g then rapidly increase to reach maximal values ranging from 2.07 to 4.43 meq/100 g of beans. Afterwards, a drastic decrease of acidity was observed at the end of the process. Five of the tested regions displayed the maximum of titratable acidity after 24 hours of fermentation while Gôh region presented its maximum acidity production after 12 h of the process (Fig. 4).

3.2 Dynamic of AAB Growth during Cocoa Fermentations

The dynamic of AAB growth during cocoa fermentations is shown in Fig. 4. The same evolution of AAB population was observed for the six studied regions. Indeed, at the beginning of the process, the cultivable amount of AAB population ranged from 4.90 to 6.56 (Log_{10} cfu g^{-1} of cocoa beans) and increase to reach 7.74-8.52 Log_{10} cfu g^{-1} at 48 to 72 h of fermentation. After, the size of AAB population decreased progressively until the end of the fermentation process with bacterial count estimated to 4.11 to 7.02 Log_{10} cfu /g of cocoa beans (Fig. 4). However, the maximum bacterial load was displayed at 48 h of fermentation for San-Pedro region while for the other areas; maximum amount was reached after 60 h (Fig. 5).

3.3 Selection of Thermotolerant AAB and Acetic Acid Production Capacity

Today, a very few data regarding thermotolerant AAB isolates implicated in cocoa fermentation have been reported [11]. The increase in temperature is a common phenomenon of cocoa fermentation process; strains implicated in this process must be able to grow under these

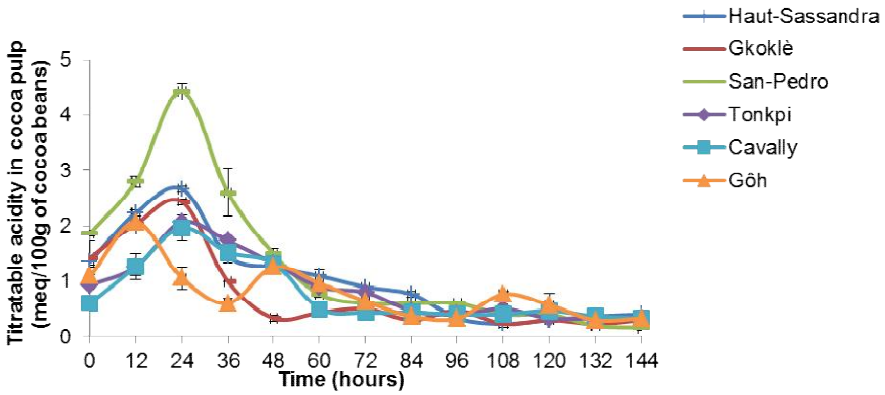

Fig. 4. Evolution of cocoa mass acidity during cocoa heap fermentation

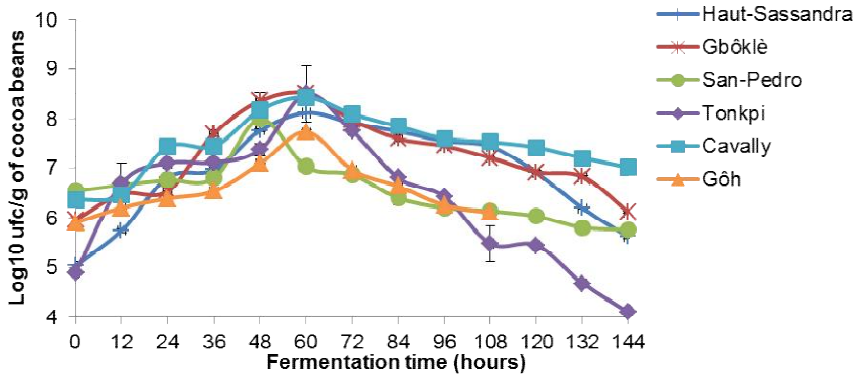

Fig. 5. Growth dynamic of AAB during cocoa heap fermentation

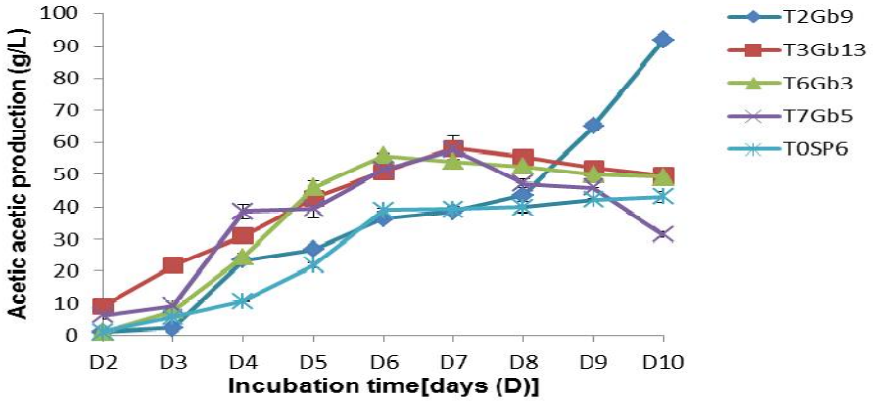

Fig. 6. Acetic acid production profiles at 30°C of five thermotolerant AAB strains isolated from cocoa fermentation

conditions. A total of 821 strains were isolated from the six regions. Among these strains, 810 were able to grow at 35°C and 117 at 40°C (Table 1). Moreover, 26 displayed growth capacity at 45°C and six (6) at 50°C. These thermotolerant strains showed a good growth from 30 to 35°C. At 35 to 45°C, the growth was variable in accordance with strains (Fig. 6). The isolate considered as thermotolerant strains (at 45°C) were only isolated in San-Pedro (10 isolates), Gboklé (13 isolates) and Haut-Sassandra (03 isolates) (Table 1).

Table 1. Growth of acetic acid bacteria at different temperatures

Temperature (°C)	Number of isolates						Total
	Regions						
	Gboklé	Haut-Sassandra	San Pédro	Gôh	Cavally	Tonpki	
50	2	2	2	0	0	0	6
45*	**13**	**3**	**10**	**0**	**0**	**0**	**26**
40	39	29	49	0	0	0	117
35	136	92	186	132	106	158	810
30	136	100	188	132	106	159	821

**: Isolates selected as thermotolerant AAB at 45 °C*

Table 2. Acetic acid production by acetic acid bacteria at different temperature

Isolates	Acetic acid production (g/L)			
	Temperature (°C)			
	30	35	40	45
T0SP1	78,30±1,37^{a}	3,30±0,30^{k}	1,60±0,17^{b}	1,20±0,30^{bcd}
T0SP2	45,00±1,50^{g}	7,20±0,30^{i}	1,50±0,30^{bcd}	0,90±0,00^{de}
T0SP4	37,60±0,87^{j}	1,20±0,30^{m}	1,10±0,17^{ef}	0,80±0,17^{e}
T0SP5	43,60±1,22^{gh}	2,30±0,17^{l}	1,20±0,00^{def}	0,80±0,17^{e}
T0SP6	39,00±0,50^{j}	13,80±0,82^{e}	1,20±0,00^{def}	1,20±0,00^{bcd}
T10Gb1	19,80±0,30^{m}	1,50±0,00^{lm}	1,30±0,17^{cde}	1,10±0,17^{cde}
T10SP5	50,70±0,30^{de}	10,20±0,60^{g}	1,80±0,00^{ab}	1,40±0,17^{bc}
T11SP9	47,30± 0,56^{f}	24,63±1,17^{a}	1,80±0,00^{ab}	1,70±0,17^{a}
T1SP3	49,80±0,20^{e}	4,80±0,72^{j}	1,30±0,34^{cde}	1,20±0,00^{bcd}
T2Gb9	38,70±1,10^{j}	1,20±0,00^{m}	1,20±0,00^{def}	0,90±0,00^{de}
T2HS4	34,20±1,80^{k}	1,50±0,00^{lm}	0,90±0,00^{f}	0,90±0,00^{de}
T3Gb13	50,70±1,37^{de}	1,70±0,10^{lm}	1,50±0,00^{bcd}	0,90±0,30^{de}
T4Gb2	35,30±2,12^{k}	7,50±0,50^{i}	1,50±0,30^{bcd}	1,20±0,00^{bcd}
T4HS15	38,63±1,68^{j}	15,00±0,60^{d}	1,50±0,00^{bcd}	0,90±0,00^{de}
T6Gb13	48,00±0,50^{f}	18,90±1,49^{c}	1,50±0,00^{bcd}	1,20±0,30^{bcd}
T6Gb3	55,80±0,60b	1,50±0,30^{lm}	1,20±0,00^{def}	1,20±0,00^{bcd}
T6Gb4	41,50±0,50^{i}	1,80±0,00^{lm}	1,80±0,00^{ab}	1,20±0,00^{bcd}
T6HS14	18,30±1,50^{m}	1,80±0,00^{lm}	1,20±0,00^{def}	0,90±0,00^{de}
T7Gb11	52,50±0,82^{d}	8,70±0,80^{h}	2,00±0,17^{a}	1,40±0,17^{bc}
T7Gb16	43,10±0,40^{hi}	1,60±0,17^{lm}	1,40±0,17^{cde}	1,50±0,00^{ab}
T7Gb5	51,50±0,46^{de}	11,80±0,79^{f}	1,6±0,17^{b}	1,3±0,17^{bc}
T8Gb13	52,20±0,70^{d}	21,90±0,30^{b}	1,5±0,30^{bcd}	1,2±0,00^{bcd}
T8Gb14	29,10±0,70^{l}	3,30±0,30^{k}	1,6±0,17^{b}	1,2±0,30^{bcd}
T8Gb4	34,80±0,30^{k}	4,50±0,30^{j}	1,4±0,17^{cde}	1,4±0,17^{bc}
T8SP6	63,60±0,60^{b}	12,00±0,30^{f}	1,60±0,17^{b}	1,2±0,00^{bcd}

Values in the same column with different superscripts are significantly different from one to another (P=.05) and expressed as mean ± S.E.M = Mean values ± Standard error of means of three experiments; Code of isolates (TiXj): T_i = time of sampling, X = Region studied [(Gb) = Gboklè, (HS) = Haut-Sassandra, (SP) = San-Pedro] and j = number of isolate.

3.4 Biochemical Identification of Thermotolerant AAB strains

Acetic acid bacteria strains were identified as Gram negative, short rod-shaped, catalase positive, oxidase negative and strict aerobic. These bacteria formed clears haloes around the colonies on the medium containing ethanol because of acetic acid production from ethanol. All the 26 isolates, which growth capacity at high temperature, were identified by biochemical tests. Among these strains, 24 were able to further oxidize acetic and lactic acid into CO_2 and H_2O and then classified as *Acetobacter* or *gluconoacetobacter* while 2 strains were not able to metabolize lactic acid; these strains belong to *Gluconobacter* genus. Some others tests below could permit to perform biochemically identification (Table 3).

Table 3. Biochemical characteristics of thermotolerant isolates from cocoa fermentation of Côte d'Ivoire

Properties / Code of isolates	T4Gb2	T7Gb5	T8Gb4	T0SP1	T2Gb9	T8SP8	T7Gb16	T8GB14	T8SP6	T2HS4	T6HS14	T10SP5	T4HS15	T1SP3	T7Gb11	T11SP9	T3Gb13	T0SP2	T0SP4	T6Gb3	T6Gb4	T6Gb13	T8Gb13	T10Gb1	T0SP5	T0SP6
Gram	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth in :																										
EtOH agar	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
EtOH (10%) medium	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidation of :																										
Ethanol to AA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acetate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+
Lactate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+
Ketogenesis from :																										
Glycerol	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	-	-	-
Mannitol	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	+	-	+	-	+	+	-	+	-
Nitrate-reduction	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Mannitol assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid production from:																										
Fructose	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	-	-	+	+	-	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	-
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Glycérol	+	+	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	+	-	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	-	+	-	-	-	-	-	-	+	+	+	+	-	-	-	+	-	-	+	+	+	-
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
Xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+
Ethanol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Voges-Proskauer reaction	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pigment production																										
on GYC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
on YEPG	-	-	-	-	-	+	+	-	+	-	-	-	-	+	+	-	-	-	-	+	-	+	+	-	-	-

Note: *-= negative and + = positive; Code of isolates (TiXj) : T_i = time of sampling, X = Region studied [(Gb) = Gboklè, (HS) = Haut-Sassandra, (SP) = San-Pedro] and j = number of isolate*

4. DISCUSSION

The variation of temperature during the cocoa fermentation process was also reported in other studies [22]. The increase of the temperature is due to the growth of cocoa microflora, notably yeasts activities which consist in the conversion of pulp sugars into ethanol and acetic acid bacteria which oxidize ethanol produced by yeasts to acetic acid. Therefore, the decrease of cocoa mass temperature during the end hours of fermentation process could be explained by a decline of the cocoa microflora activities. The results of pH during cocoa fermentation were similar to those recorded in different traditional spontaneous cocoa fermentations in Côte d'Ivoire [12,23]. Moreover, for some of the studied regions, the pH becomes neutral or alkaline at the end of the fermentation process. This phenomenon was generally observed in Côte d'Ivoire and in Malaysia [24,25]. The high acidity observed at the beginning of fermentation process is due to the presence of citric acid in mucilage of fresh cocoa beans and to organic acids (mainly acetic acid and lactic acid) produced by lactic and acetic acid bacteria [26]. Also, the diffusion of produced organic acids inside the cotyledons strongly contributes to the increase in pH value as the decreased acidity of fermenting heap [27].

The variations in fermentative parameters were similar to those of many authors in other countries, but differences were observed from a region to another [28]. This difference of cocoa fermentation parameters between the regions could be linked to the nature of the cocoa fermentation microorganisms that depend to the climate conditions of the studied regions. Indeed, the regions of Haut Sassandra and Gôh are located in the Centre-West zone were the climate is equatorial transition climate with 950-1600 mm/year rainfall. For San-Pedro and Gboklé belonging to the South-West zone, the climate is coastal and equatorial with 1300-2400 mm/year. For the West zone (Cavally and Tonpki regions) the climate is mountainous with 1500-2200 mm/year rainfall [29]. The increase of cocoa mucilage pH values, as well as the decrease of the acidity of the fermenting heap, are mainly due to the assimilation of initial citric acid by LAB [30] and yeasts species involved in the fermentation process [8].

The enumeration of AAB population during the different cocoa fermentations indicated that these microorganisms were present during all the process with a maximum growth obtained between 48 and 72 hours. These results were different from those reported by [31]. In fact, these authors did not detect AAB at the beginning of fermentation. These findings confirm that the AAB species involved in the cocoa fermentation are specific to each cocoa producing region, thus leading to a variability of cocoa beans quality from one region to another. Moreover, the presence of acetic acid bacteria at the beginning of fermentation process could indicate the ability of these strains to grow in the presence of high acid and low oxygen levels probably due to adaptation phenomenon to cocoa fermentation conditions. This capacity could be an interesting technological property for these AAB strains to improve cocoa fermentation process and beans quality. Indeed, AAB is essential for fermentation process by the production of acetic acid which contribute strongly to beans biochemical compounds modification and production of desirable chocolate flavor. In this context, acetic acid production at beginning of this process will contribute rapidly to death of embryo and consequently to the reduction of cocoa fermentation duration which is one of the major objective in cocoa beans quality improvement. Also, the variation of the appearance time of the growth peaks and their sizes could be related to variability in the mucilage composition. This is due to the period of microorganisms adaptation to conditions prevailing in cocoa fermentation and to the microbial variability observed between the regions too [32,33]. The differences observed in the variation of pH values, temperature, and titratable acidity between the six regions could be related to the different microorganisms including that of acetic acid bacteria involved in cocoa fermentation process [34]. The delays observed between maximum temperature and growth could be explained by the microbial variability existing in the six cocoa producing regions [33].

For all tested strains, the resistance to high temperature decreased with increasing the incubation temperature. Similar effects of increasing of temperature on AAB growth capacity have been reported [35]. However, the ability of these strains to grow at high temperatures (45 or 50°C) is greater than that recorded in AAB strains isolated from fermenting fruit and rice vinegar for which the highest growth temperature ranged from 37 to 41°C. This difference could indicate that thermotolerance capacity result undoubtedly from an adaptation of

fermentation conditions [36]. Indeed, AAB has been reported to exhibit phenotype instability, which can occur by either, temporal acclimation or heritable adaptation [37].

As acetic acid is a key metabolite to obtain good cocoa quality, capacity of these strains to produce acid in culture medium under high temperature was evaluated. Usually, AAB isolates which were able to grow at 37 to 41°C were regarded as thermotolerant strains [38]. In this study we selected only those 26 strains with growth capacity at 45°C for evaluation of acid production capacity because the maximal temperature during cocoa fermentation ranges from 45 and 50°C. Among the 26 isolates with growth capacity at high temperature, 5 strains displayed a good capacity to produce acetic acid at 35°C (Table 2). These 5 isolates kept 35 to 50 % of production capacity at 30°C (Fig. 5). This technological property could be used in the vinegar industry. However, it appears that the in vitro acidifying activity of thermotolerant, AAB strains decreased considerably at 40 and 45°C. These results suggest that the yield of acetic acid necessary for complete fermentation should be supplied by AAB strains before the fermenting mass reach these temperature [12]. Also, this decline in acetic acid production could depend on alcohol evaporation and acetic acid losses occurred if too high temperature is used [39]. Acidification is one of the most relevant properties in cocoa fermentation since production of acetic acid during cocoa fermentation allows the development of chocolate flavor and aroma [40]. In this context, a beneficial effect on quality product may occur when fermenting cocoa beans include a population of AAB presenting a high capacity of acidification. Therefore, the Gboklè and San Pedro regions, which displayed a high rate of acid-producing thermotolerant strains, could lead to the best cocoa beans quality at the end of fermentation process. The acetic acid production during cocoa fermentation goes with an increase in temperature of fermenting mass. So, making research to find thermotolerant acetic acid bacteria involved in cocoa fermentation could be helpful in finding a potential microbial starter for cocoa fermentation improvement leading to good flavor of chocolate

Moreover, 25 strains displayed growth capacity in medium containing 10% ethanol. The same results were reported by [11]. In generally, AAB strains exhibit three growth phases in ethanol-containing medium; an ethanol oxidation phase, acetic acid resistance phase, and acetate over oxidation phase [41]. In the ethanol oxidation phase, cells oxidize ethanol to acetic acid via acetaldehyde with membrane-bound alcohol and aldehyde dehydrogenases linked to the respiratory chain. In the acetic acid resistance phase, cells resist auto-produced acetic acid using several mechanisms as proton motive force-dependent and ABC-transporter-like efflux pump system for acetic acid, production of pellicle polysaccharide and additionally by changes in membrane lipid composition [42]. In the over oxidation phase, cells assimilate acetate by oxidizing it to CO_2 via the tricarboxylic acid cycle [43].

In all case, these isolates could be used as starters in fermentation for the standardization of cocoa quality since some authors [44] used some AAB strains tolerating only 6% ethanol concentration as starter in cocoa fermentation. The feature of alcohol tolerance (10%) presented by AAB strains is relatively more elevated; suggesting that a high yield of alcohol from yeasts [4] could not be susceptible to limit the growth of AAB during the process. However, effect of this high alcohol concentration could be evaluated on acid production of these strains before use as starter in cocoa fermentation process. Nevertheless, the growth capacity in medium containing 10% ethanol coupled with acid production capacity under high temperature (35°C) could be an interesting performance for vinegar industry since some AAB strains widely used for vinegar production lose this activity in this condition (ethanol 10%) [45]. Thus, most agricultural activities in Côte d'Ivoire which generate large quantities of products unexploited (banana or others fruits rich in organic substances) which constitute a substantial economic loss and a real environmental issue could be converted in vinegar by using these strains to avoid human and animal health risks [46].

Among the tested strains, 17 were able to produce acid from fructose while 14 displayed this capacity from glucose and 8 from sucrose. The ability of acetic bacteria to breakdown these three sugars is not surprising since according to [47], AAB strongly oxidize various carbohydrates including sugars, sugar alcohols, and ethanol with the production of acetic acid as the major end product. This special type of metabolism differentiates them from all other bacteria [47]. Generally, the cocoa pulp constituting the substrate of cocoa fermentation contains mainly glucose, sucrose and fructose which are to be

fermented into ethanol by yeast and at term, into acetic acid by acetic acid bacteria [4]. These essential reactions allow to development of specific flavor and aroma of the cocoa beans and chocolate [48]. Moreover, degradation of various sugars could allow to the reduction of cocoa fermentation duration and production of a various aldehydic, ketogenic and other volatile compounds which strongly impact cocoa beans final flavor [20].

In addition, presence of brown pigment was detected for 8 isolates. These strains could belong to genera *Gluconobacter* or *Gluconoacetobacter* while the strain T6HS14 which was able to produce acetoin belong probably *Acetobacte*r genus. The strain T6HS14 could improve cocoa beans quality by using as starter in cocoa fermentation assay since production of acetoin from lactate has double advantage in cocoa fermentation process. The first one is to reduce the lactate concentration which is undesirable in cocoa beans and the second one is to contribute to the formation of the cocoa beans aroma [49].

A total of 3 genera namely *Acetobacter, Gluconobacter* and *Gluconacetobacter* were revealed according to the biochemical tests results. Moreover, the tested strains were dominated by genera *Acetobacter* and *Gluconacetobacter*. *Acetobacter* and *Gluconobacter* genera are frequently isolated from cocoa fermentation [50] in the world and seemed to be mainly AAB genera which are adapted to fermentations conditions. In general, Acetic acid bacteria are classified into two groups, *Acetobacter*/ *Gluconacetobacter* and *Gluconobacter,* based on their abilities to over oxidize acetate or lactate and the positions of their flagella [51]. Moreover, *Acetobacter* and *Gluconacetobacter* are well known to their high acetic acid production capacity more than *Gluconobacter* strain frequently used in other biotechnological applications such L-ascorbic acid (vitamin C) and DHA production [47]. Therefore dominance of these *Acetobacter* and *Gluconacetobacter* in AAB tested could be interesting since high acetic acid level in the cocoa fermenting heap is desirable.

4. CONCLUSION

Twenty six acetic acid bacteria with growth capacity at 45°C, involved in cocoa fermentation of six major cocoa producing region of Côte d'Ivoire, have been isolated. Moreover, six (6) of them were able to grow at 50°C. These isolates displayed high acid production capacity at 30 and 35°C but this performance drastically decrease at 45°C and is totally inhibited at 50°C. These thermotolerant AAB isolates were constituted of three genera namely *Acetobacter*, *Gluconobacter* and *Gluconoacetobacter*. In addition, all thermotolerant strains displayed grow capacity under high ethanol conditions and some isolates showed various compounds (acetoin, ketones and brown pigment) production capacity which could contribute to merchant beans quality as formation of the aroma in cocoa fermentation. This study outlined a presence of thermotolerant AAB involved in Ivoirian cocoa fermentation and showed the possibility to use among them some strains as potential starter for improvement of cocoa fermentation. However, their ability to grow and produce acid under fermentative stress conditions must be evaluated before selecting the most valuable strain as potential starters. Further, a concise molecular identification would be perform on these thermotolerant strains because phenotypic characterization of the strains only reveal physiological differences.

COMPETING INTERESTS

Authors have declared that no competing interests exist

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