

Analysis of some functional properties of acetic acid bacteria involved in Ivorian cocoa fermentation

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ABSTRACT

Objective: To investigate some functional properties of acetic acid bacteria (AAB), involved in Côte d'Ivoire cocoa fermentation.

Methods and results: Six day heap fermentation on banana leaves was conducted at farm level and AAB growth was monitored during this process at 24 h interval by numeration on plate agar. Functional properties such as acid production, thermotolerance, resistance to alcoholic stress carbon metabolism and over oxidation of acetic acid were conducted on either plate agar or liquid medium. During fermentation, AAB reached maximum load at 72 h, corresponding to a high temperature 44 °C of fermenting mass and a pH round 4.45. All the 86 strains isolated proved to be thermotolerant with ability to grow up to 45 °C and 63 % of them showed particularly high tolerance to alcohol up to 15 %. The temperature between 30 and 40 °C and alcohol at high concentration (15 %) did not notably affect acid production. However, beyond 40 °C, the acidification ability of the most acidifying strains was strongly affected. A high proportion of strains 80 %, were able to further oxidize acetic acid into water and carbon dioxide and subsequently belong to *Acetobacter* genus. The strains displayed a poor carbon metabolism profile with the capacity to utilize only glucose as carbon source.

Conclusion and application of findings: This study shows that Ivorian AAB fermenting mass possesses some technological traits potentially useful for their utilization as valuable starters in cocoa fermentation.

Key words: Acetic acid bacteria, alcohol-tolerance, cacao, fermentation, thermotolerance.

INTRODUCTION

Cocoa fermentation is a crucial step in the process of transformation of cocoa into chocolate (Biehl *et al.*, 1993; Schwan and Wheals, 2004). This process involves mainly yeasts and bacteria including *Bacillus*, acetic acid bacteria (AAB) and lactic acid bacteria (Ardhana and Fleet, 2003; Schwan and Wheals, 2004; Ouattara *et al.*, 2008; Papalexandratou *et al.*, 2011). Microbial activity during cocoa fermentation causes various

biochemical reactions, which influence greatly the quality of fermented dried cocoa bean and chocolate (Schwan, 1998; Jinap *et al.*, 2003).

Although the entire physiological role of microorganisms involved in cocoa fermentation is not completely elucidated, it is well established that acetic acid bacteria take a very important role in this process. Hence, during cocoa fermentation, acetic acid bacteria oxidize ethanol produced by

yeasts, into acetic acid which penetrates into beans and provokes the lowering of the inner pH. Additionally, the exothermic reactions of AAB increase the temperature of the fermenting mass. The low inner pH of bean combined with the heat, triggers the activation of endogenous enzymes, mainly proteolytic enzymes (Kirchhoff *et al.*, 1989; Amin *et al.*, 1998), but also aminopeptidase, invertase, polyphenol oxidase and glycosidases (Hansen *et al.*, 1998). This results in cascade reactions responsible for the final quality of the fermented beans and chocolate (Biehl *et al.*, 1993; De Brito *et al.*, 2000; Schwan and Wheals, 2004). Accordingly, any cocoa fermentation cannot be correctly processed without the presence of acetic acid bacteria. Since cocoa fermentation remains difficult to control, many studies have been undertaken in order to improve this process (Passos *et al.*, 1984; Hansen *et al.*, 1998; Nielsen *et al.*, 2005; Koen *et al.*, 2012). In this context, the use of starter microbial culture is assumed the most promising approach. Hence, several microbial cultures, including acetic acid bacteria have been assayed on the farms to assess their potential as starters (Schwan, 1998; Lefeber *et al.*, 2010; Lefeber *et al.*, 2011; Papalexandratou *et al.*, 2011). These assays are mainly to analyze the effect of chosen strains, on the quality of

fermented product. A deep understanding of the wild type microbial consortium, notably its functional and technologic properties, is therefore indispensable for optimum screening of starter strains, improving fermentation and delivering cocoa of first grade. Yet many studies focus essentially on identification of microbiota from cocoa fermenting bean in different countries such as Ghana (Nielsen *et al.*, 2005; Camu *et al.*, 2007), Brazil (Papalexandratou *et al.*, 2011; Lefeber *et al.*, 2011), Malaysia and Trinidad (Carr and Davies, 1980), have been reported. On the other hand, since geographic location is known to have an influence on the composition and characteristic of microbial consortium responsible for cocoa fermentation (Schwan and Wheals, 2004), it is surprising that very little is known on microbiota involved in Côte d'Ivoire cocoa fermentation. Nevertheless, Côte d'Ivoire stands as the first cocoa producer in the world with up to 37 % of the global production. The few relevant reports on Côte d'Ivoire cocoa microbiota remains the recent study on fungal flora (Guehi *et al.*, 2010) and pectinolytic *Bacillus* (Ouattara *et al.*, 2008). Here, this study reports some characteristic and functional properties of one of the most important microbial actor namely; acetic acid bacteria involved in Côte d'Ivoire cocoa fermentation.

MATERIAL AND METHODS

Fermentation conditions: Cocoa pods were harvested from Agboville (geographic coordinates 5°59' North 4°28' West), situated at 79 km from Abidjan (Côte d'Ivoire). Beans were removed from pods and fermented traditionally by heap fermentation for six days. The fermenting mass about 100 kg, set on banana leaves and covered with banana leaves was constituted of mixed genotypes (Forastero, Trinitario, and Criollo cultivars). Samples of fermenting cocoa bean were taken according to a fixed time schedule, notably at the start of the fermentation (0 h) and after 24; 48; 72; 96 h; 120 and 144 h of fermentation. pH and temperature were also regularly recorded directly at 15 cm depth on the fermenting heap, with portable pH-meter and thermometer.

Acetic acid bacteria isolation and numeration: After appropriate dilution in sterile saline, the fresh fermentation samples were plated on potato medium as

described by Duthathai et Pathom-Aree (2007). The medium containing 0.5 % D-glucose, 1 % yeasts extract, 1 % peptone, 2 % glycerol, 1.5 % potato and 4 % ethanol (v/v), was supplemented with 0.0016 % bromocresol green to monitor pH variation and nystatin (50 µg/ml) to inhibit fungal growth. The culture was incubated at 30 °C to enable colony count enumeration (expressed as CFU per gram cocoa pulp-bean mass), as described previously (Lefeber *et al.*, 2012). Acetic acid bacteria were identified as Gram and oxydase negative (short) rod-shaped, catalase positive and obligatory aerobic. The strains isolated were stored at -80 °C in LB medium supplemented with glycerol 20 % in Eppendorf tubes, for further studies.

Evaluation of acidification capacity: Acidification capacity of bacterial strains was evaluated as previously described by Aydin *et al.* (2009) with slight modification. Hestrin-Schramm (HS) agar containing

0.05 % D-glucose, 0.5 % yeasts extract, 0.3 % casein peptone, 2 % glycerol, 1.5 % calcium carbonate, 1.5 % ethanol (v/v) and 1.5 % agar, supplemented with 0.0016 % bromocresol green, pH 6.8, was spot inoculated with pure 18-24 h pre-culture of bacterial strain and incubated at 30 °C for 48 h. Acid production was monitored by formation of yellow zone around the spot. Acidification capacity of strains was evaluated by measuring the yellow zone diameter. To analyze the effect of temperature on acid production, cultures were incubated at 30; 35; 40 and 45 °C and then, the resulting diameter of yellow zone was measured.

Analysis of strains resistance to alcoholic stress:

The analysis of strains resistance to alcoholic shock was carried out on HS medium supplemented with different final concentrations (v/v) of alcohol in the range (5; 10; 15 and 20 %). To prepare the alcoholic medium, HS medium was cooled after autoclaving and maintained in liquid state at 45 °C in water bath and then appropriate quantity of alcohol was aseptically added to the medium to obtain the fixed concentration. Purified cultures were streaked onto the HS-agar plate and incubated for 48 h à 30 °C. A negative control was prepared in the same condition excepted that the streak was performed with sterilized material. The capacity of strain to resist to alcoholic stress is assessed by the growth of colony and the presence of yellow zone, along the streak. **Analysis of carbon metabolism of strains isolated:** The isolates were analyzed for their ability to produce acid by catabolizing glucose, fructose,

saccharose and citrate which are the main carbohydrates contained in the cocoa pulp. The study of glucose, fructose and saccharose metabolism was performed in HS broth containing the appropriate carbohydrate at 1 % as sole carbon source. Citrate utilization was tested using citrate broth (0.1 % $\text{NH}_4\text{H}_2\text{PO}_4$, 0.1 % KH_2PO_4 , 0.5 % NaCl, 0.02 % MgSO_4 , 0.2% sodium citrate, 0.008 % bromothymol blue, pH 6.8) (Aydin *et al.*, 2009). The liquid medium was inoculated with pure 18-24h pre-culture of AAB and incubated at 30 °C for 48 h. A negative control was prepared in the same conditions excepted that it was not inoculated with the microbial culture. The capacity of strains to metabolize the carbon source is assessed by the presence of turbidity and the change of medium color due to pH lowering, comparatively to the negative control.

Over oxidation capacity: The test of over oxidation was carried out in HS broth medium containing 0.05 % glucose and 1 % acetic acid as carbon source. Addition of acetic acid to the HS broth provokes the change of the green medium color into yellow. Five millilitres of medium contained in a 20 ml flask were inoculated with the bacterial pre-culture and incubated at 30 °C for 48 h. Over oxidation of acetic acid into CO_2 and H_2O was assessed by the change of medium color back to green, due to pH rising. All the assays in this study unless otherwise specified, were performed in triplicate, and the coefficient of variation was less than 8 %.

RESULTS AND DISCUSSION

The temperature of fermenting cocoa mass was 29 °C at the start of the process and progressively rose to reach a peak at 45 °C within 48-72 h (Fig. 1). Then the remaining time, a gradual decrease of temperature was observed, dropping at 36 °C at the end of fermentation. The same profile of temperature and pH variation has

been regularly recorded in cocoa fermenting mass in many countries (Schwan and Wheals, 2004; Papalexandratou *et al.*, 2011; Camu *et al.*, 2007; Guehi *et al.*, 2010) indicating that the increase of both parameters constitutes an inherent property of cocoa fermentation worldwide.

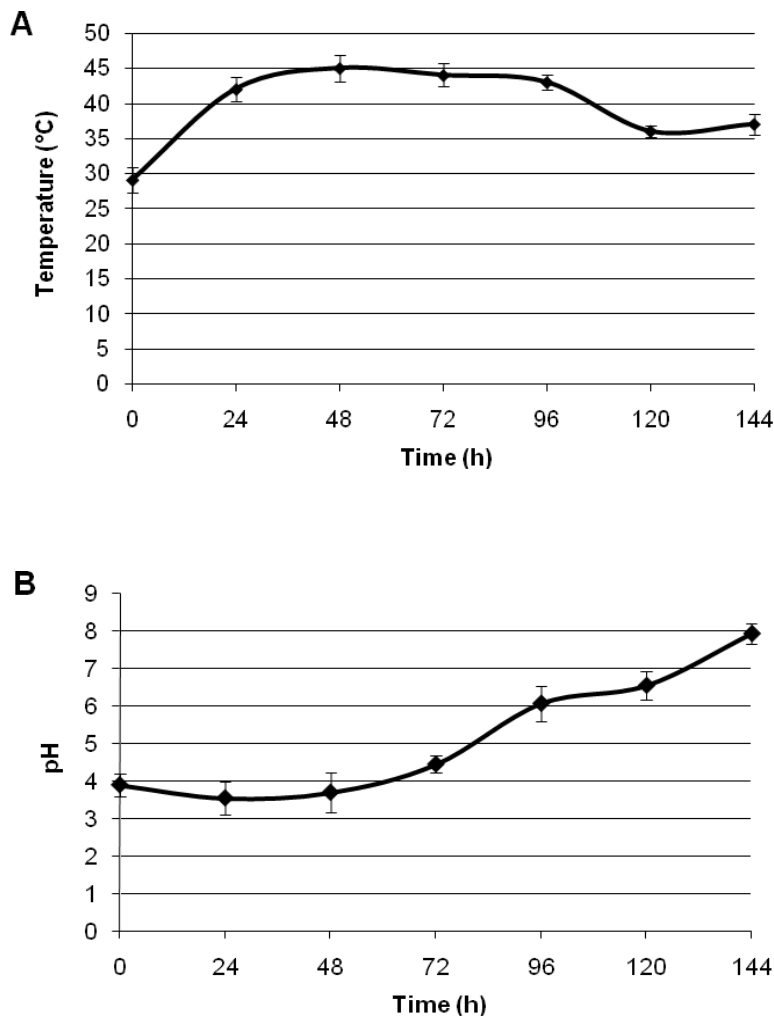


Figure 1: Evolution of temperature (A) and pH (B) during cocoa fermentation.

Concerning pH, the fermentation began with a pH 3.9 and seems to slightly decrease within the first 48 h (Fig. 2). Then, this pH increased sharply to reach 4.45 at 72 h and 6.07 at 96 h (Fig. 2). The pH continuously increasing during the fermentation gets its maximum (7.9) at the end of the process. Guehi *et al.* (2010) also reported an alkaline pH (8.5) at the end of spontaneous cocoa fermentation in Côte d'Ivoire. This result is particularly surprising since, it is very often observed that pH of cocoa fermentation is maximum toward the

end, but remains in acidic range (Schwan, 1998; Schwan and Wheals, 2004; Camu *et al.*, 2007). We could not explain the reason for what, sometime pH become alkaline in cocoa fermentation, but this seems to be a particularity of Côte d'Ivoire cocoa fermentation, since alkaline pH has not been yet reported in other country. The variation of temperature and pH of cocoa fermenting mass is known to be closely linked to the microbial growth dynamic (Schwan and Wheals, 2004).

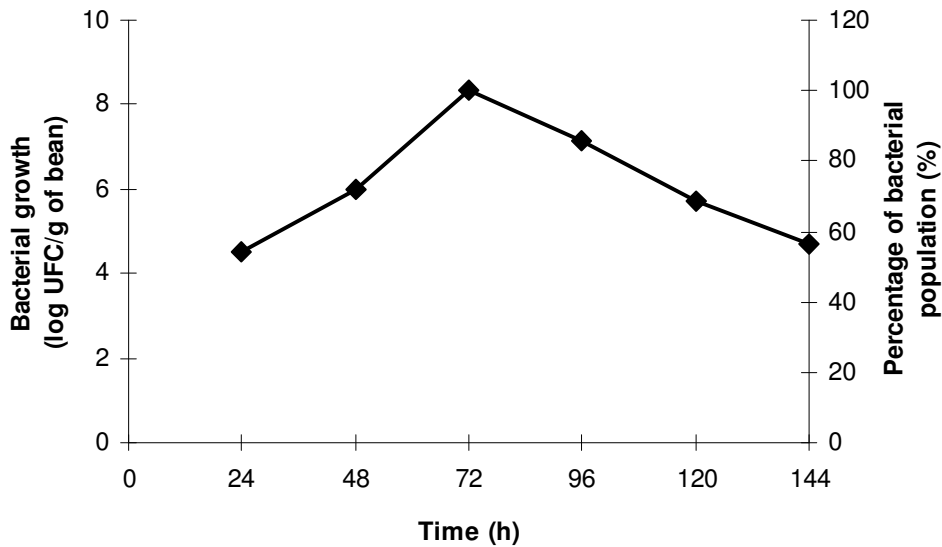


Figure 2: Acetic acid bacteria growth dynamic during cocoa fermentation. Samples of fermenting bean were harvested at 24 h interval, and microbial numeration was performed by decimal dilution method on potato medium as described by Duthatai and Pathom-Aree, (2007).

The dynamic of acetic acid bacteria population obtained from numeration during fermentation shows a very low and undetectable level of bacterial load at the beginning (Fig. 3), since no colony corresponding to AAB was identified on the plate at this time. Then after 24 h, the bacterial load increased rapidly to reach a peak of 13.10^6 UFC/g of bean at 72 h (Fig. 3). A

decrease of microbial growth was observed after 72 to a low population ($3.8.10^5$ UFC/g of cocoa bean) at the end of the process. The same growth pattern of AAB with maximum population occurring at 66-88 h, has been observed by Pereira *et al.* (2012) in Mexico, Schwan (1998) in Brazil and Camu *et al.* (2007) in Ghana.

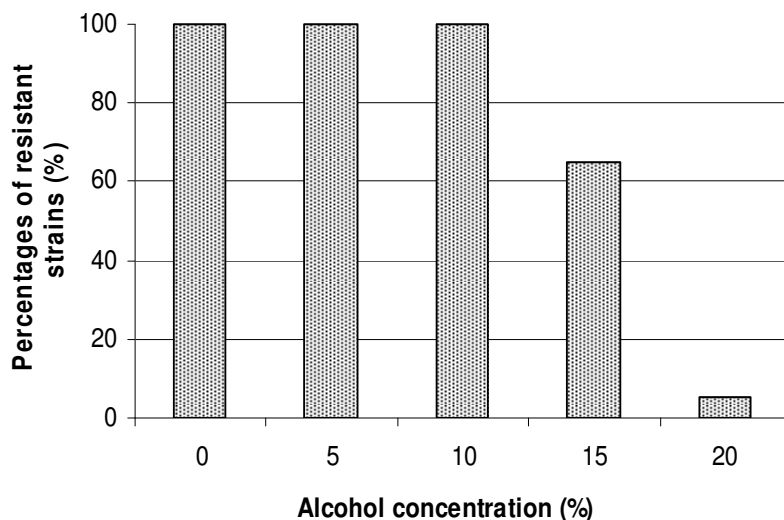


Figure 3: Effect of alcoholic stress on the growth of Acetic acid bacteria.

Alcoholic stress conditions were created with agar medium containing different alcohol concentrations. Pure cultures were streaked onto the medium and incubated for 48 h à 30 °C. This study also observed that the maximum population of AAB corresponds to a high temperature of fermenting mass. This is known to be due to the bioconversion of ethanol into acetic acid, an exothermic reaction which is responsible for the increase of the temperature (Adams, 1998). Furthermore, the peak of temperature in cocoa fermentation conditions may correspond to the end of AAB role, since effective decrease of AAB population begins after this peak. Although AAB are usually mesophilic microorganisms with optimum growth temperature between 25 and 30 °C (De Ory *et al.*, 1998; Adachi *et al.*, 2003), AAB strains isolated in this study show ability to grow beyond 40 °C. This indicates a thermotolerance which result undoubtedly from an adaptation to cocoa fermentation conditions. Acidification is one of the most relevant properties in cocoa fermentation since it influences greatly the quality of fermented bean and chocolate (Schwan and Wheals, 2004). The production of acetic acid during cocoa fermentation allows the development of

chocolate flavour and aroma (Biehl *et al.*, 1993; De Brito *et al.*, 2000). Hence, strong acidification should be a desired parameter for starter strain screening and improvement of fermentation. The 86 AAB strains isolated were analyzed and screened for their acidification capacity. All the strains showed naturally acidification capacity but with different levels. The yellow halo diameters round the colonies ranged from 6 to 25 mm. Twenty strains producing halo diameter between 16 and 25 mm showed the most important acidification capacity, while 48 strains produced middle acidity with 10-15 mm and 18 strains showed low acidification. As the temperature of fermentation is subjected to fluctuation, its effect on acidification capacity was investigated using the most acidifying strains as model. Table 1 shows three patterns of acid production with temperature variation. The first pattern observed with strains BA123 and BA74, concerns a regular decrease on acid production with the augmentation of temperature from 30 to 40 °C (Table 1). The second pattern of acid production observed with strains BA125, BA121 and BA11 concerned an increase of acid production when the temperature rises from 30 to 35°C and a decrease at 40 °C (Table 1).

Table 1: Acidic zone diameter produced during AAB growth *in vitro* under different temperatures

Temperature (°C)	Diameter of acidification zone (mm)						
	strains						
	BA123	BA74	BA125	BA121	BA94	BA35	BA11
30	25 ±2.365	22 ±1.352	21 ±2.108	20 ±2.001	19 ±2.884	18 ±0.815	16 ±1.368
35	20 ±1.369	21 ±2.481	22.5 ±2.412	24 ±1.771	19 ±0.969	18 ±2.397	18 ±0.962
40	15 ±1.741	9 ±1.205	20.5 ±1.532	18 ±1.235	16 ±1.526	15 ±1.691	12 ±0.897
45	0	0	0	0	0	0	0

The last pattern observed with strains BA94 and BA35 describes a constant acid production between 30 and 35 °C and a decrease at 40 °C. Although the temperature proved to influence negatively the acidification capacity at 40 °C, strains display more than 80 % of initial acid zone diameter at 30 °C. The results indicate that all AAB strains isolated possess the capacity to produce acid from ethanol but with different level; some being more effective. Indeed, a beneficial effect on quality product is liable to occur when fermenting cocoa beans include an important

population of AAB presenting a high capacity of acidification. However, it appears that AAB strains were scarcely able to grow at 45 °C and subsequently lost their acidifying activity *in vitro*, although they were isolated from fermenting mass at 45 °C. This suggests that the yield of acetic acid necessary for complete fermentation should be supplied by AAB strains before the fermenting mass reach 45 °C. Since a strong acidification capacity of AAB is related to resistance to high alcohol concentration, the tolerance to alcohol is one of the functional properties on which the screening

of AAB starter culture is based (Schwan, 1998). Additionally, the resistance to alcohol of strains should allow the rapid consumption and disappearance of alcohol in the fermenting mass and facilitate the growth of the remaining microbiota. The results from this study show that all the 86 strains of AAB isolated, are resistant to alcohol at concentration up to 10 %, as indicated by the growth of colony and the pH change of the culture medium (Fig. 2). This resistance decreased when the concentration of alcohol increase at 15 %, but remains relatively high with 53 strains presenting growth ability. However, 5 strains proved to be resistant to alcohol at 20 % concentration. Among the strains belonging to the most important acid producer, 13 were able to grow on medium with 15 % alcohol concentration while only one strain was resistant to 20 % concentration. Additionally, it was observed in the conditions of this experimentation that, when the strains were able to grow on alcoholic medium, they almost produce the same quantity of acid since the yellow halo diameter did not vary with alcohol concentration. To date, the tolerance to alcohol of AAB from cocoa fermentation has not been yet investigated. Previously, Du Toit and Pretorius (2002) observed that AAB remained viable in wine with 14 % of ethanol, while Gullo (2004) reported a growth of AAB in 10 % ethanol. Furthermore, Schwan (1998) used some AAB strains tolerating only 6 % ethanol as starter in cocoa fermentation. The feature of alcohol tolerance (up to 20 %) presented by AAB strains is relatively more elevated; suggesting that a high yield of alcohol from yeasts (Schwan and Wheals, 2004) could not be susceptible to limit the growth of AAB during cocoa fermentation. This is also strongly supported by the fact

that no notable variation was observed in the acidification capacity of strains even at high alcohol concentration. Strains were also analyzed for acid production from different carbon sources namely glucose, fructose, sucrose and citrate. The results indicate that AAB isolated failed to produce acid from fructose, sucrose and citric acid. Only glucose was successfully used by bacteria studied for acid production. In spite of this characteristic, AAB growth during cocoa fermentation should not be limited by an eventual depletion of glucose, due to its intense utilization by the whole microbiota, because of the preference of AAB strains for ethanol as oxidation substrate (Lefeber et al., 2010). In contrast, some AAB strains isolated from wastes of vinegar fermentation were observed to utilize both glucose and sucrose (Aydin et al., 2009). Moreover, over oxidation test performed on the 20 most acidifying strains show that 16 strains were able to overoxidize acetic acid and to provoke a de-acidification by changing back the yellow culture medium into green within 48 h. In this study, AAB strains isolated from Ivoirian cocoa fermenting bean in Ivory Coast proved to be thermotolerant with ability to grow up to 45 °C. These strains are characterized by a high tolerance to alcohol up to 20 % with different levels of acidification capacity. The temperature proved to affect strongly the acidification ability of strains beyond 40 °C. A high proportion of strains 80 %, were able to further oxidize acetic acid into water and dioxide carbon and belonging to *Acetobacter* genus. This study demonstrates that AAB involved in Côte d'Ivoire cocoa fermentation have interesting potentialities and indicate the relevance of screening for starters culture development.

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