

Effects of culture conditions on acetic acid production by bacteria isolated from Ivoirian fermenting cocoa (*Theobroma cacao* L.) beans

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ABSTRACT

Objectives: This study investigates the acidification capacity under various culture conditions of high acetic acid producer AAB strains previously isolated from Ivoirian cocoa beans fermentation.

Methodology and Results: Effect of culture conditions was studied in agar medium and acid production was monitored by measuring the clear halo diameter during incubation. All tested strains showed acetic acid production at 30, 35 and 40 °C. Moreover, at initial concentration 0.1 - 0.4 %, lactic and citric acids stimulated acidification capacity of these strains with increase rate ranged from 50 to 100 % while acetic acid reduced this capacity. In addition, maximum acetic acid production capacity was obtained for strains 123 D; 56 AB and 49 D at 8 % ethanol initial concentration.

Conclusions and application of findings: This study shows that all tested strains are able to produce acetic acid under certain culture conditions similar to cocoa fermentation stress. However, cocoa fermentation assay is needed to better estimate the performance of selected strains.

Keywords: Acetic acid bacteria, acetic acid production, Cocoa fermentation, culture conditions

INTRODUCTION

Acetic acid bacteria (AAB) are Gram-negative bacteria with the ability to oxidize ethanol to acetic acid (Matsushita *et al.*, 1994; Sievers and Swings, 2005; Sharafi *et al.*, 2010; Kersters *et al.*, 2006). In cocoa bean fermentation, the first step in chocolate production, AAB play an important role for obtaining a well-fermented cocoa (Schwan and Wheals, 2004; Romero-Cortes *et al.*, 2012). Generally, AAB emerged after 24 h of fermentation when air comes in fermentation heap (Nielsen *et al.*, 2007) and then oxidize ethanol previously

produced by yeasts to acetic acid by exothermic reaction. This acid diffuses into beans and in combination with heat produced by this exothermic bioconversion causes the death of the seed embryo as well as the end of fermentation. These biochemical changes are leading to the formation of precursor molecules that are necessary for the development of a characteristic aroma, flavour and colour of beans (Hansen *et al.*, 1998; Thompson *et al.*, 2001). These properties are further developed during drying, roasting, and final processing of

well-fermented cocoa beans (Thompson *et al.*, 2001). Therefore, activities of AAB during cocoa fermentation are essential for the production of high-quality cocoa (Quesnel, 1965). On the other hand, cocoa fermentation remains difficult to control leading to inconsistent production of cocoa quality. In this context, many studies suggest the use of microbial starter culture as a best approach to improve fermentation process (Passos *et al.*, 1984; Hansen *et al.*, 1998; Hashim *et al.*, 1998; Nielsen *et al.*, 2005; Papalexandratou *et al.*, 2011). Therefore, several microbial cultures, including acetic acid bacteria have been assayed to assess their potential as starters and their effect on the

quality of fermented product (Schwan, 1998; Lefeber *et al.*, 2010; Lefeber *et al.*, 2011; Papalexandratou *et al.*, 2011). In this context, selected strains should be able to grow and produce metabolite essential for cocoa high quality under heap fermentation stress conditions mainly high temperature, variation of acids and ethanol concentrations. Previous studies have already evaluated the capacity of AAB strains to grow under fermentations conditions (Soumahoro *et al.*, 2015). The aim of this work was to analyse the capacity of these isolates to produce acetic acid under different fermentations stress conditions.

MATERIAL AND METHODS

Bacterial strains : Five (5) AAB strains including *Gluconobacter oxydans* strain (49 D), *A. pasteurianus* (123 D), *A. peroxydans* (56 AB), *A. aceti* (139 D) and *A. pasteurianus* (121 D) isolates recovered previously from Côte d'Ivoire cocoa fermentation were tested in this study. These strains were selected because of their high capacity to produce acetic acid in solid and liquid media (Soumahoro *et al.*, 2015).

Analysis of acid production under various culture conditions

Effects of temperature and pH on acetic acid production: Effects of temperature and pH were evaluated in Hestrin-Schramm (HS) agar medium supplemented with green bromocresol and 4 % ethanol as described by Andelib and Nuram (2009). The medium was spot inoculated with pure 24 h pre-culture of bacterial strain and incubated at different temperatures (30, 35, 40, 45 and 50 °C). To evaluate the effect of pH on acetic acid production, the HS medium was adjusted to different pH (4; 5; 6; 7 and 8). All cultures were incubated during 10 days at 30 °C in

aerobic condition. The resulting diameter of yellow zone was measured daily. Acidifying capacity of strains was assessed by acid forming colony characterized by a clear halo with a diameter related to the amount of acid produced.

Effect of alcoholic and acid initial concentration on acetic acid production: Evaluation of acetic acid production capacity to ethanol, lactic acid, acetic acid and citric acid stress was carried out on HS agar medium as described by Andelib and Nuram (2009). Stress media were prepared using HS medium supplemented aseptically with alcohol and acids after sterilization. Their final concentrations (v/v) were ranging from 0 to 20. The negative control with 4 % alcohol did not contain the compound studied. The medium was spot inoculated as previously described and plates were incubated at 30 °C for 10 days in aerobic conditions. The capacity of strains to produce acetic acid in alcoholic and acid stress conditions was assessed as previously described.

RESULTS AND DISCUSSION

Effect of temperature and pH on acetic acid production: The effect of temperature variation on acetic acid production capacity for 49 D strain is available in figure 1a. The results showed a progressive increase of clear zone diameter during the first 7 days incubation at 30, 35 and 40 °C. Then a stationary phase was observed until the end of incubation (Figure 1a). These results were same for all tested isolates. These findings are interesting because the cocoa fermentation is on average 6-7 days (Yao *et al.*, 2014).

These findings suggest that the five tested strains in cocoa fermentation essay could continuously product acetic acid during the process. However, when the samples were incubated at 40 °C a long lag time (2 days) was observed (Figure 1 a). Moryadee *et al.* (2008) reported an acetic acid bacteria strain isolated from fruit that produced acetic acid at 37 °C with long lag time (3 days). Saeki *et al.* (1997) found also that the lag phase for ethanol oxidation was elevated (at 37 °C) and they suggested that the low productivity observed

at this temperature might be attributed to the long time of adaptation of strains. In the context of cocoa fermentation, this adaptation time may promote reduction of acetic acid amount in cocoa beans fermenting heap and strongly influence the final cocoa quality. Indeed, Biehl *et al.* (1985) reported that high acetic acid amount is required for activation of the endoproteases activities and synthesis of amino acids, which are characteristics chocolate aromas precursors. Our results also showed that no acetic acid production was observed at temperature above 45 °C. Yet, in previous studies, Soumahoro *et al.* (2015) demonstrated that all tested strains are able to growth at 45 °C and strains 49 D, 139 D and 56 AB at 50 °C. On the other hand, Saeki *et al.* (1997) demonstrated the increase in stability with increase of temperature (above 50 °C) of enzymes notably alcohol dehydrogenase and aldehyde dehydrogenase implicated in conversion of ethanol into acetic acid (Tayama *et al.*, 1989). Therefore, incapacity of these strains to produce acetic acid at 45 °C is probably due to several factors unexplained in our knowledge especially since Moghadami *et al.* (2013) have demonstrated that thermotolerant strains have high ethanol oxidation rate. Moreover, increase of temperature during cocoa fermentation is undissociable

factor of this process. Generally, the temperature of the fermenting cocoa mass reaches 35 °C in the first 24 hours of fermentation corresponding to ethanol production by yeast strains. After 36 to 72 hours of cocoa fermentation, the bioconversions of ethanol in acetic acid caused increase of temperature to a peak at about 45 °C value (Schawn, 1998; Fowler, 2009; Samagaci *et al.*, 2014). Therefore, a high AAB activity in cocoa fermenting heap could lead to inhibition of acetic acid production capacity and consequently a decrease of fermented cocoa quality. As both high temperatures and high acid production are important for successful cocoa beans fermentation and obtaining of cocoa quality, further investigations are needed to understand the inability of these thermotolerant strains to produce acetic acid and to improve their acidification capacity under high temperature conditions. Figure 1b shows the effect of variation of pH values on the acetic acid production capacity of strain 121 D. This result is same for all tested isolates. The results indicated that acetic acid optimal production was observed at initial pH 5 (with 3.5 cm as maximum clear halo diameter) probably because optimum pH for the growth of acetic acid bacteria is range from pH 5 to 6.3 (Holt *et al.*, 1994).

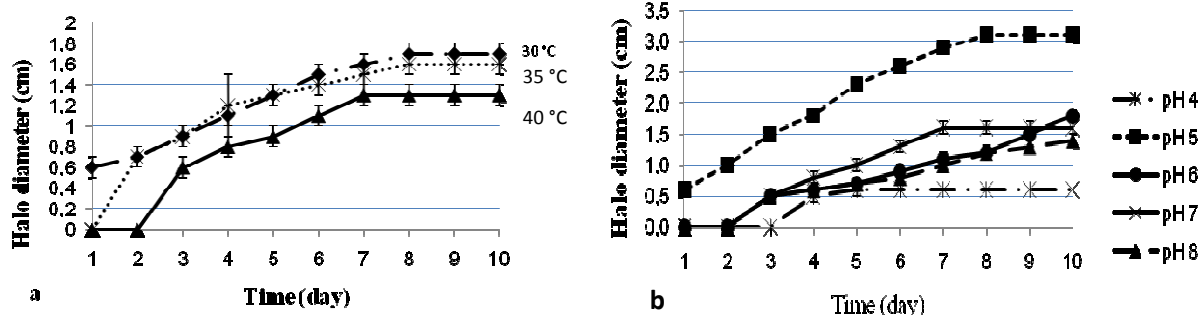


Figure 1: Effect of variation of temperature (a) and pH (b) on acidification capacity of strains 49 D (a) and 121 D (b)

The medium HS containing 4 % of ethanol was spot inoculated with tested strain and incubated at different temperatures (30, 35, 40, 45 and 50 °C). The effect of pH was evaluated in HS medium adjusted to different pH (4; 5; 6; 7 and 8). Acidifying capability of strains was assessed by acid forming colony characterized by a clear halo with a diameter related to the amount of acid produced after incubation. Furthermore, the lower activity is observed after 3 days lag time for pH 4 culture medium (Figure 1b). In addition, no acetic acid production was obtained during the 2 first days of

incubation at pH 6, pH 7 and pH 8. During cocoa fermentation, pH increased from 3.88–4.2 at start of fermentation to 4.8–5.02 after 3 days and 4.15–8 at the end of the seventh day (Samagaci *et al.*, 2014; Afoakwa *et al.*, 2013; Ardhana and Fleet, 2003; Guehi *et al.*, 2010). On the other hand, acetic acid bacteria reach a maximum growth after three days of fermentation when fermenting heap pH value around 5 (Kouamé *et al.* 2015; Yao *et al.*, 2014). These data clearly explains why Afoakwa *et al.* (2013) found that acidity level, due probably to acetic acid, was highest at

3 days of fermentation. In addition, in previous studies, Soumahoro et al. (2015) reported a good growth capacity of these strains at pH values ranged 3 to 7. Consequently, variation of the pH values during a cocoa fermentation essay could not affect acetic acid production capacity of tested strains.

Effect of initial ethanol concentration on acetic acid production: Results for strain 49 D after ten days incubation is shown in Figure 2. Table 1 shows clear halo diameters for all strains after 10 days of incubation. Maximum acid production capacity was obtained with 4 % ethanol as initial concentration for strains 121 D and 139 D and with 8% for the isolates *A. Pasteurianus* (123 D), *A. peroxydans* (56 AB) and *G. oxydans* (49 D) (Table 1). These results are consistent with those reported by Romoreo-Cortes et al. (2012)

and Ardhana and fleet (2003) with AAB strains isolates from cocoa beans heap fermenting. These results also are interesting because ethanol is a major metabolite of cocoa pulp fermentation (Roelofsen, 1958) and the maximum rate of ethanol produced by the yeast is around 8 % during this process (Camu et al., 2008; Lefeber et al., 2012; Ho et al., 2014). Moreover, at ethanol initial concentration of 12-15 %, a low effect on acetic acid production capacity of strain 56 AB was observed comparatively to the control whose reduction rate was estimated to 45 -85 % (Figure 2). These results are same for the all tested strains. So, these isolates could be used in combination with highly ethanol producing yeast isolates to improve the final fermenting cocoa quality.

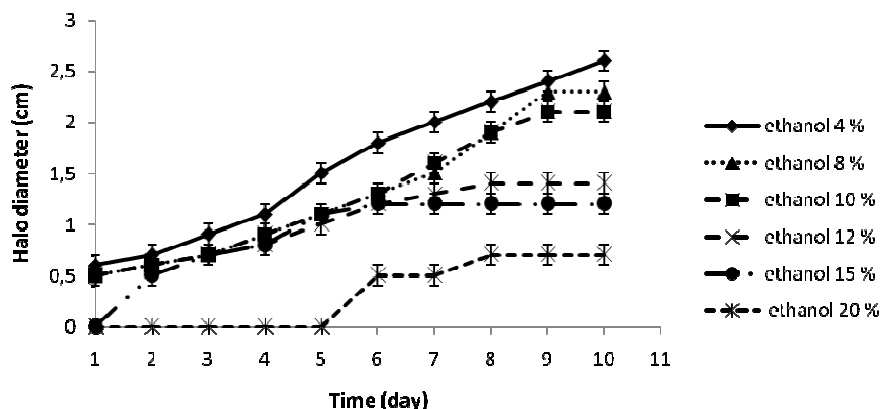


Figure 2: Effect of initial ethanol concentration on acetic acid production

Various concentrations of ethanol (4, 8, 10, 15 and 20 %) were added to the culture medium before the tested strain was inoculated by spot. Acidification capacity

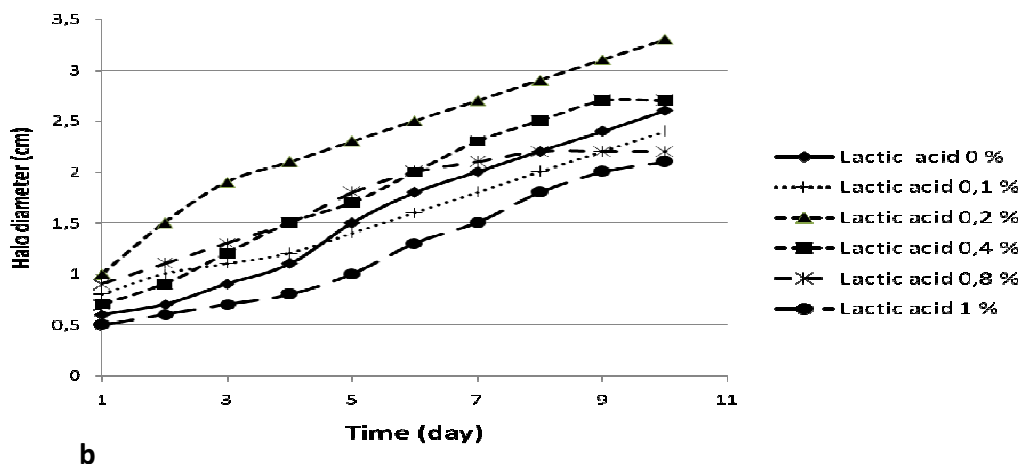
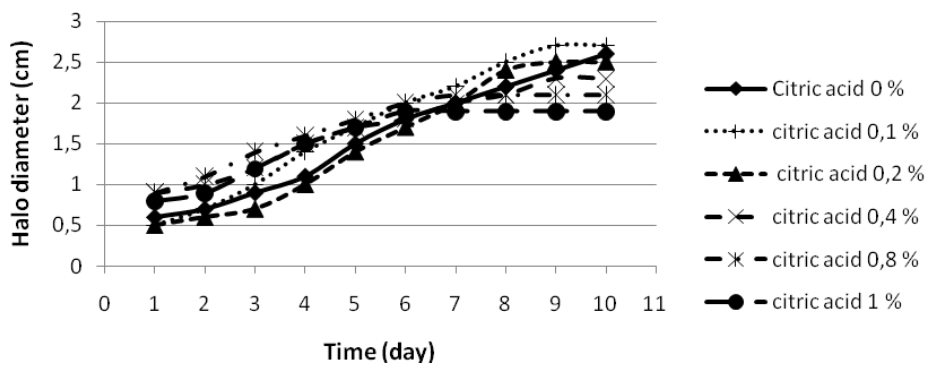
was evaluated after incubation by measuring of yellow zone diameter.

Table 1: Effect of initial ethanol concentration on acetic acid production after 10 days incubation

	Halo diameter (cm)					
	4 %	8 %	10 %	12 %	15 %	20 %
121D (<i>A. pasterianus</i>)	2.5 ± 0.1	2.2 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	0.7 ± 0.1	0
123D (<i>A. pasterianus</i>)	2.2 ± 0.1	2.6 ± 0.1	2.1 ± 0.1	1.8 ± 0.1	1.4 ± 0.1	0 ± 0.1
56AB (<i>A. peroxydans</i>)	1.9 ± 0.1	2.5 ± 0.1	1.8 ± 0.1	1.6 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
139D (<i>A. acetii</i>)	2.6 ± 0.1	2.3 ± 0.1	2.1 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	0.7 ± 0.1
49D (<i>G. oxydans</i>)	2.2 ± 0.1	2.3 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	1.5 ± 0.1	0 ± 0

Effect of acids initial concentration on AA production capacity: The influence of organic acids on the acid production capacity was performed in the HS medium supplemented with 0-1% of citric, lactic and acetic acids. The results showed that at 0.1 and 0.4% initial concentration, lactic acid promoted acidification capacity of the tested strain with increase rate ranged from 50 to 100 % comparatively to the control (0% of lactic acid). Likewise, at 1 % initial concentration, citric acid promoted acetic acid production in culture medium (figure 3). However, unlike these acids, acetic acid reduced this capacity with decrease rate reached 100 % after one-day incubation (Figure 3). After this, acetic acid amount gradually increased in culture medium until the end of incubation. These results suggested that acetic acid produced by AAB during cocoa fermentation is probably a temporary inhibition factor of enzymes involved in conversion of ethanol to acetic acid. On the over hand, several authors have reported changes in acids concentration in the pulp during fermentation

process with values ranged from 1-3 % (Galvez *et al.*, 2007; Camu *et al.*, 2008; Lefeber *et al.*, 2011; Papalexandratou *et al.*, 2011), 1-2 % (Quesnel, 1965) and 0.6-2.9 % (Ho *et al.*, 2014) respectively for citric, acetic and lactic acids. Consequently, this suggested that such conditions might prevent the production of acetic acid by these isolates during cocoa fermentation assay since Soumahoro *et al.* (2015) found in previous study incapacity of all tested strains to grow at acid initial concentration above 1 %. However, it is well known that AAB are involved in acetic acid production during fermentative process under these conditions (Schwan and Wheals, 2004) suggesting an adaptation capacity to the cocoa fermentation stress conditions. Therefore, procedures for selection of microorganisms strains as cultures starter, should take into account these factors. In this context, cocoa fermentation assay is needed to better estimate the acetic acid production capacity of our selected strains.



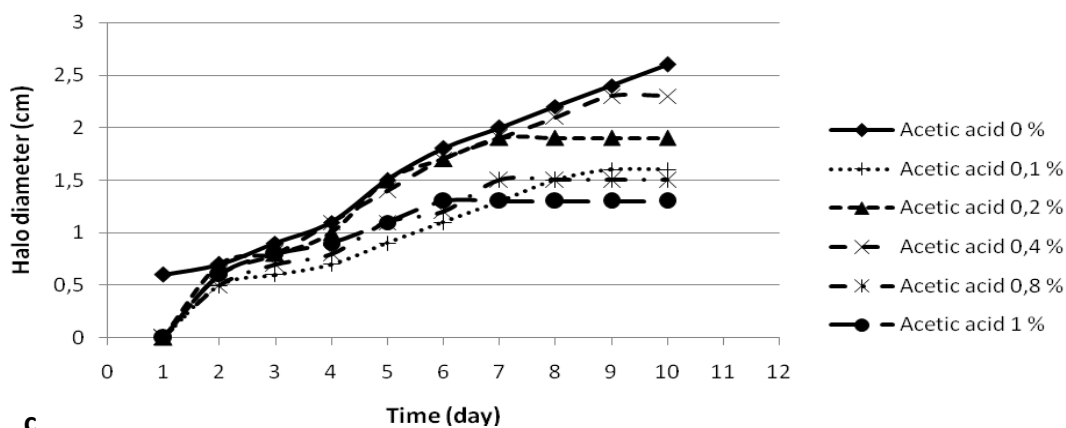


Figure 3: Effect of initial acid concentration on acetic acid production. (a): citric acid, (b): lactic acid, (c): acetic acid

In conclusion, the tested strains were able to produce acetic acid under different culture conditions. In addition, strains 123 D, 56 AB, 139 D and 49 D revealed their maximum acidification capacity under

these stress conditions. Therefore, these isolates could be use as starter in fermentation essay to improve the fermenting cocoa final quality.

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