Influence of Acetic Acid Bacteria on the Acidity of the Cocoa Beans During Fermentation

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Introduction

Cocoa (*Theobroma cacao*) is a crop that has a long tradition in the history of Mexico (1). Currently, from cocoa was obtained four intermediate products (cocoa liquor, cocoa butter, cocoa mass and cocoa powder and chocolate. However, for these products can be exploited, the cocoa beans should be treated by a cured process. The stages of this process are: harvest, fermentation, drying and storage. Fermentation is the principal stage, because the changes that originate during this stage are fundamentals for the appearance of the typical precursors of aroma and flavor of cocoa (2, 3, 4, 5, 6). The succession of microorganisms involved in fermentation begins with the yeast followed by lactic acid bacteria (LAB), acetic acid bacteria (AAB) and finally bacilli (7). However, in some fermentations is observed that yeasts, LAB and AAB exhibit the same behavior since the start of the fermentation (8, 9, 10). The concentration of each of these microorganisms varies during the days it takes for the fermentation and between each fermentation batch. The selection of each one of it, is mainly due to the resistance and adaptation developed during this stage (11). During the fermentation step, increasing the aeration of the fermenting mass and the temperature reaches 37°C, which favors the growth of AAB and start to be the dominant microorganisms (12). These are responsible for the oxidation of ethanol to acetic acid, which is later oxidized to carbon dioxide and water (13, 14). The accumulation of acetic acid in the dicotyledon is slow in the early hours but increases after the third day (8, 10, 11) maintaining a ratio to the end of fermentation. Furthermore, the diffusion of acetic acid into the grain and consumption of citric acid which surrounds the grain causing pH changes (11, 15) which vary according to the variety, location and type of fermentation (16). In Mexico, not enough is known of AAB species involved in fermentation and the characteristics that contribute to the fermentation of cocoa. The aim of this study was to isolate and identify molecularly the AAB present during the traditional fermentation of cocoa and quantify the production of acid in the grain and the produced by each of the AAB *in vitro*.

Materials and Methods
Raw material

The cocoa bean used in this study was provided by the "Asociación Local Agrícola de Productores de cacao" of the municipality of Huimanguillo, Tabasco. The cocoa beans contained in the industrial fermenting boxes (1 ton approx.) were mixed every 24 h by the beneficiaries. The sample recollection was performed at three different sites of each box (surface, middle and bottom), every 12 h, the grains were placed in sterile bags.

Microbiological analyzes
Sample preparation

In sterile bag was placed one cocoa grain by each 10 mL of NaCl (0.85%) and the bag was massaged until the pulp was completely removed of the grain. Subsequently, dilutions were made (10^{-4} a 10^{-6}) with the same physiological solution (NaCl, 0.85%) for each of the samples. The dilutions were dependent on the microorganism and the number of CFU reported (10, 17).

Growth conditions for AAB isolated during the traditional fermentation of cocoa

250 µL of the samples diluted were taken every 12 h (as mentioned in the previous section). For the isolation and quantification of the AAB, were used the Acetobacter/Gluconobacter (A/G) and modified CARR media.

To observe the growth and isolating the microorganisms, was used the scanning method with handle glass. Subsequently, the Petri dishes were incubated at 37°C for 24 h. The test was performed by duplicate.

pH determination

10 g of mucilage, shell with remains of mucilage, dicotyledon and whole grains were taken. Dicotyledon and whole grains were frozen with liquid nitrogen and pulverized in a coffee grinder (Krups model F203). The samples were diluted in 90 mL of distilled water, placed in thread flasks were carried and boiling during 15 min, subsequently, the samples were cooled to room temperature and the measurement was performed using a potentiometer (OAKTON pH500 S).

Acetic acid extraction

Approximately 20 g sample of cocoa previously pulverized with liquid nitrogen and 100 mL of distilled water were placed in a 125 mL Erlenmeyer flask. The extraction was performed in an ultrasonic bath (Westprime Systems model LR84943), during 30 min. The methanol extract obtained was filtered with Whatman paper No. 4. Acetic acid was quantified by gas chromatography (10).

DNA extraction, PCR and sequencing and molecular characterization of AAB

DNA Extraction of microorganisms isolated were performed as described previously Romero *et al*. in 2012 (18). A region of nuclear DNA, containing the ITS1-ITS2 regions and the 16S gene, was amplified by PCR using 6 different pairs of primers and amplification conditions (18). The resulting products were purified with the Geneclean® II kit (Bio101 Inc., California, USA) according to the manufacturer's protocol. DNA samples were sequenced by the Instituto de Biotecnología, Cuernavaca, Mor., Mexico.

DNA sequences from each AAB strain were assembled by use of Chromas 1.45 (School of Health science, Griffi th University, Gold Coast Campus, Southport, Queensland, Australia), and then aligned using the ClustalX inference package (19). The sequences were subjected to the basic local alignment search tool (BLAST) available at http://blast.ncbi.nlm.nih.gov/Blast.cgi against sequences available in GenBank. Phylogenetic analysis of the aligned sequences was done by Neighbour-Joining analysis with 100 bootstrap replicates and was supported by bootstrap method. Unrooted phylogenetic tree was constructed with help from the PhyloDraw program 0.8 (20).

Quantification of acetic acid produced by the AAB

For quantification of the acetic acid, were used 50 mL of sample, was titled with NaOH 0.1 Normal using phenolphthalein as indicator (21).

Results and Discussion

Evolution of the AAB during fermentation of cocoa

During development of the traditional cocoa fermentation was observed the same behavior in the three boxes studied, although each one started the fermentation at different time. The AAB showed their maximum growth from the second day of fermentation, but decreased around the third day, this growth could be related to the ventilation (17).

Evolution of acetic acid production and pH during the cocoa fermentation

The results clearly shows that the acetic acid increased after 48h; however, the maximum concentration obtained from the cocoa bean was lower than that reported by other authors (10, 17), who reported a decrease immediately after reaching the maximum concentration of this acid. The high concentration of acetic acid, at the end of fermentation, can be related to the species or strain of the AAB found at this stage. According to some authors (21), not all strains of the same species have the ability to oxidize acetic acid to CO_2 and H_2O.

Figure 1, shows clearly the pH decrease in the different samples after increased acetic acid. The pH of the mucilage beginning about 4, while the mucilage with shell started with a pH value of 3.5. The pH value of

mucilage correspond to the same value reported by García *et al.*, in 2005 (22) of a cocoa fermentation from Jalpa de Mendez, in Tabasco, México.
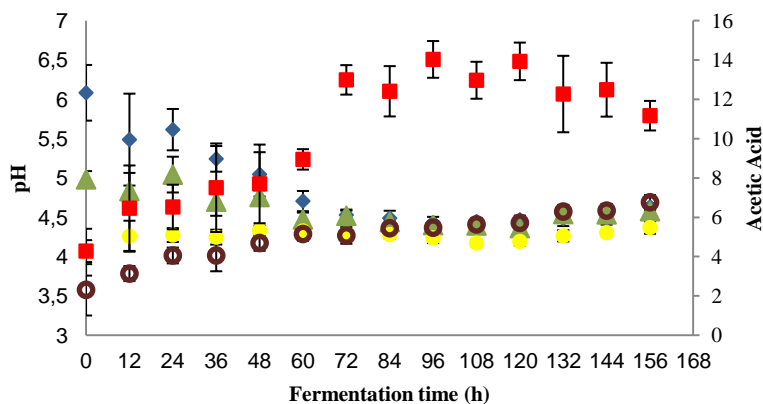

Figure 1. Evolution of acetic acid production and pH during the cocoa fermentation. ■ Acetic acid. ♦ Dicotyledon. ▲ Grain. ● Mucilage and ⊙ Mucilage with head.

However, these data vary with those reported by Ardhana and Fleet (11), this difference may be due because the cocoa beans used in this study contained less citric acid, which is reported to be consumed by microorganisms at the beginning of fermentation (7). With the progress of fermentation was observed a slight increase in pH in the mucilage. This change in pH is attributed to yeast, as are the main degraders of citric acid (23). As shown, although the shell is very thin, retains a degree of acidity, since the pH of the mucilage with shell was slightly more acidic than the mucilage at the beginning of the fermentation, but starting from 60 hours all samples followed the same behavior. In the case of the dicotyledon and the whole grain the pH decreased from 6.5 and 5, respectively to 4.5. The decrease in pH in the dicotyledon is mainly due to the diffusion of metabolites produced by microorganisms, which are absorbed by the dicotyledon among those that stand out acetic acid and lactic acid produced by the AAB and LAB (24).

Molecular characterization of isolates AAB

Specific amplification of the intergenic sequence spanning the rDNA of the 16S gene was achieved by using the primers ITS1 and ITS2. Two fragments were amplified of 750 and 678 bp for ITV75 and ITV114 strains, respectively. The sequences were deposited in GenBank bases with the accession number JF930132for strain ITV75 and JF930134 for strain ITV114. There was no amplification for strains ITV55, ITV61, ITV82 and ITV98. The lack of amplification of the ITS sequences from these strains is likely due to the lack of recognition of the primer ITS2 (25). The BLAST search for ITS sequences of strains ITV75and ITV114, showed homology (98%) with the sequence of strain LMG 1663 of *Acetobacter tropicalis* (FR716480) reported in the NCBI database. The homology of the strains in comparison to other sequences was less than 70%. For identification of the strains ITV55, ITV61, ITV82, ITV98 and to confirm the identification of strains ITV75 and ITV114, the DNA sequences of the 16S gene were amplified by PCR using primers AC1 and AC3. Obtaining the following fragments: 414 bp (ITV55), 836 bp (ITV61), 819 bp (ITV82), 834 bp (ITV98), 407 bp (ITV75), and 412 bp (ITV114). The sequences were deposited in GenBank bases with the following accession numbers: ITV55 (JF930135), ITV61 (JF930136), ITV75 (JF930133), ITV82 (JF930137), ITV98 (JF930138) and ITV114 (JF930145).

BLAST analysis of the region 16S-23S of the sequences of the strains ITV75 and ITV114 revealed the highest homology (98 to 100%) with strains of *A. tropicalis*, which is shown in the phylogenetic tree generated in this study (Figure 2).
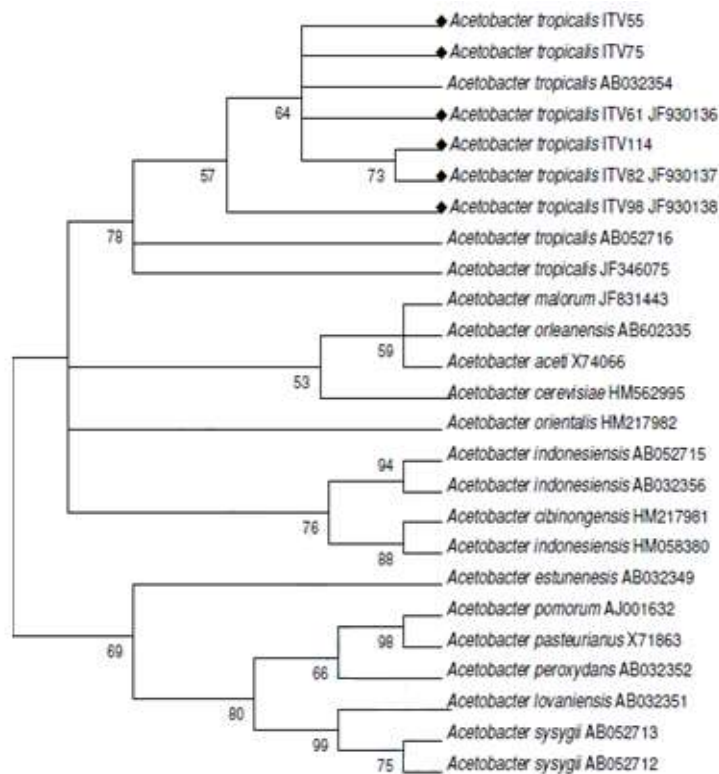
Figure 2. Phylogenetic tree based on 16S rDNA sequence showing the relationship of strains isolated from cocoa fermentation with *Acetobacter tropicalis*.

Phylogenetic analysis revealed that *Acetobacter* strains isolated in this study are aligned close to the strains of *A. tropicalis* from GenBank (AB052716, JF346075, AM748710, and AB03254). *A. tropicalis* is often found in the fermentation of cocoa beans (26). The strains identified in this study differ with the strains identified in the fermentation of cocoa (*Acetobacter pasteurianus*, *A. ghanensis*, *A. senegalensis* and *A. lovaniensis*) made in Indonesia (9). This difference could be related to the diversity of microorganisms and environmental conditions.

Acetic acid production during the traditional fermentation and produced by the strain ITV61
Comparing the acetic acid produced during cocoa fermentation and the produced by the strain ITV61, the increase of acetic acid during the traditional fermentation was after 60 h, however, the acetic acid production by *A. tropicalis* (ITV61) increased after 48 h (0.2% to 2.41 ± 0.11%) under the conditions used (18). However, this strain was able to oxidize it to CO_2 and H_2O (21). Although *A. tropicalis* has been reported as the most predominant microorganism fermentation in Ghana (27), there is insufficient information about the production of acetic acid of this species in the cocoa bean.
In conclusion the results obtained, allow us to propose that in the future, one could select strains that are most important during the process of fermentation and inoculated to avoid compromising the quality of fermented cocoa beans.

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