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Full Length Research Paper

Isolation and characterization of acetic acid bacteria in cocoa fermentation

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Acetic acid bacteria (AAB) from traditional indigenous process of cocoa bean fermentation were studied at a location in Huimanguillo, Tabasco, Mexico. High counts were detected at the beginning of the fermentation (5.7+ or - 0.26 cfu/g dry matter) and were present throughout fermentation time. Six AAB strains were isolated and characterized by morphological, biochemical and molecular methods. Morphological and biochemical methods were inconclusive for final identification of bacteria. However, the amplification of 16S rDNA of the strains by polymerase chain reaction (PCR) and the Blast analysis of the obtained nucleotide sequences showed homology (99 to 100%) with *Acetobacter tropicalis.* Because the production of acetic acid is an important factor in the development of chocolate flavour precursors we studied the capacities of the strains to produce acetic acid. *A. tropicalis* ITV61 reached 2.5% (v/v) acetic acid production in 9-h on potato broth medium supplemented with ethanol (4% v/v) while the other *A. tropicalis* strains accumulated low acetic acid concentrations (> 0.2%). *A. tropicalis* ITV61 strain was able to grow at 4, and 7% ethanol. Tolerance of the strain *A. tropicalis* ITV61 to acetic acid is not affected at concentrations of 1% from the beginning of fermentation.

Key words: *Acetobacter,* cocoa, fermentation, isolation, molecular characterization.

INTRODUCTION

Cocoa is one of the many foods that rely on a microbial curing process for flavor development. The main reason for fermentation of cocoa is to induce biochemical transformations within the beans that lead to formation of the color, aroma, and flavor precursors of chocolate (Thompson et al., 2001). A microbial consortium: yeast, *Lactobacillus*, acetic and spore-forming bacteria have been identified (Schwan and Wheals, 2004). During fermenta-tion yeasts dominate for the first 24 h. As the pulp disappears acetic acid bacteria (AAB) start to dominate, temperature rises to 50°C and the heat and acid result in chemical reactions in the beans known as curing (Schwan, 1998). In this step AAB oxidize the ethanol produced previously by yeast to acetic acid. The effect of acetic acid and raise of the temperature cause death of the seed embryo as well as the end of fermentation (Cleenwerck et al., 2007). Some authors have suggested that *Acetobacter pasteuriansis* is one of the main species of Acetic acid bacteria (AAB) involved in spontaneous fermentation by its acid and heat resistance, and its preferential growth in the presence of lactic acid and mannitol (Camu et al., 2008). Additionally other AAB species have been isolated from cocoa fermentation, identified by biochemical tests and it has been suggested to participate in a major way in the cocoa fermentation (Lagunes et al., 2007). On the other hand, in other areas, AAB diversity has been well studied, however little is known about of AAB related to cocoa fermentation and few strains have been identified by molecular methods (Camu et al., 2008). Alternatively, cocoa bean fermen-tation is spontaneous and microbial flora differs from country to country and even from farm to farm. The purpose of this study was to isolate, characterize by biochemical and molecular methods the acetic acid bacteria of cacao beans during the process of fermentation at a commercial plantation in Huimanguillo,

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Tabasco, Mexico. Additionally we have studied the ability of the isolated AAB to produce acetic acid *in vitro* and its ethanol tolerance.

MATERIALS AND METHODS

Fermentation monitoring and sampling

Cocoa bean fermentations were conducted as traditional indigenous process at a location in Huimanguillo, Tabasco, Mexico. Samples (1 kg) were collected at the top, middle and bottom layers of the cocoa mass from three boxes (100 × 100 × 100 cm) every 12 h for 6 days.

Microbiological analyses

Samples (10 g) were collected in sterile plastic bags, homogenized using a stomacher in sterile 0.85% (w/v) NaCl solution. Series of dilution up to 10^{-6} were carried out. The final dilution was spread in Petri dishes containing modified CAAR Agar (CAAR) (expressed as g/L: glucose, 3; $CaCO_3$, 10; bromothymol blue, 0.04; yeast extract, 10; agar, 20 and ethanol 17.5 ml/L) and AG Agar (expressed as g/L: glucose, 100; $CaCO_3$, 10; yeast extract 10; and agar, 25). Petri dishes were incubated in triplicate (37°C, 24 h). To obtain the number of bacteria the average was multiplied by the dilution. Cell counts were expressed as CFU/g of cocoa dry matter (DM). The colonies with yellow zone and clearing of calcium carbonate on the agar plates were selected for further experiments.

Morphological, physiological, and biochemical properties

AAB were tested to allow biochemical identification. The tests used were: catalase, cellulose production, oxidation of glycerol (Moryadee and Pathom, 2008) Gram staining, glucose oxidation, growth on 10% ethanol (Lisdiyanti et al., 2001), acetate, oxidation of ethanol, calcium acetate, dihydroxyacetone production from glycerol (Aydin and Deveci, 2009), production of water-soluble brown pigments and ability to oxydize sodium acetate (Seearunruangchai et al., 2004), growth and oxidize of methanol (Cleenwerck et al*.,* 2007). Acid production from glucose (Yamada et al., 1999). Cellulose test has been carried out using a Lugol's iodine stain followed by 60% sulphuric acid on pellicles from liquid cultures (Passmore and Carr, 1975). Other assimilation properties were tested with the use of API 20C AUX galleries (Bio-Merieux®). The API tests were exploited with APIWEB bacterial identification software. For the presence of flagella: several colonies of *A. tropicalis* ITV61 were transferred to 5 ml of broth and filtered through a 0.22 μm Millipore filter. The cells were fixed in the filter with 10% formaldehyde in phosphate buffer (pH 7.0) at room temperature for 15 min (Bozzola, 2007). Then several 5 ml of tannic acid (3% w/v) was applied and kept for 72 h at room temperature. Scanning electron microscope (SEM) picture was taken with an electronic microsope (JEOL; JSM-5600LV) at the Institute of Ecology (INECOL, Xalapa, Veracruz. Mexico).

Molecular characterization

AAB were cultivated in potato medium at room temperature under shaking at 220 rpm and 37°C. The composition of potato medium (expressed as g/L in distilled water) was as follows: glycerol, 20; polypeptone, 10; yeast extract, 10; glucose, 5, and 100 ml of potato extract (Nanda et al., 2001). Cells were grown overnight, recovered and washed twice with TE buffer (Tris-HCl 10 mM, EDTA 1 mM). DNA was extracted by the sonication method described by Fykse et al. (2003) and purified with the Bio101 kit (Hercules. CA).

PCR primers used to amplify the 16S-23S spacer region of the ITV75, and ITV114 strains were selected on the basis of the sequences of 16S (its1, 5´-ACCTGCGGCTGGATCACCTCC-3´) and 23S (its2, 5´-CCGAATGCCCTTATCGCGCTC-3´) rDNA genes conserved among acetic acid bacteria (Ruiz et al., 2000). Oligonucleotides primers used to amplify part of the 16S rDNA gene of the strains ITV55, ITV61, ITV82, ITV98, and ITV114 were selected from conserved regions of rDNA bacterial sequences. The sequences of primers were Ac1: 5´GCTGGCGGCATGCTTAACACAT-3´ and Ac3: 5´-AACCACATGCTCCACCGCTTG-3´ (Poblet et al., 2000). PCR amplification was carried out in 50 μL samples consisting of 5 μL DNA bacterial extract and 45 μL amplification mixture, which contained 15 pmol of each primer, 200 μM of each of the four dNTPs, 5 μL 10 X amplification buffer, and 5 U *Taq* DNA polymerase (Promega, Madison, WI. U.S.A.). The reaction was performed in a PCR thermocycler (Biorad, Hercules. CA., U.S.A.). For the amplification of the 16S intergenic spacer, the samples were incubated for 5 min at 94°C to denature the target DNA and then cycled 35 times at 94°C for 30 s, 65°C for 30 s and 72°C for 1 min. The samples were then incubated for 7 min at 72°C for a final extension and were maintained at 4°C until tested. For the amplification of the 16S, the DNA was denatured at 94°C for 5 min and amplified 35 cycles at 94°C for 1 min, 62°C for 2 min and 72°C for 2 min. A final extension incubation of 10 min at 72°C was included. The resulting products were purified with the Geneclean® II kit (Bio101, CA., U.S.A.) according to the manufacturer's protocol. DNA was sequenced by the Biotechnology Institute, Cuernavaca, Mor. Mexico. The sequences were run by the Blast program against the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and DNA sequences were submitted to GenBank where the accession numbers were obtained. The high homology sequences obtained and 16S-23S sequences of higher homology were used to construct Neighbour-joining (NJ) phylogenetic trees (Saitou and Nei, 1987). The alignment of the sequences and the phylogenetic tree construction were carried out with the ClustalW2 software (http://www.ebi.ac.uk). Trees were constructed using MEGA ver. 4.0 (Tamura et al., 2007) with 1000 bootstrap replicate.

Acetic acid production of AAB strains

Acetic acid bacteria (6.0×10^7 cell) were inoculated in 250 ml Erlenmeyer flask containing 50 ml of potato broth sterile medium (1.0% D-glucose, 1.0% glycerol, 0.2% polypeptone, 0.2% yeast extract, 10% potato extract, 4.0% of ethanol). Ethanol tolerance was evaluated by adding ethanol to 7 and 10%, while tolerance to acetic acid was evaluated by adding 1 and 2% acetic acid to the culture medium. Fermentation was carried out at room temperature during 10 days and samples (2 ml) were collected every 24 h. Acetic acid content was determined by titration with 0.1 N NaOH against phenolphthalein as an indicator (Nanda et al., 2001).

Acetic acid determination in cocoa beans

Cocoa bean samples were frozen with liquid nitrogen and grounded in a Krups ModF203 miller (Millville, NJ., U.S.A.). Samples were collected (10 g) and sonicated (Westprime Systems, Fullerton, CA., U.S.A.) for 20 min in distilled water (50 ml). The solution obtained was filtered through a Whatman No. 4 filter paper. The supernatant acidity was measured with 0.1 N NaOH and phenolphthalein as

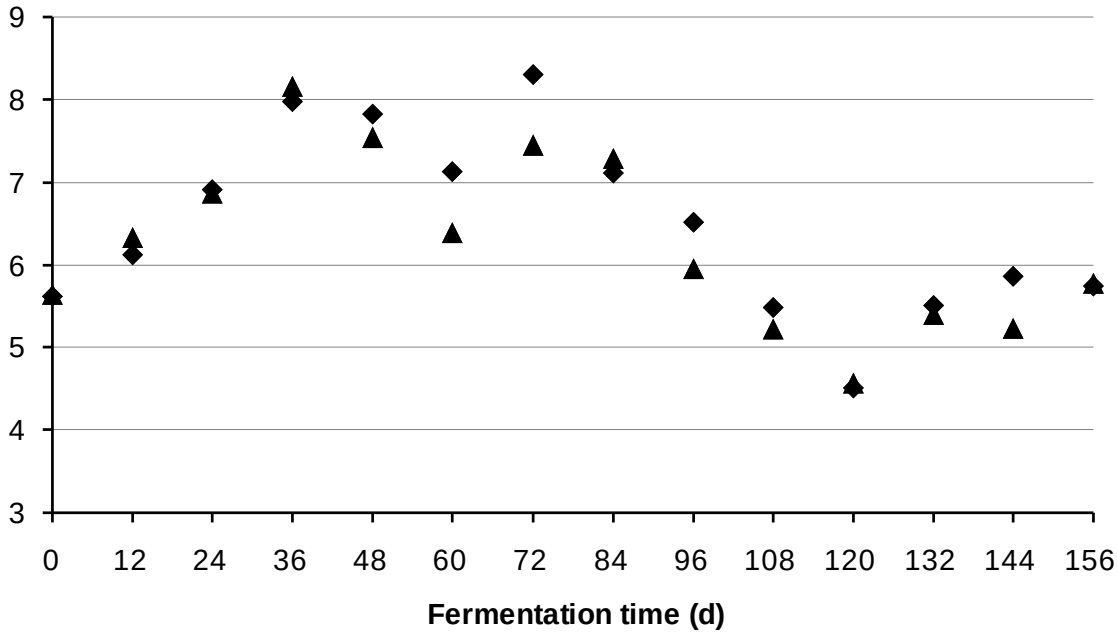

Figure 1. Changes of acetic acid bacteria during cocoa bean fermentation (υ AG medium σCAAR medium).

described Nanda et al. (2001).

RESULTS AND DISCUSSION

Changes in population of AAB during fermentation

Cocoa beans were fermented as traditional indigenous process in Huimanguillo, Tabasco. AAB were present throughout the fermentation time (Figure 1). At the beginning of fermentation, high counts of AAB were detected (5.7 ± 0.26 CFU/g of DM), which show that it did not seem to be any lag phase. AAB counts were similar using two agar culture media, AG, and CAAR. The AAB maximum cell count was observed between 36 and 72 h (8.16 ± 0.43 to 7.45 ± CFU/ g DM). After 72 h a decrease was observed. Our results are consistent with those reported by Ardhana and Fleet (2003). This group reported that BAA contributed from the begining of cocoa fermentation. The decrease in the AAB population after 72 h suggests that in that period a significant decrease in ethanol concentration in cocoa beans has ocurred as a result of consumption by the AAB, and a high concentration of acetic acid has been synthesized. These results are consistent with those reported by Lagunes et al. (2007). They studied the microflora of cocoa fermentation in the Dominican Republic and reported a peak of ethanol concentration at 48 h a very significant decrease at 72 h and at a time a higher acetic acid concentration. This group reported maximum AAB population of $1.5 \pm 1 \times 10^8$ CFU g^{-1} DM. at 48 h. Our results were similar to that observed by Camu et al. (2008) who report AAB counts since the beginning of fermentation of 5.4 to 6.5 log CFU g^{-1} DM, showing a considerable increase from 30 to 72 h (6.8 to 7.6 log CFU g^{-1}DM). In contrast, Ardhana and Fleet (2003), found AAB populations of 10^5 to 10^6 CFU/g DM at 12 h fermentation before declining sharply.

Morphological characteristics

The six strains were preliminarily identified as acetic acid bacteria because they produce acetic acid and detected in the culture medium. All the strains were analyzed using Gram staining. Five strains were Gram-negative (strains ITV61, ITV75 ITV82, ITV98 and ITV114) and two were Gram variable (strains ITV55 and ITV 102).

Scanning electron microscope (SEM) of the strain ITV61 showed ellipsoidal-shape, singly, in pairs or rarely chain. There were not present of flagella (Figure 2). The size of AAB bacteria observed was of 0.4 to 0.6 by 1.4 to 1.6 μm and was slightly different to that reported previously (De Ley et al., 1984; Nanda et al., 2001). In agreement to the previous works the presence of flagella is an important characteristic that differentiates *Acetobacter* and *Gluconobacter* genus, both found in cocoa fermentation. *Gluconobacter* is a genus that may have single flagella but normally have three to eight polar flagella (Yamada et al., 1999). Despite the fact that flagella were not observed in the SEM photograph, it is possible that the strain ITV61 is not-motile which coincides with some AAB strains reported by other authors (De Ley et al., 1984; Kadere et al., 2008).

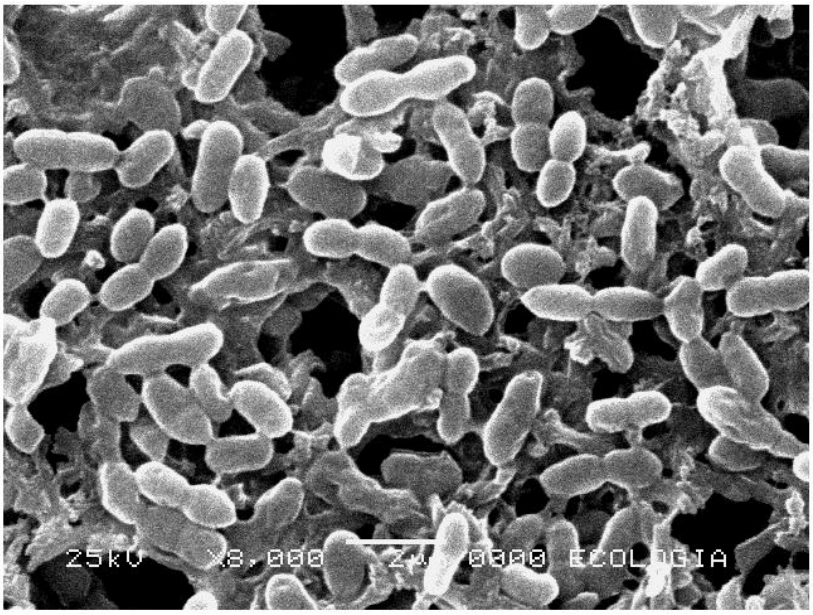

Figure 2. SEM image of *A. tropicalis* ITV61 showing details of the bacterial cells.

Biochemical identification of AAB

AAB strains were subjected to biochemical tests shown in Table 1. All strains were able to oxidize ethanol but were oxidase negative. Moreover all the strains were incapable to hydrolyze cellulose and produce brown pigments. However, in other biochemical tests, strains had different characteristics between them. For example, in contrast to other AAB strains tested, strain 114 had a negative reaction with catalase. On the other hand API system tests (API 20 AUX) showed that the strains ITV55, ITV61, ITV102, and ITV114 were not able to use any carbohydrate as substrate. In general, according to the biochemical characteristics of *Acetobacter* strains shown in Table 1, ITV114 strain was identified as *A. pasterianus*, the identification of the other *Acetobacter* strains was not clear. Given these results we decided to use molecular methods to identify AAB strains.

Molecular characterization of AAB isolates

Specific amplification of the intergenic sequence spanning the 16S rDNAs was achieved by using primers its1 and its2. Two amplified fragments were obtained, 750 and 678 bp for strains ITV75 and ITV114, respectively. Sequences were deposited in GenBank database with accession numbers JF930132 (ITV75), and JF930134 (ITV114). There was no amplification of the strains ITV55, ITV61, ITV82, and ITV98. The lack of amplification of the ITS sequence of these strains is probably due to failure of primer its2 to anneal (Ruiz et al., 2000). The BLAST search of the ITS sequence of the strains ITV75 and ITV114, showed the highest similarity (98%) with *Acetobacter tropicalis* FR716480 sequence (strain LMG 1663) reported in NCBI data base. The homology of the strains compared to other sequences was less than 70%.

To identify the strains ITV55, ITV61, ITV82, ITV 98 and to confirm the identity of the strains ITV75 and ITV114, the 16S DNA sequences were amplified by PCR using the primers Ac1 and Ac3. The following fragments were obtained: 414 bp (ITV 55), 836bp (IT V61), 819 bp (ITV 82), 834 bp (ITV 98), and 407pb (ITV75), and 412 pb (ITV114). The sequences were deposited in the GenBank database with the following access numbers: ITV55 (JF930135), ITV61 (JF930136), ITV75 (JF930133), ITV82 (JF930137), ITV98 (JF930138) and ITV114 (JF930145). The BLAST analysis of the 16S-23S sequence of the strains ITV75 and ITV114 revealed the highest similarity (98 to 100%) with *A. tropicalis* strains shown in the phylogenetic tree generated in this study (Figure 3). Phylogenetic analysis revealed that *Acetobacter* strains isolated in this study are located close to *A. tropicalis* strains (AB052716, JF346075, AM748710, and AB03254). *A. tropicalis* is frequently found in the fermentation of cocoa beans (Schwan et al., 2004). The strains identified in our study differ with the strains identified from the fermentation of cocoa beans in Indonesia by Camu et al. (2008). This group isolated AAB using four different cultivation media and reported a validation of strains with 16S rDNA gene sequencing. The strains they identified were *A. pasteurianus*, *Acetobacter ghanensis*, *Acetobacter senegalensis* and *Acetobacter lovaniensis.* As the fermentation is spontaneous, the difference of strains identified in our

Table 1. Biochemical and API tests of the *A. tropicalis* strains.

Test		Strain isolated					
		55	**61**	**75**	**82**	**98**	**114**
Catalase		W	+	+	W	+	-
Oxidase		-	-	-	-	-	-
Dihydroxyacetone from glycerol		-	-	-	-	+	-
Growth on ethanol 10 %		+	-	-	+	-	-
Growth on methanol		+	W	+	+	-	-
Ability to oxidize glycerol		-	-	-	+	+	-
Ability to oxidize ethanol		+	+	+	+	+	+
Ability to oxidize methanol		+	+	-	-	-	-
Ability to oxidize sodium acetate		-	-	+	-	-	-
Production of brown soluble pigment		-	-	-	-	-	-
Celulose production		-	-	-	-	-	-
API 20 C AUX	none	-	-	-	-	-	-
	D-Glucose	-	-	-	-	+	-
	Glycerol	-	-	-	+	+	-
	Calcium 2-keto-gluconate	-	-	-	+	-	-
	L- Arabinose	-	-	+	-	-	-
	D-Xilose	-	-	+	-	-	-
	Adonitol	-	-	-	-	-	-
	Xilitol	-	-	-	-	-	-
	D-Galactose	-	-	-	+	+	-
	Inositol	-	-	-	-	+	-
	D-Sorbitol	-	-	-	-	-	-
	Methyl-αD-glucopyranoside	-	-	-	-	-	-
	N-Acetyl-glucosamine	-	-	-	-	+	-
	D-Cellobiose	-	-	-	-	+	-
	D-Latose (bovine Origin)	-	-	-	-	-	-
	D-Maltose	-	-	-	-	-	-
	D-Saccharose	-	-	-	+	+	-
	D-Trehalose	-	-	-	-	+	-
	D-Melezitose	-	-	-	-	-	-
	D-Raffinose	-	-	-	-	+	-
	* Weak						

study and those identified by this group could be due to the difference of the microorganisms found in the environment.

Acid acetic production of AAB

The combination of acetic acid and alcohol is essential for cocoa bean death and fermentation (Moreton, 1985). To evaluate the ability to produce acetic acid *A. tropicalis* strains were grown in potato broth medium supplemented with 4% (v/v) ethanol and acetic acid production was determined (Figure 4). *A. tropicalis* ITV61 acetic acid production was increased from 2 to 7 h and at 9 h reached 2.5% (v/v). Strains ITV55, ITV75, ITV82, ITV98 and ITV114 accumulated low acetic acid concentrations (>0.2% v/v). The fact that AAB identified as *A. tropicalis* have different characteristics in their acetic acid production has been described previously. Nanda et al. (2001), isolated strains of *A. pasterianus* from the moromi of komesu and kurosu and reported that the strains produced around 4% of acetic acid, but, differed strongly in the ability to oxidize acetic acid to CO_2 and H_2O. Our results suggest that the *A. tropicalis* ITV61 is dominant AAB in cocoa fermentation. Our results are opposite to those obtained by other authors who report *A. pasterianus* as the dominant AAB. *A. pasterianus* have been reported in cocoa beans fermentation in Ghana and Brazil by Garcia et al. (2010) and in East Java, Indonesia by Camu et al. (2008). In Ghana a AAB strain demonstrated to be tolerant of high concentrations of ethanol (10%) and was detected throughout the cocoa

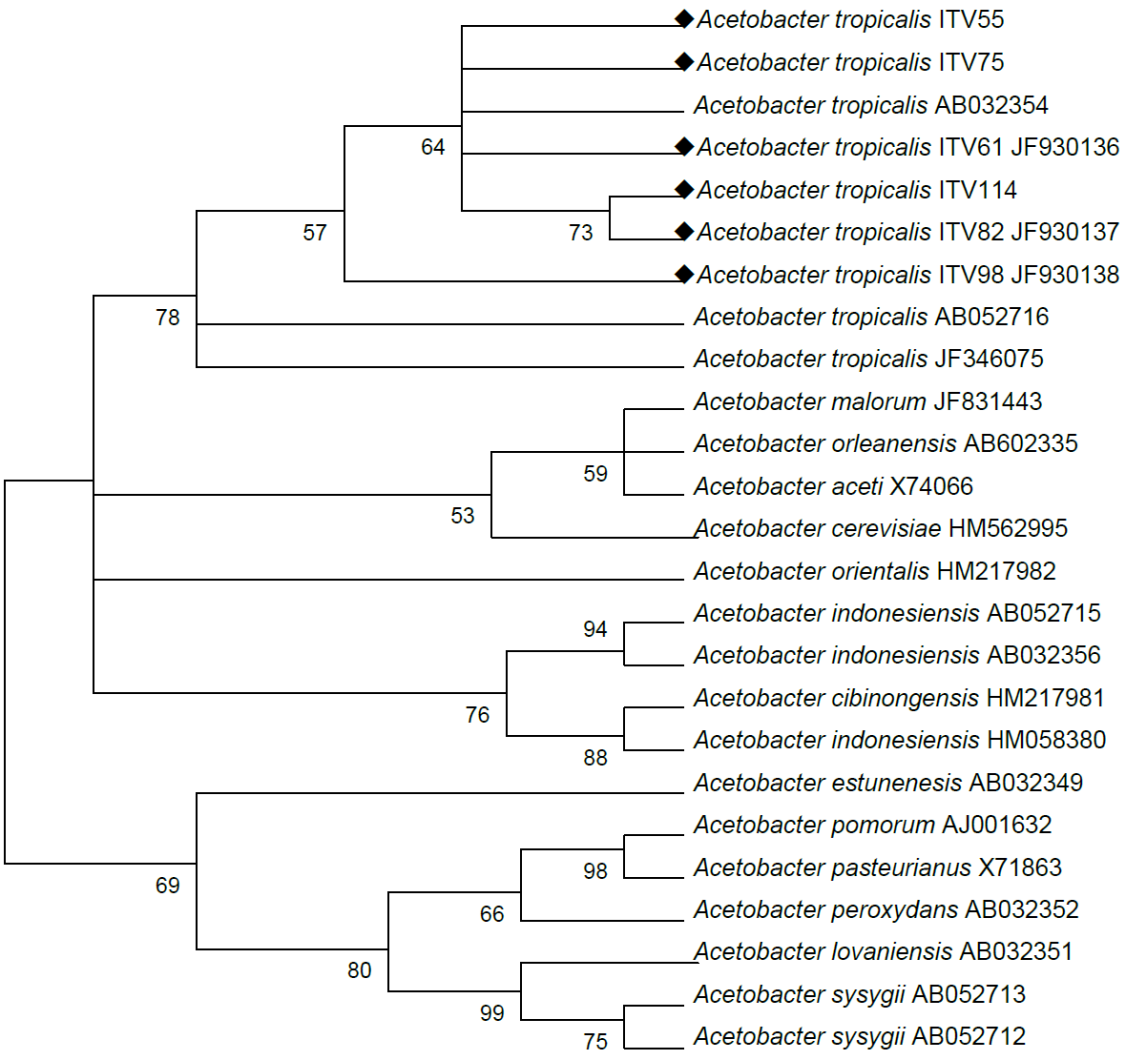

Figure 3. Phylogenetic tree based on 16S rDNA sequence showing the relationship of *Acetobacter tropicalis* isolates (υ) with related strains. Significant bootstrap probabilities values are indicated at the branching points.

fermentation (Ardhana and Fleet, 2003). On the other hand *A. pasteurianus* and *A. tropicalis* have been reported to predominate in traditional fermentations in Ghana (Nielsen et al., 2006). Other not dominant AAB could play a role in the synthesis of compounds related to the aroma and taste of cocoa such as aldehydic and ketogenic compounds and other volatile products (Drysdale and Fleet, 1988).

In order to determine the ability of *A. tropicalis* ITV61 to produce acetic acid from ethanol, kinetics of acetic acid production were performed with 4, 7, and 10% (v/v) of ethanol in the culture medium. Acetic acid production was less than 0.1% (v/v) at 7 and 10% ethanol concentrations (Figure 5). Ethanol concentrations close to 4% seems to be the maximum tolerated by the *A. tropicalis* ITV61 strain to produce acetic acid. These limits are consistent with those reported of 5% during the fermentation of cocoa (Ardhana and Fleet, 2003).

A. tropicalis ITV61 ethanol and acetic acid tolerance

In traditional cocoa fermentation there is a microbial natural selection by acidity and ethanol tolerance. The ethanol concentration differs between cocoa fermentations. There are reports of less that 1% (Lagunes et al., 2007; Schwan, 1998) to concentrations around 5 to 6% (Ardhana and Fleet, 2003). We study the *A. tropicalis* ITV61 ethanol tolerance at 0, 4, 7 and 10% (v/v) concentrations. The strain was able to grow at 0, 4 and 7% of ethanol. However only produce acetic acid at 4% of ethanol (2.38 ± 0.11%) and weakly from 7% ethanol (0.05%± 0.001). Some AAB have shown to tolerate high ethanol concentrations. *A. pasteurianus* is tolerant at 10% ethanol concentration, whereas *A. aceti* has not ability to grow at ethanol concentrations above 7% (Ardhana and Fleet, 2003). High ethanol concentrations (65 to 80%) are used as disinfectant by their capacity

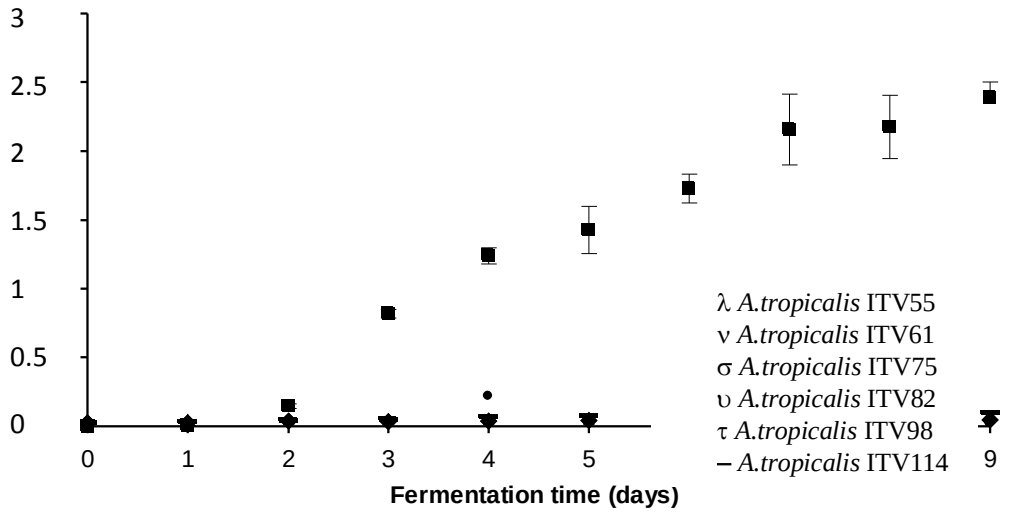

Figure 4. Acetic acid production of AAB isolates in potato broth medium supplemeted with 4% (v/v) of ethanol. C. acetic acid concentration. C0. Initial acetic acid concentration.

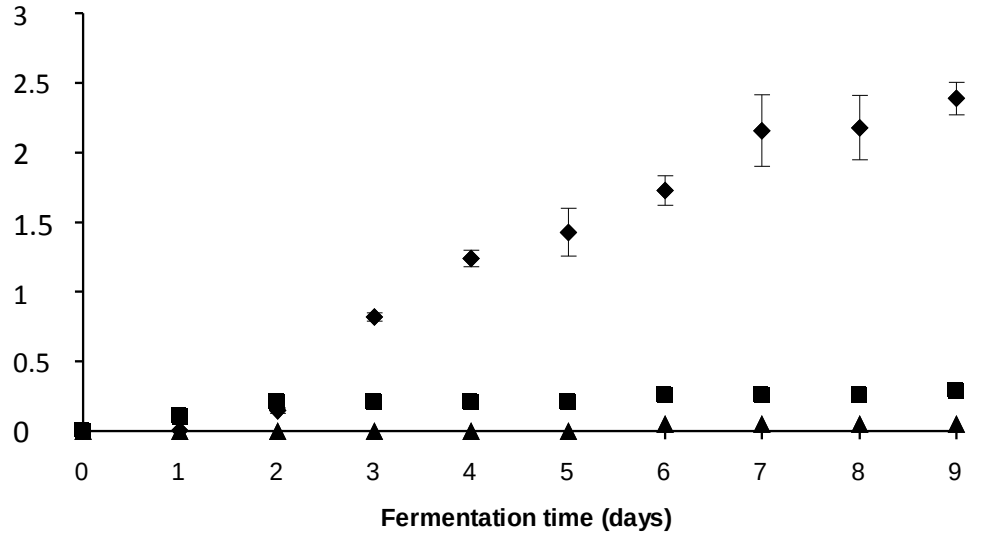

Figure 5. Ethanol tolerance to acetic acid production of *A. tropicalis* ITV61.
♦ 4%, ■ 7%, and ▲ 10% of ethanol concentration.
C=acetic acid concentration C0=initial acetic acid concentration

to dissolve lipids and denature proteins (Madigan et al., 2009). In this sense higher ethanol concentrations could be an important factor for low AAB strains growth.

In order to know the *A. tropicalis* ITV61 acetic acid tolerance, the microorganism was grown in culture medium of potato dextrose supplemented with 1 and 2% (v/v) acetic acid. Tolerance of the strain *A. tropicalis* ITV61 to acetic acid is not affected at concentrations of 1% from the beginning of fermentation; it produces 2.4% (v/v) acetic acid. However at levels of 2% acetic acid from the beginning of fermentation *A tropicalis* ITV 61 does not have the ability to produce acetic acid (Figure 6).

Conclusion

In this study we have identified from the cocoa beans fermentation, six strains of *A. tropicalis* and we have shown that one of them (ITV61) have higher capacity to produce acetic acid, suggesting that is dominant in the fermentation. This strain is tolerant to the conditions of concentration of ethanol and acetic acid that have been

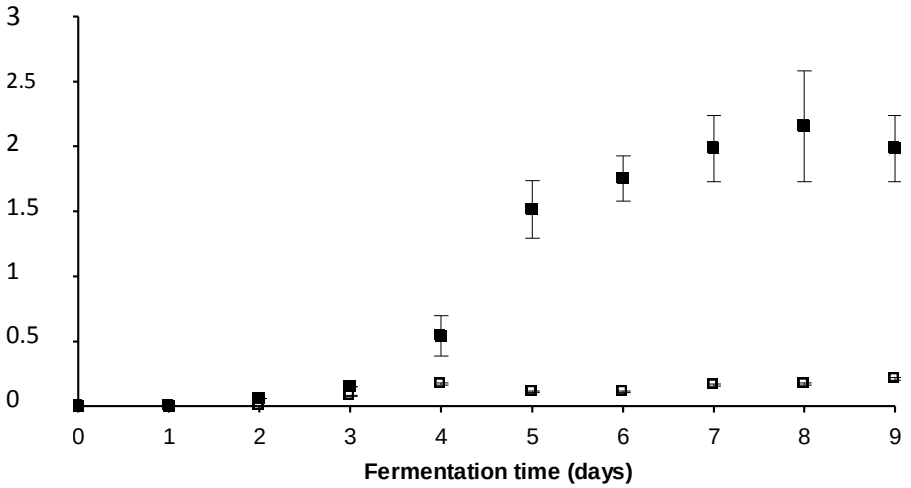

Figure 6. Acetic acid tolerance to acetic acid production of *A. tropicalis* ITV61. ■ 1% and ♦ 2% of acetic acid concentration.C = acetic acid concentration C0=initial acetic acid concentration

reported previously in the fermentation of cocoa.

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