

## ORIGINAL ARTICLE

# Diversity of yeasts during fermentation of cocoa from two sites in the Brazilian Amazon

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## ABSTRACT

The purpose of this study was to identify the yeasts involved in spontaneous fermentation of cocoa from the Brazilian Amazon region. The fermentation process was carried out experimentally with cocoa seeds from two sites (Medicilândia and Tucumã), State of Pará, northern Brazil, during a six-day period. Totals of 44 yeasts were isolated from Medicilândia and 29 from Tucumã. Molecular identification was carried out by sequencing the D1/D2 region fragment of the rRNA 26S gene, expanded with universal primers for the NL1GC and LS2 eukaryotes. *Pichia manshurica* and *Saccharomyces cerevisiae* were identified in Medicilândia and five yeast species (*Pichia fermentans*, *P. kudriavzevii*, *P. manshurica*, *S. cerevisiae* and *Zygosaccharomyces bailii*) were identified in Tucumã. The results showed that *P. manshurica* and *S. cerevisiae* may have potential for use as starter cultures in future studies to improve the quality of cocoa seeds fermented in the Brazilian Amazon region.

**KEYWORDS:** accession; molecular identification; cocoa beans; *Pichia Manshurica*; *Saccharomyces cerevisiae*; *Theobroma cacao*

## Diversidade de leveduras presentes na fermentação de cacau de duas localidades na Amazônia brasileira

### RESUMO

A proposta deste estudo foi identificar as leveduras envolvidas na fermentação espontânea de cacau da Amazônia brasileira. A fermentação foi realizada em Medicilândia e Tucumã, Pará, Brasil, durante 6 dias. Em total foram obtidos 44 isolados de leveduras de Medicilândia e 29 de Tucumã. A identificação molecular foi realizada por sequenciamento do fragmento da região D1/D2 do gene rRNA 26S, amplificado com primers universais para eucariotos NL1GC e LS2. Em Medicilândia, foram identificadas *Pichia manshurica* e *Saccharomyces cerevisiae*. Em Tucumã foram identificadas cinco espécies (*Pichia fermentans*, *P. kudriavzevii*, *P. manshurica*, *S. cerevisiae* e *Zygosaccharomyces bailii*). Os resultados sugerem que *P. manshurica* e *S. cerevisiae* podem ter potencial para uso como culturas *starter* em estudos futuros, para melhorar a qualidade das sementes de cacau fermentadas na Amazônia brasileira.

**PALAVRAS-CHAVE:** acesso; identificação molecular; *Pichia manshurica*; *Saccharomyces cerevisiae*; *Theobroma cacao*

## INTRODUCTION

Up to the mid-1990s, Brazilian cocoa was cultivated almost exclusively in the northeastern state of Bahia. However, since the onset of the witch's broom disease, which devastated cocoa plantations in Bahia in the 1980's and early 1990's, the state of Pará, in the Brazilian Amazon region, became an increasingly important source of cocoa (Castelo and Almeida 2015). In 2016, Pará produced 115,127 tons of fermented cocoa seeds (CEPLAC 2016), and to date it is the more important cocoa producer state in Brazil (IBGE 2017).

The fermentation of cocoa seeds is a natural and spontaneous process that is carried out by a succession of microorganisms (Afoakwa *et al.* 2013; Kadow *et al.* 2013; Krahmer *et al.* 2015). Cocoa fermentation is carried out by yeasts and by acetic and lactic acid bacteria. Yeasts play an essential role in fermentation, since these microorganisms are responsible for the production of ethanol from the fermentable sugars present in the pulp of the seeds, also facilitating the intake of oxygen through degradation of the pulp (Ho *et al.* 2014). Low quantities of oxygen allow for the growth of lactic and acetic acid bacteria, and for the release

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of enzymes that carry out a range of biochemical reactions and which are also producers of higher esters and alcohols, reported as volatile compounds that are essential for the aroma and the flavour of chocolate (Schwan and Wheals 2004; Camu *et al.* 2008; Owusu *et al.* 2011). The influence of the region of origin of the cocoa pods, the fermentation methods used in local producer farms, and the role of microorganisms present during fermentation on the quality of the fermented cocoa seeds have been widely investigated in Cuba, Ecuador, Indonesia and Malaysia (Papalexandratou *et al.* 2011; Papalexandratou *et al.* 2013; Fernández-Maura *et al.* 2016). However, there are no reports in the literature about the microflora present during cocoa fermentation in the Amazon region.

The greatest cocoa producer in Pará is the municipality of Medicilândia (Castelo and Almeida 2015; TNC 2015; CEPLAC 2016), including a significant production of organic cocoa in agroforestry systems (Vronski and Olimpio 2016). Most cocoa in Pará is produced in small family farms (TNC 2018) that still use artisanal processing methods. As a consequence of the lack of adequate processing standards, the cocoa from Pará receives low quality classifications, even though the beans have superior intrinsic characteristics of fusion point and fat content (Valente 2012). Considering that the fermentation processes used are rudimentary, with no knowledge of the active microflora (S.F.O. Almeida, personal observation), the objective of this study was to carry out the molecular identification of the diversity of yeasts present during cocoa fermentation in cocoa seeds from Medicilândia and the municipality of Tucumã, an upcoming cacao producer in southeastern Pará (CEPLAC 2016).

## MATERIAL AND METHODS

### Fermentation

Fermentation experiments were carried out in the cities of Medicilândia (03°26'46"S, 52°53'20"W) and Tucumã (06°44'51"S, 51°09'40"W), Pará State, Brazil. The distance between the two cities is of 963 km. The fermentation processes in each locality were carried out in duplicate in June and July 2014, and all analyses were carried out in the Laboratory for Food Science, Technology and Engineering (LCTEA) of Universidade Federal do Pará - UFPA.

The cocoa pods (belonging to the Forastero group) were manually opened with stainless steel knives 24 hours after harvest, and approximately 70 kg of seeds were placed in a wooden box with a volume of 0.063 m<sup>3</sup> and dimensions of 1.0 m (length) × 0.30 m (width) × 0.25 m (height). The boxes were covered with banana leaves (approximately 2 kg) and burlap bags to maintain the heat produced during fermentation. The fermentation process was monitored for six days. After the first 48 hours of fermentation, the cocoa seeds were turned daily in the boxes to allow for oxygenation and to facilitate adaptation of the acetic bacteria to the medium. Samples of approximately 300 g (consisting of 20 g portions)

were removed from different points in the fermentation box after 0, 24, 48, 72, 96, 120 and 144 hours, placed in sterile polyethylene bags, and frozen immediately after collection.

The temperature was measured during fermentation (at 0, 24, 48, 72, 96, 120 and 144 hours) by insertion of a skewer-type digital thermometer (Incoterm®, model 9791.16.1.00, Porto Alegre, RS, Brazil) into the fermenting mass at different points of the fermentation box (surface, middle and bottom). The pH (970.21, AOAC) was measured according to the Official Methods for Cocoa and its Products (Horwitz 2006).

Isolation, purification and maintenance of isolates

The frozen samples were thawed at room temperature (24 °C), the husks and embryos discarded, and the cotyledons ground using an analytical mill (IKA®, mod. A11b). A 25 g portion of cocoa seeds was aseptically removed from the 300 g sample, transferred to a Stomacher bag and homogenised in 225 mL of peptone water at 1% w/v. Serial decimal dilutions were then prepared up to 10<sup>-7</sup>. Aliquots of 1 mL were plated using the spread plate technique with Petri dishes (n = 3) containing potato dextrose agar and the addition of 1% tartaric acid w/v, and incubated at 30 °C for one to four days. After the incubation period, the yeast colonies were counted using the standard plate counting method to determine the number of colony forming units per gram of seeds (CFU g<sup>-1</sup>) for each point in the fermentation time (Daniel *et al.* 2009). Colonies showing differing morphological characteristics (shape, size and colour) were submitted to purification, taking at least five colonies from each plate.

The isolates were purified in malt extract agar with the addition of 10% tartaric acid. The purified yeast colonies were transferred to YEPD agar (15 gL<sup>-1</sup> bacteriological agar; 5 gL<sup>-1</sup> yeast extract; 20 gL<sup>-1</sup> glucose and 10 gL<sup>-1</sup> peptone) with the addition of 1 mL chloramphenicol /100 mL of medium, and incubated for two days at 30 °C to obtain pure isolates. After the purification step, the isolates were maintained in YEPD medium at 4 °C (APHA 2001).

### DNA extraction and PCR reaction

For the extraction and purification of yeast genomic DNA, a portion of each isolated lineage was transferred to a conical flask containing 50 mL of YEPD broth, and incubated in an orbital shaker (Lucadema®, mod. Luca-223, Campinas, SP, Brazil) at 150 rpm and 30 °C for 12 hours. Aliquots of 1 mL were transferred to conical tubes, centrifuged at 4,000 rpm for 5 minutes, the supernatant discarded and the DNA extracted and purified using the AxyPrep Multisource Genomic DNA Miniprep kit (Axygen, Biosciences®, USA) according to the manufacturer's instructions. The DNA was stored at -20 °C. A 250 bp fragment of the D1/D2 region of the 26S rRNA gene was amplified with universal primers for gc- NL1 eukaryotes (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA TAT CAA TAA GCG GAG GAA AAG-3') and

LS2 (5'-ATT CCC AAA CAA CTC GAC TC-3' Invitrogen®) (Cocolin-Bisson and Mills 2000).

For the PCR we used a mix consisting of 7.4 µL of ultrapure water, 5 µL of betaine, 2.5 µL of 10 x buffer, 1 µL of dNTPs (10 mM); 2.5 µL of each primer, 2 V of magnesium chloride - MgCl<sub>2</sub>, 0.1 µL of Taq DNA polymerase and 2 µL of DNA, giving a total reaction volume of 25 µL. The PCRs were carried out in an Amplitherm® Thermocycler and amplified using the following parameters: initial denaturation at 94 °C for 3 minutes, 30 cycles at 94 °C for 35 seconds, 52 °C for 60 seconds and 72 °C for 75 seconds, followed by a final extension at 72 °C for 10 minutes.

To verify the amplification and the quantity and integrity of the extracted DNA, the PCR products were subjected to electrophoresis in 2% agarose gel.

### Sample sequencing

The PCR products were purified sequentially with 65% isopropanol and 70% ethanol at 4,000 rpm and 10 °C for 45 min and 10 min, respectively. The cycle sequencing reactions were then carried out with ~20 ng of amplified DNA using the Big Dye Terminator kit v. 3.1 (ThermoFisher®), as recommended by the manufacturer. The bi-directional reactions were sequenced and analysed in a DNA Analyzer ABI 3730 (ThermoFisher®, Carlsbad, CA, USA). Sequence assembly with MAFFT v. 7.2 (Katoh and Standley 2013) was based on maximum likelihood carried out using the software Geneious R10 (Biomatters), and the similarity analysis by BLAST searches in the GenBank (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Guindon and Gascuel 2003).

## RESULTS

### Fermentation parameters

As expected, the pH and temperature profiles of the cocoa seeds varied during the fermentation process (Figure 1). The

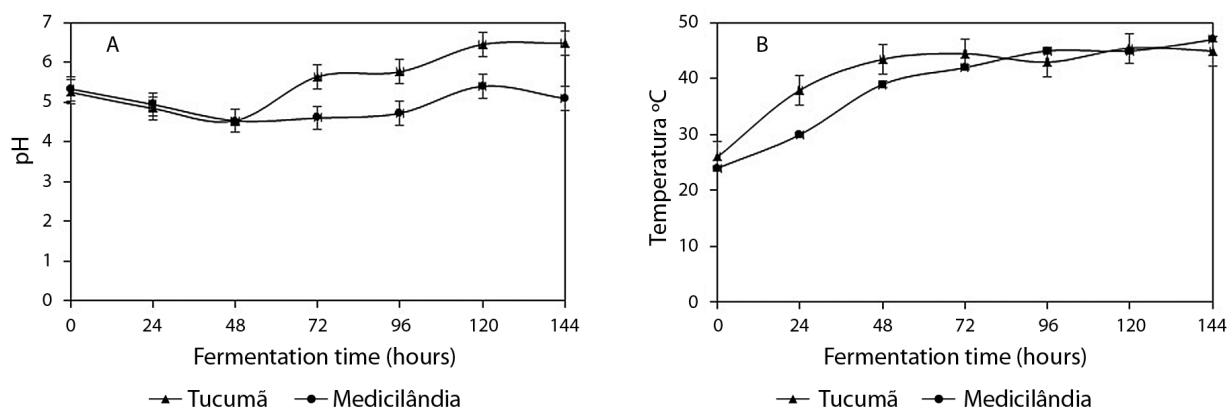
initial pH values were 5.33 and 5.25 for the cocoa seeds from Medicilândia and Tucumã, respectively. In both cases the pH decreased up to 48 hours, followed by an increase up to the end of the fermentation. The pH values at the end of fermentation were 5.0 and 6.48 in Medicilândia and Tucumã, respectively.

The average temperature profiles for the cocoa seeds from both locations showed a significant increase after the first 48 hours of fermentation, with temperatures above 40 °C, mainly due to the acetic bacteria metabolism, which is an exothermic process (Ho *et al.* 2014). In Medicilândia, the highest mean temperature was recorded after 144 hours, when it reached 47 °C (Figure 1B), whereas in Tucumã, the highest mean temperature of 45.5 °C was reached after 120 hours.

### Yeast diversity

Overall 73 yeast isolates were obtained from the two localities (44 in Medicilândia and 29 in Tucumã). The colonies showed morphological differences in colour (white and yellowish-white), general appearance (smooth and rough surfaces) and gloss (bright and opaque). Under the microscope, the yeast cells presented round and oval shapes. After 48 and 120 hours of fermentation only a small number of isolates were obtained at the two localities, which can be attributed to changes and adaptations in the microbial groups.

Yeast count at 0 and 144 hours of fermentation was 10<sup>6</sup> CFU.g<sup>-1</sup> in Medicilândia, while in Tucumã the initial yeast count was 10<sup>8</sup> CFU.g<sup>-1</sup> and reached between 10<sup>5</sup> and 10<sup>7</sup> CFU.g<sup>-1</sup> at the end of the fermentation process (Table 1), confirming the presence of yeasts throughout the entire fermentation process, and not just in the first few hours. *Pichia* and *Saccharomyces* yeasts were predominant at both localities (Table 2). In Medicilândia, two species were identified, *Saccharomyces cerevisiae* (63.4%) and *Pichia manshurica* (36.6%). During the first 24 hours of fermentation, *S. cerevisiae* was dominant among 21 identified isolates. After 96 hours, however, *P. manshurica* showed to be more adapted



**Figure 1.** Average pH (A) and temperature (B) values during the fermentation process of cocoa seeds in the localities of Medicilândia and Tucumã (Pará state, Brazilian Amazon region).

**Table 1.** Average total yeast count (CFU/g cocoa seeds) during cocoa fermentation in two localities in Pará state, Brazilian Amazon region.

Locality	Fermentation time (hours)							
	0	24	48	72	96	120	144	168
Tucumã	1.6x10 <sup>8</sup>	1x10 <sup>7</sup>	5x10 <sup>6</sup>	5x10 <sup>5</sup>	5.1x10 <sup>5</sup>	1.3x10 <sup>7</sup>	1.5x10 <sup>7</sup>	1x10 <sup>7</sup>
Medicilândia	1.8x10 <sup>6</sup>	5.7x10 <sup>5</sup>	6x10 <sup>3</sup>	1x10 <sup>5</sup>	1.3x10 <sup>5</sup>	1x10 <sup>6</sup>	3x10 <sup>6</sup>	1x10 <sup>6</sup>

**Table 2.** Distribution of the dominant yeast species isolated during the fermentation of cocoa seeds in Medicilândia and Tucumã, state of Pará, in the Brazilian Amazon region. The number of isolates per yeast species is shown for samples taken at different fermentation times at each locality. Accession numbers of reference sequences in GenBank are indicated. % identity = identity coincidence between two sequences estimated by BLAST; E value = probability of a random correspondence with a GenBank sequence.

Yeast species	Accession number	% identity	E value	Fermentation time (hours)							
				0	24	48	72	96	120	144	
Medicilândia											
<i>Pichia manshurica</i>	KU316788.1	100	6e-119	1	2	-	-	3	5	4	
<i>Saccharomyces cerevisiae</i>	EU441887.1	99	8e-132	4	17	-	-	1	2	2	
Tucumã											
<i>Pichia fermentans</i>	KU820954.1	100	8e-117	1	-	-	-	1	-	-	
<i>Pichia kudriavzevii</i>	KU316741.1	100	5e-92	-	-	-	1	-	-	-	
<i>Pichia manshurica</i>	KU316788.1	100	5e-119	-	1	-	-	3	1	1	
<i>Saccharomyces cerevisiae</i>	EU441887.1	99	8e-132	1	2	-	-	1	-	-	
<i>Zygosaccharomyces bailii</i>	KJ433981.1	100	2e-123	-	2	-	-	-	-	-	

to the environment, and was the dominant species among a total of 12 isolates. In Tucumã, the diversity of yeasts was greater than in Medicilândia, with five identified species (*Pichia fermentans*, *P. kudriavzevii*, *P. manshurica*, *S. cerevisiae* and *Zygosaccharomyces bailii*). *Pichia manshurica* showed the highest number of isolates (3) throughout the fermentation process. Two *Zygosaccharomyces bailii* isolates were obtained from the 24 hour sample, and contamination by filamentous fungi was observed after 96 hours of fermentation. The role played by filamentous fungi in the microbial succession during the fermentation of cocoa seeds is not well known.

The GenBank accession numbers of the nucleotide sequences determined in this study are MF043900 to MF043908 and MF043882 to MF043899.

## DISCUSSION

The decrease in pH within the first 48 hours of fermentation was the result of the permeation of organic acids from the seed core. The sugars present in the pulp (specially glucose, sucrose and fructose) are primary substrates for yeasts and lactic acid bacteria. The ethanol produced by the yeasts serves as a substrate for acetic acid bacteria, which metabolize the ethanol produced by the yeasts (Galvéz *et al.* 2007). The increase in pH after 48 hours of fermentation was due to the consumption of the citric acid present in the pulp by the yeasts (Galvéz *et al.* 2007; Lefeber *et al.* 2011; Moreira *et al.* 2013). The more neutral pH values found in the present study were similar to those reported in studies on cocoa fermentation carried out in the Ivory Coast and Cuba (Samagaci *et al.* 2014;

Fernández-Maura *et al.* 2016). Cocoa seeds with a final pH below 4.5 show the potential to yield the characteristic flavour of chocolate, while this potential is significantly increased when the pH ranges from 5.0 to 5.5 (Amin *et al.* 2002). The pH values found in the cocoa seeds from both locations can be considered as low acidic seeds, and they produce a 'milder' chocolate with international notoriety in the manufacture of high quality products (Amin *et al.* 2002).

The temperature progression in our study was considered satisfactory for a successful fermentation process (Camu *et al.* 2007, 2008), and was similar to that reported in other studies (Papalexandratou *et al.* 2011; Moreira *et al.* 2013), who found a mean maximum temperature of 46.8 °C after 144 hours of fermentation as a result of microbial metabolic activity.

The presence and growth of yeasts during fermentation can be affected by many factors, such as the initial microflora, the use of starter cultures, the chemical composition of the cocoa seeds, the fermentation temperature and the interactions between different microorganisms (Gomes 2007). Arana-Sánchez *et al.* (2015) and Menezes *et al.* (2016) reported microbial counts of around 10<sup>8</sup> and 10<sup>9</sup> for *Saccharomyces* during the fermentation of cocoa in Mexico and Brazil, which is similar to those found in the present work. In a study carried out in Australia, values of 10<sup>6</sup> and 10<sup>7</sup> CFUg<sup>-1</sup> were reported after 72 hours of cocoa fermentation (Ho *et al.* 2014), suggesting that cocoa fermentation in other climate zones can affect microbial growth. *Pichia manshurica* is able to metabolize some acids, including lactic acid (Franco and Pérez-Díaz 2012), which could explain the predominance of this species in the last days of the cocoa fermentation process.

*Saccharomyces cerevisiae* is the most commonly reported yeast during alcoholic fermentation in several fermentation processes, due to its ability to produce and tolerate high concentrations of ethanol and sugars in an acid medium (Zinnai *et al.* 2013). In Brazil and in other countries (Ghana, Mexico, Malaysia), *S. cerevisiae* is the dominant species in the fermentation of cocoa, due to its ability to adapt to changes in pH and temperature, and also due to its low nutritional needs (Papalexandratou *et al.* 2011; Pereira *et al.* 2012; Moreira *et al.* 2013; Papalexandratou *et al.* 2013; Arana-Sánchez *et al.* 2015; Batista *et al.* 2015). *Pichia manshurica*, on the other hand, was only known from cocoa fermentation in one study carried out in Cuba (Fernández-Maura *et al.* 2016). Our results suggest that both *Saccharomyces cerevisiae* and *Pichia manshurica* are important yeasts in cocoa fermentation in the Amazon region, and their presence throughout the fermentation processes highlights the resistance of these yeasts to the environmental conditions during fermentation. *Zygosaccharomyces bailii* strains, which were isolated only from the initial 24 hours of fermentation in Tucumã, has been reported to produce unwanted changes in food, specially in wine fermentation, due to excessive production of carbon dioxide (CO<sub>2</sub>) and alteration of sensory characteristics (Zuehlke *et al.* 2013).

## CONCLUSIONS

We identified five yeast species during the spontaneous fermentation process of cocoa seeds in two localities in Pará state, in the Brazilian Amazon. The detection of *Pichia manshurica* is unprecedented in cocoa fermentations in this region. *Saccharomyces cerevisiae* and *P. manshurica* should be further examined for their role in the quality of cocoa from the Brazilian Amazon and their potential for the development of starter cultures.

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