

# Fermentation of cacao (*Theobroma cacao* L.) seeds with a hybrid *Kluyveromyces marxianus* strain improved product quality attributes

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## Keywords

chocolate; cocoa; DGGE; sweatings.

## Abstract

Fermentation of *Theobroma cacao* (cacao) seeds is an absolute requirement for the full development of chocolate flavor precursors. An adequate aeration of the fermenting cacao seed mass is a fundamental prerequisite for a satisfactory fermentation. Here, we evaluated whether a controlled inoculation of cacao seed fermentation using a *Kluyveromyces marxianus* hybrid yeast strain, with an increased pectinolytic activity, would improve an earlier liquid drainage ('sweatings') from the fermentation mass, developing a superior final product quality. Inoculation with *K. marxianus* increased by one third the volume of drained liquid and affected the microorganism population structure during fermentation, which was detectable up to the end of the process. Introduction of the hybrid yeast affected the profile of total seed protein degradation evaluated by polyacrylamide gel electrophoresis, with improved seed protein degradation, and reduction of titrable acidity. Sensorial evaluation of the chocolate obtained from beans fermented with the *K. marxianus* inoculation was more accepted by analysts in comparison with the one from cocoa obtained through natural fermentation. The increase in mass aeration during the first 24 h seemed to be fundamental for the improvement of fermentation quality, demonstrating the potential application of this improved hybrid yeast strain with superior exogenous pectinolytic activity.

## Introduction

*Theobroma cacao* L. (cacao) seeds are the unique source of cocoa butter and solids, essential raw materials for chocolate production (Pires *et al.*, 1998). The development of chocolate flavor requires that seeds are fermented and dried (also known as cacao beans), which are essential for obtaining flavor precursors, which are fully expressed later during the roasting process (Lopez & Dimick, 1995). Cacao seeds are surrounded by an aromatic mucilaginous pulp, which arises from the seed teguments (technically an aril) (Figueira *et al.*, 1993). The seed pulp represents around 40% of the seed fresh weight (FW), and is composed of a spongy parenchymatous tissue containing cell sap, which is rich in sugars (10–13% glucose and fructose; 0.7% sucrose), salts

(8–10%), pentosans (2–3% pectin), organic acids (1–2%) and 0.6% proteins (Roelofsen, 1958; Lopez, 1986; Schwan & Wheals, 2004). During on-farm processing, the seed-surrounding pulp is degraded by fermentation, hydrolyzed by microorganisms, producing a running liquid pulp known in the industry as 'sweatings.' Fermentation was once thought to be simply an easy way to remove the pulp to facilitate drying, but its importance to cacao quality has been well established (Lopez, 1986).

Traditionally, cacao seed fermentation is a noncontrolled process initiated by microorganisms naturally occurring at fermentation sites, including yeasts, lactic and acetic bacteria, *Bacilli*, and filamentous fungi (Roelofsen, 1958; Ostovar & Keeney, 1973; Schwan, 1998). These fermenting organisms utilize the seed pulp as a carbon and nitrogen source.

At the onset of fermentation, the presence of the seed pulp reduces oxygen diffusion within the fermenting seed mass, creating anaerobic conditions (Schwan *et al.*, 1995). During this stage, yeasts and lactic bacteria consume pulp sugars and organic acids, producing ethanol and lactate (Lopez, 1986). However, after the first 48 h, pectinolytic yeasts degrade the seed pulp, draining the liquid trapped in the parenchymatous pulp, increasing aeration and favoring the establishment of acetic bacteria. Production of acetate from ethanol oxidation produces carbon dioxide and heat, gradually increasing the temperature of the fermenting seed mass, which can reach values close to 50 °C (Schwan & Wheals, 2004). Heat and acetic acid activity cause death of the seed embryos, with consequent loss of the membrane-selective permeability (Forsyth & Quesnel, 1963; Biehl *et al.*, 1982a). After embryo death, seed enzymes (e.g. proteases, polyphenol oxidases) and substrates (e.g. anthocyanins, flavanols, phenols and storage proteins), previously separated in specialized cells and/or compartments, interact and react in a specific manner in response to heat and the decrease in pH (Thompson *et al.*, 2001). Anthocyanin degradation and polyphenol oxidation result in browning of the typical violet cotyledons, while oxidation and condensation of polyphenols reduce the astringency usually found in nonfermented cacao seeds (Forsyth *et al.*, 1958). Amino acids generated during fermentation by proteolysis of vicilin storage proteins, by natural seed-occurring aspartic proteinases and carboxipeptidases (Laloi *et al.*, 2002), appear to be the precursors of chocolate flavor (Rohan, 1965; Biehl *et al.*, 1982a; Lerceteanu *et al.*, 1999), after reacting with reducing sugars during roasting in a Maillard reaction (Voigt *et al.*, 1994a, b). These changes detected in fermented cacao beans, together with residual lactic and acetic acid, known as fixed and volatile acidity, respectively, can be used as an index for evaluation of fermentation success and seed quality (Bonvehí & Coll, 1997). Therefore, the products from fermentation (ethanol, acetate and lactate) plus heat directly affect the bean components, causing important biochemical changes leading to the development of typical chocolate color, flavor, and aroma precursors (Thompson *et al.*, 2001).

Fermentation conditions can reduce the quality of resulting beans, such as an increase in nonvolatile acidity, a serious defect for many manufacturers (Jinap & Zeslinda, 1995). In Brazil, fermented seeds traditionally contain high levels of acidity, depreciated in the world market, but apparently not caused by local fermenting microbial communities (Schwan & Wheals, 2004). The diversity of yeasts associated with cacao seed fermentation is heterogeneous, varying in terms of location, producing country, climate conditions and fermentation method and duration (Schwan *et al.*, 1995; Ardhana & Fleet, 2003; Jespersen *et al.*, 2005). One of the recognized problems in reducing cacao quality is

the excess of seed pulp, which conditions more nutrients (e.g. excess of fermentable sugars) and decreases oxygen diffusion in the fermenting mass, leading to a slow fermentation and excess production of lactate and ethanol (Biehl *et al.*, 1991; Jinap & Zeslinda, 1995). Reduced initial diffusion of oxygen favors the production of lactic acid, and during the intermediate phase of fermentation, decreases the oxidation of acetate to CO<sub>2</sub> and water by acetic bacteria, increasing the residual acidity of seeds. The technique of periodically turning the fermenting seeds favors aeration, reducing residual levels of acetate and lactate in fermented beans (Thompson *et al.*, 2001), an effect that can be improved by partial removal of pulp before fermentation. Pressing freshly harvested seeds can reduce pulp by up to 10–15% FW; alternatively, increasing aeration during the initial stages of fermentation (Wood, 1985) and seed centrifugation or washing can give similar results (Schwan & Wheals, 2004). Mechanical removal of pulp can be improved by the addition of industrial pectin-degrading enzymes (Wood, 1985). However, mechanical or enzymatic extraction of seed pulp increases the cost of production, and it has not been viable in large-scale operations. One possibility would be the adoption of artificial inoculation of cacao fermenting seeds with yeasts that secrete pectinolytic enzymes (Freire *et al.*, 1999).

Many yeast species have been identified from cacao fermentation (Nielsen *et al.*, 2007), including *Kluyveromyces marxianus*, *Saccharomyces cerevisiae* var. *chevalieri*, *Candida rugopelliculosa* and *Kluyveromyces thermotolerans*, all of which display relevant pectinolytic activity (Lehrian & Patterson, 1983; Sanchez *et al.*, 1985; Schwan & Wheals, 2004; Nielsen *et al.*, 2007). However, the insufficient activity of these wild pectinolytic strains in natural fermentation limits the amount of drained pulp ('sweatings'), because of the limited starting inoculum (Sanchez *et al.*, 1985; Buamah *et al.*, 1997). Artificial inoculation of cacao fermentations has been evaluated to improve the production of 'sweatings' (Sanchez *et al.*, 1985; Buamah *et al.*, 1997). Under laboratory conditions, artificial inoculation of fermenting cacao seeds with strains of *S. cerevisiae* var. *chevalieri* and *Kluyveromyces fragilis* with pectinolytic activity improved liquid draining, while improving the final product quality (Sanchez *et al.*, 1985; Buamah *et al.*, 1997). However, quality improvement obtained from artificial inoculation in small-scale laboratory fermentations has been questioned (Schwan & Wheals, 2004).

Considering that reduction in seed pulp volume could improve the final quality, the objective of this work was to evaluate whether artificial inoculation with a *K. marxianus* hybrid strain with high pectinolytic activity would increase liquid draining from fermenting seed mass, with a consequent improvement in the final bean quality in comparison with natural conditions.

## Materials and methods

### Microorganisms and hybrid development

The yeast *K. marxianus* hybrid strain MMIII-41 was obtained from endocrossing between the *K. marxianus* parental strains IZ1339 and IZ619 using a breeding protocol (Mortimer & Hawthorne, 1975) to select for higher extracellular inulinase activity (Laguna, 1986) at the Laboratory of Yeast Genetics, Universidade de São Paulo, Brazil. Briefly, the strains IZ1339 and IZ619 were induced to sporulate on RA media (0.02% Raffinose, 0.3% potassium acetate, pH 7.0) for 7 days. Cultures were centrifuged at 1300 g and treated with 10 U of Zymolyase 5000 (Seikagaku Kogyo Co Ltd, Tokyo, Japan) at 0.1% yeast cell FW, for 10 min at 30 °C to release ascospores. The spores were then centrifuged and resuspended in 0.9% NaCl twice. The spore suspensions from both parents were treated with a 15 W ultraviolet light (253 nm) and screened for auxotrophic mutants according to Snow (1966). Haploid mutants from each parental strain were selected for Arginine- or Methionine-auxotrophy, and tested for inulinase activity; those with improved activity in comparison with the parental strains were crossed to obtain hybrids. The hybrids were then selected on Yeast Nitrogen Base media (Becton Dickinson Microbiology Systems, MD) without amino acids. Hybrids with higher inulinase activity than the parental strains were induced to sporulate. The processes of haploid production, crossing and selection were repeated twice, in a total of three crossing and selection cycles (MMI, MMII and MMIII), with the selection of the hybrid strain MMIII-41 at the third cycle. Cultures were preserved in 15% glycerol at –80 °C.

An aliquot from the glycerol stock of the strain MMIII-41 was grown in 250 mL of YEP + sucrose medium (1% peptone, 1% yeast extract and 2% sucrose) in an orbital shaker at 200 r.p.m. at 30 °C for 24 h. This culture was divided over eight flasks containing 1 L YEP + sucrose medium each and grown at 200 r.p.m. at 30 °C for 48 h. Cultures were then centrifuged at 2500 g for 10 min, and the pellet cells were combined and resuspended in 40 mL of YEP + sucrose medium and stored at 4 °C until used to inoculate two cacao fermentation replicates.

Pure cultures of *S. cerevisiae* (Fleischmann & Royal Ltda., São Paulo, Brazil), *Hansenula anomala* (IZ1420), *K. marxianus* (MMIII-41), *Endomycopsis fibuligera* (CBS2527), *Candida pseudotropicalis* (IOC2697), *Pichia membranifaciens* (IZ379), and *Candida tropicalis* (IZ1824) were from the stock collection of the Laboratory of Yeast Genetics, originally obtained from the collection of the 'Instituto Zimotécnico' (IZ), Universidade de São Paulo, Brazil. The cultures were maintained in 15% glycerol at –80 °C. An aliquot (1 mL) of each species was inoculated in 50 mL YEPD medium (1% peptone, 1% yeast extract, 2% dextrose) and grown at

200 r.p.m. at 30 °C for 24 h. The cultures were centrifuged at 3000 g for 10 min, and cell pellets were used for DNA extraction.

### Evaluation of pectinolytic activity of the MMIII-41 hybrid and the parental strains

Isolated colonies from the strains IZ1339, IZ619 and MMIII-41 were cultivated on 10 mL of YEP medium (1% peptone, 1% yeast extract, 2% sucrose) at 30 °C for 24 h at 200 r.p.m. One milliliter from each of these cultures was then used to inoculate 100 mL of YEP medium with pectin (1% peptone, 1% yeast extract 2% pectin), and grown at 30 °C for 48 h at 200 r.p.m. The yeast cultures were then centrifuged at 2500 g for 20 min at 4 °C, and the culture supernatants were used to estimate pectinolytic activity using a polygalacturonase assay. Polygalacturonase activity was determined by reacting 1 mL of supernatant in 5 mL 0.5 M potassium phosphate buffer (pH 7.0) containing 0.1% pectin (w/v) at 40 °C for 10 min. Boiled supernatants were evaluated as negative controls. Protein concentration was determined according to Bradford (1976). Total reducing sugars in solution were determined in 1-mL aliquots according to Somogy (1945) and Nelson (1944), measuring changes in optical density by spectrophotometry at 520 nm. Galacturonic acid (Sigma) was used as a standard, and 1 U of polygalacturonase activity was defined as the amount of the enzyme that catalyzed the formation of 1 µmol of galacturonate per microgram of protein per minute at 40 °C. The experiment was performed in triplicate for each yeast strain.

### Fermentation experiment

The experiment was conducted at a cacao farm located in Itamaraju municipality, Bahia state, Brazil. Fully mature cacao pods were harvested and broken open with machetes, and seeds were manually removed and transported to the site of fermentation on the same day. Seeds were mixed and divided into four batches of 45 kg. Two batches were used for natural fermentation, while the other two were inoculated with MMIII-41 yeast lineage at a rate of 0.1% FW, equivalent to  $2.6 \times 10^6$  cells kg<sup>-1</sup> of cacao seeds with 90% cell viability. New plastic laundry baskets (1 m height, 0.3 m smaller diameter and 0.5 m higher diameter) (Clapperton *et al.*, 1994; Figueira *et al.*, 1997) were cleaned with detergent and rinsed with tap water, before they were separately filled with each of the fermentation treatments. The baskets had holes at the bottom from where liquids could drain to individual collecting vessels. The baskets were covered with banana leaves to reduce heat loss, and fermentation proceeded for 144 h, with the cacao seed mass being turned at 48, 72, 96, 120 and 144 h. Afterwards, fermented seeds were dried on a stainless-steel screen of an artificial convection platform dryer (Wood, 1985) for 12 h with periodic cacao

bean turning. During fermentation, temperature was monitored every 24 h at three heights (0, 50 and 90 cm from bottom), each at three sites. Ambient temperature was also monitored at the same time. Volume of drained liquid 'sweatings' was measured, and seed samples (50 g FW) were collected at every 24 h up to 120 h after fermentation began. Samples were used for total seed protein extraction and profiling by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and for DNA isolation from fermenting pulp to analyze the yeast genetic diversity using denaturing gradient gel electrophoresis (DGGE). A 2-kg sample of dried fermented beans was obtained for evaluation of quality using the bean-cut test (Wood, 1985) of pH and total acidity. These bean samples were later roasted and ground to produce cocoa liquor and chocolate products for sensorial analyses (described below).

#### DNA extraction from yeast pure cultures and from fermenting cacao seed pulp

The cell pellets from *C. pseudotropicalis*, *C. tropicalis*, *E. fibuligera*, *H. anomala*, *K. marxianus* (MMIII-41), *S. cerevisiae* and *P. membranefaciens* were used for DNA extraction using the Genomic DNA Purification kit (Fermentas, Ontario, Canada). DNA was also isolated from fermenting seed pulp samples using a suspension of five seeds in 30 mL saline solution (0.85% NaCl) containing 15% glycerol. Three-milliliter suspension aliquots from each treatment were spun at 12 000 g for 10 min, and the pellet was used to extract DNA using the Genomic DNA Purification kit as above. Extractions were conducted in duplicate.

#### Amplification reactions from fermenting seed pulp for DGGE analyses

DNA from fermenting cacao seed pulp and from the yeast pure cultures (as positive controls) were analyzed. Amplification of the D1 domain of the 26S ribosomal gene was based on a PCR nested approach (Kurtzman & Robnett, 1998; Masoud *et al.*, 2004). The amplification reactions contained 25 ng of DNA, 1.5 mM MgCl<sub>2</sub>, 100 μM of each dNTP, 0.2 μM of each primer and 1.5 U *Taq* polymerase in 50 mM KCl, 20 mM Tris-HCl pH 8.4 buffer (Invitrogen, São Paulo, Brazil) in a final volume of 50 μL. Amplifications were conducted on a GeneAmp 9600 thermocycler (Applied Biosystems, Foster City, CA), programmed with 35 cycles of 60 s at 94 °C, 45 s at 50 °C and 60 s at 72 °C, followed by a final cycle of 7 min at 72 °C. The first reaction used the external primers NL4 (5'-GGTCCGTGTTTCAAGACGG-3') and NL1 (5'-GCCATATCAATAAGCGGAGGAAAG-3') amplifying a c. 600-bp fragment (Kurtzman & Robnett, 1998). One-microliter aliquots from each reaction were then used in reamplification reactions under the same conditions, but using the internal primers NL1-GC (5'-CGC

CCGCCGCGCGGGCGGGCGGGGCGGGGGCCATATCAATAAGCGGAGGAAAG-3') and LS2 (5'-ATTTCCTCAAACAACTCGACTC-3') (Kurtzman & Robnett, 1998; Cocolin *et al.*, 2000) amplifying a c. 250-bp fragment. Reactions were conducted in duplicate. Amplification products were first analyzed in 1.5% agarose gels stained with ethidium bromide, followed by analyses by DGGE according to Muyzer & Smalla (1998), using a CBS Scientific DGGE system (CBS Scientific, Del Mar, CA). The 8% (w/v) polyacrylamide gel (37.5:1 acrylamide:bisacrylamide) was prepared using 1 × TAE (40 mM Tris, pH 8.5; 1 mM EDTA; 20 mM acetic acid) using a GM-40 Gradient Maker (CBS Scientific) in a 20–80% denaturing range [100% denaturing corresponding to 7 M urea and 40% (v/v) formamide]. DGGE was conducted at constant 70 V for 16 h at 60 °C. The gel was then stained with SYBR-Green Nucleic Acid Stain (Bio-Rad, Hercules, CA) for 20 min and photographed in an ultraviolet transilluminator using the Kodak Digital Science DC120 system (Eastman Kodak, New Haven, CT). Band profiles were converted into binary matrix (absence × presence), which was analyzed using NTSYS-PC (Exeter Software, Setauket, NY). Similarity was estimated based on the Dice coefficient, while grouping used UPGMA (unweighted pair group method with arithmetic average clustering algorithm).

#### Total seed protein analyses by SDS-PAGE

The sample of seeds collected during fermentation and after drying was analyzed for total protein profiles. Seeds (2 g) were freeze-dried for 24 h and ground in liquid nitrogen. 200-mg samples of seeds were extracted in 500-μL dissociation buffer (0.5 M Tris-HCl, pH 6.8; 18% glycerol; 20% SDS; 0.6 M β-mercaptoethanol; 0.05% blue bromophenol). Samples were boiled for 5 min, spun at 12 000 g for 10 min and the supernatant was used to determine the protein concentration (Bradford, 1976). Around 6 μg of total protein was loaded onto SDS-PAGE (3% stacking and 12.5% running gel) and run at 50 mA for 2 h at 10 °C (Laemmli, 1970). The gels were stained with 0.1% Coomassie brilliant blue.

#### Quality evaluation of fermented seeds, cocoa liquor and chocolate production

Three random samples of 100 cacao beans from each treatment were analyzed by the cut-test according to local regulations ('Resolução no. 160, Conselho Nacional de Comércio Exterior'; Brasil, 1988). Fermented and dried beans were processed separately and broken into 'nibs' in a knife mill (ICMA, Campinas, SP, Brazil). The nibs were then roasted in a PRE 17 Probat-Werke laboratory roaster at 150 °C for 38 min, according to the parameters defined by Fadini (1998), before being milled and refined in a Draiswerk three-roll refiner (Draiswerk Inc., Mahwah, NJ) down

to a 40- $\mu\text{m}$  granulometry into cocoa liquor. The liquors were conched for 3 h at 70 °C in a longitudinal mini-conche (Friwessa, Parsippany, NJ). Dark chocolates were prepared with 56% cocoa liquor, 43.6% of milled sugar and 0.4% of soy lecithin according to the conventional small-scale method (Luccas *et al.*, 2002) by mixing ingredients in a planetary mixer (model K5SS; Kitchen-Aid, St Joseph, MI), refining and conching for 12 h at 70 °C in the Friwessa mini-conche, and tempering in a ACMC laboratory temper (ACMC, Bohemia, NY). Chocolates were molded in polycarbonate molds and cooled in a cooler tunnel (Siaht, Jundiaí, SP, Brazil) (Beckett, 1994). Chocolates were produced in batches of 700 g. Liquor and chocolate production were conducted at the 'Instituto de Tecnologia de Alimentos – ITAL,' Campinas, São Paulo, Brazil.

### Chocolate sensorial analyses

The chocolates were analyzed using an acceptability test with 30 invited nontrained panelists (age 18–60 years). Samples were evaluated for aroma, flavor and general impression using a 9-cm unstructured line hedonic scale ranging from 'extremely dislike' to 'extremely like' (Meilgaard *et al.*, 1988). Tests were carried out in individual air-conditioned booths (23 °C) using a complete balanced block (MacFie *et al.*, 1989) and sequentially monadic form. The results were analyzed by analysis of variance (ANOVA) using two factors (consumer and sample) and Tukey's honestly significant difference (HSD) for hedonic scales. The statistical analyses were carried out using the software STATISTICAL ANALYSIS SYSTEM v. 8.6e (SAS, 1993).

## Results

### Pectinolytic activity of the MMIII-41 hybrid and the parental strains

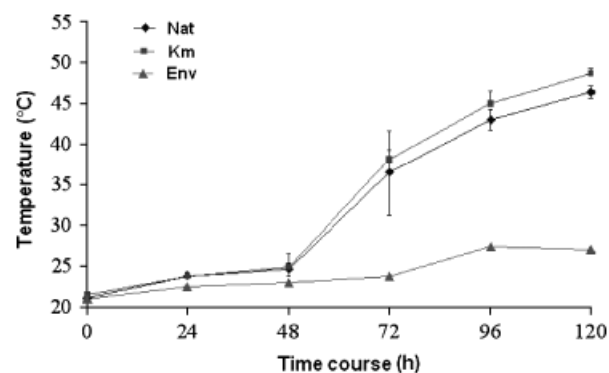
The parental strain IZ1339 and IZ1619 presented a pectinolytic activity of  $5.98 \pm 0.33$  and  $7.61 \pm 0.09$  U ( $\mu\text{M}$  galacturonic acid  $\mu\text{g}^{-1}$  protein  $\text{min}^{-1}$  at 40 °C), respectively. The resulting MMIII-41 hybrid strain obtained from crossing and selection revealed a pectinolytic activity of  $34.80 \pm 0.64$  U.

## Fermentation

Both treatments exhibited a normal fermentation, with a typical increase in temperature and producing beans with normal external visual appearance. The temperature of the fermenting cacao seed mass under natural conditions or inoculated with *K. marxianus* began to increase 24 h after the beginning of fermentation (Fig. 1), in comparison with ambient temperature ( $24.1 \pm 2.6$  °C), becoming more evident after 72 h. In general, temperatures from *K. marxianus* inoculated seed mass were 2° above, but not statistically different from the natural fermentation treatment after 72 h (Fig. 1). The total volume of drained liquid from the fermenting seed masses ('sweatings') was on average 11.8% of the seed fresh weight (5.30 L from 45 kg seeds) for the natural fermentation and 14% (6.29 L from 45 kg seeds) for the treatment inoculated with *K. marxianus* (Table 1), which were not statistically different. The largest volume of liquid drain occurred 24 h after fermentation started, when 59.4% of the total volume (3.15 from 5.30 L) was lost from the natural fermentation and 66.7% (4.20 from 6.29 L) from the inoculated one (Table 1).

### DNA profiling by DGGE from yeasts from fermenting pulp

According to the established banding pattern using distinct yeast species, it was possible to detect *K. marxianus* in the



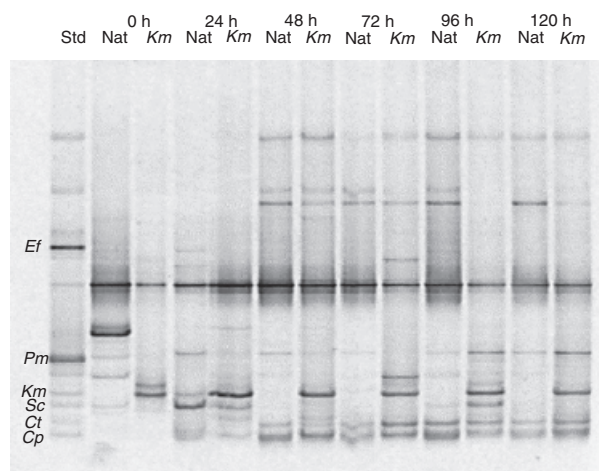
**Fig. 1.** Temperature (°C) inside the fermentation seed mass (natural fermentation, Nat; fermentation inoculated with *Kluyveromyces marxianus*, Km) and the surrounding environment (Env) measured at 24-h intervals. Presented values are average of readings at three heights within the fermentation basket (lower, middle, top).

**Table 1.** Volume of drained liquid intervals and total accumulation ('sweatings') from cacao seed fermentation at 24-h intervals

Treatment	Liquid drained volume accumulated for each 24 h (L)				Total volume accumulated (L)
	1 h	24 h	48 h	72 h	
Natural	1.05 ± 0.07	3.15 ± 0.49	0.73 ± 0.18	0.32 ± 0.14	5.30 ± 0.53
Inoculated with <i>K. marxianus</i>	1.20 ± 0.1	4.20 ± 0.42	0.73 ± 0.18	0.13 ± 0.05	6.29 ± 0.65

All values in the table did not differ significantly.

inoculated fermentation at all the sampled periods (Fig. 2) and at 24 h for the natural fermentation treatment. Based on the DGGE profiles, migration patterns similar to *C. tropicalis* (Ct), *C. pseudotropicalis* (Cp), *K. marxianus* (Km) and *S. cerevisiae* (Sc) were also detected in the natural fermentation. Under natural conditions, the putative fragment from *K. marxianus* could be detected at 24 and 96 h, whereas the

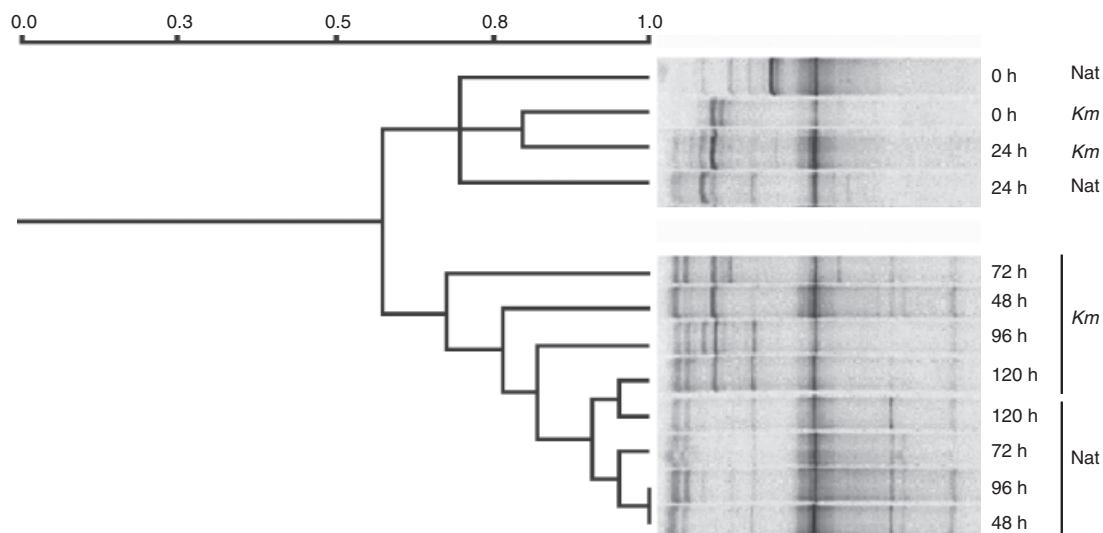


**Fig. 2.** DGGE profiles from amplified fragments of the 26S ribosomal gene derived from yeast of the fermenting pulp. Samples from natural fermentation (Nat) or fermentation inoculated with *Kluyveromyces marxianus* (Km). Yeasts were obtained from fermenting pulp extracted by washing five seeds with saline solution at 0, 24, 48, 72 and 120 h. Standards were derived from a mixture of DNA extracted from pure culture from yeast species *Endomycopsis fibuligera* (Ef), *Pichia membranifaciens* (Pm), *K. marxianus* (Km), *Saccharomyces cerevisiae* (Sc), *Candida tropicalis* (Ct) and *Candida pseudotropicalis* (Cp).

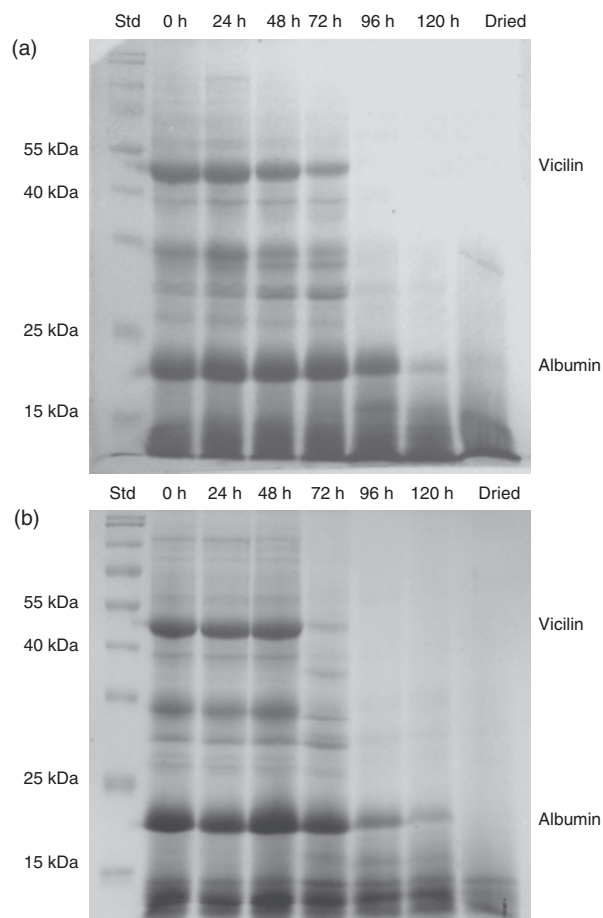
fragment corresponding to the migrational behavior of *S. cerevisiae* was detected only at 24 h. Fragments resembling the ones from the fragment corresponding to the migrational behavior of *C. tropicalis* and *C. pseudotropicalis* could be detected at both fermentation treatments between 48 and 120 h. The evaluation of the grouping generated by the UPGMA analysis based on similarity data estimated by the proportion of shared fragments between DGGE banding patterns for each treatment allowed the identification of two arbitrarily large groups (Fig. 3). The first group represented DGGE profiles from samples at the beginning of fermentation (0 and 24 h for both treatments), while the second group derived from the populations arising after increased mass aeration that occurred because of increased liquid drainage after 24 h and/or by physical turning of the fermentation mass after 48 h. The second group of profiles was more complex, containing an arbitrary subgroup joining more similar sample profiles from natural fermentation, sampled at 48, 72, 96 and 120 h, and another less similar cluster formed by DGGE patterns from samples fermented with *K. marxianus* inoculation, collected at 48, 72 and 96 h.

### Degradation of cotyledonary proteins

For both fermentation treatments, all seed high-molecular-weight proteins were apparently degraded after 120 h, while small proteins and/or oligopeptides (< 15 kDa) resulting from digestion remained (Fig. 4). The 21 kDa trypsin-inhibitor albumin protein was detected even at 120 h in both treatments, while the main storage protein globulins (vicilin) disappeared after 72 h of fermentation. Degradation of the seed proteins occurred first for the treatment



**Fig. 3.** Dendrogram derived from a similarity matrix obtained from scoring DGGE fragment profiles for fragments' absence or presence from the population obtained from natural fermentation (Nat) or from inoculated with *Kluyveromyces marxianus* (Km). Yeasts were obtained from fermenting pulp extracted by washing five seeds with saline solution at 0, 24, 48, 72 and 120 h.



**Fig. 4.** Evaluation of protein degradation during fermentation and drying of cacao seeds under natural conditions (a) or when inoculated with *Kluyveromyces marxianus* (b) by total seed protein profiles evaluated by SDS-PAGE. Lanes represent standard (Std) molecular weight; samples collected at 0, 24, 48, 72, 96 and 120 h during fermentation and after drying. As a reference, a class of proteins with a molecular weight similar to one vicilin (47 kDa) and albumin (21 kDa) was used.

with *K. marxianus* inoculation in comparison with the natural fermentation, more noticeable after 72 h of fermentation. On the other hand, no major difference in protein profiles could be observed between treatments after the beans were dried (Fig. 4).

### Quality evaluation of fermented beans and chocolate

The acidity of the fermented and dried cacao beans obtained from the conventional fermentation was  $21.7 \pm 0.5$  mEq-NaOH  $100 \text{ g}^{-1}$  beans, statistically different from the beans obtained from *K. marxianus*-inoculated fermentations ( $16.9 \pm 0.2$  mEq NaOH  $100 \text{ g}^{-1}$  beans), but the differences in pH (4.68 and 4.92, respectively) were not significant. In terms of color development, *K. marxianus* fermentation displayed a significantly higher percentage of brown and

**Table 2.** Results from the bean-cut test from natural or inoculated fermentation, based on cotyledon color (brown, brownish-purple and purple) or cotyledon morphology (fermented, partially fermented, less fermented)

Treatment	Categories of bean-cut test		
	Brown (%)	Brownish-purple (%)	Purple (%)
Natural	$9 \pm 2^a$	$46 \pm 3^a$	$46 \pm 4^a$
Inoculated with <i>K. marxianus</i>	$15 \pm 2^b$	$44 \pm 3^a$	$42 \pm 3^a$
MDS	3	4	4
Treatment	Categories of bean-cut test		
	Fermented	Partially fermented	Unfermented
Natural	$31 \pm 2^a$	$39 \pm 3^a$	$30 \pm 5^a$
Inoculated with <i>K. marxianus</i>	$36 \pm 2^b$	$33 \pm 2^b$	$31 \pm 2^a$
MDS	3	5	6

<sup>a,b</sup>Values within the same column and displaying the same letter did not differ significantly from each other based on Tukey's test at 5% probability.

MDS, minimum significant difference.

**Table 3.** Sensorial analyses of the chocolates produced from cacao beans from natural or inoculated fermentation

Treatment	Sensorial attributes		
	Aroma*	Flavor*	General acceptability*
Natural	$6.7 \pm 1.1^a$	$4.6 \pm 0.6^a$	$5.0 \pm 0.5^b$
Inoculated with <i>K. marxianus</i>	$7.0 \pm 1.3^a$	$5.4 \pm 0.4^b$	$5.8 \pm 0.3^a$
MDS	0.4	0.6	0.6

\*Aroma, flavor and general impression were evaluated using a 9-cm unstructured line hedonic scale ranging from 'extremely dislike' to 'extremely like'.

<sup>a,b</sup>Values within the same column and displaying the same letter did not significantly differ from each other based on Tukey's test at 5% probability.

MDS, minimum significant difference.

fermented seeds, with a significant reduction in the rate of partially fermented seeds (Table 2).

Sensorial analysis of the chocolate produced from both fermentation treatments revealed statistical differences in terms of the flavor attributes and global acceptability, with better results for the seeds fermented with *K. marxianus* (Table 3). For aroma, there was no statistical significance (Table 3).

### Discussion

A complete and efficient fermentation of cacao seeds is required to fully develop chocolate flavor, and proper

aeration of the fermentation mass is a critical factor for a successful process. Seed pulp represents around 40% of the seed fresh weight, and it usually restricts aeration at the beginning of fermentation. During fermentation, gravity causes mass flow drainage of seed-pulp liquids resulting from physical or biological rupture of cells from the pulp parenchymatous tissue, naturally increasing aeration. Conventionally, aeration is promoted by the periodical turning of the fermenting seed mass, but it can be increased by partial removal of seed pulp before fermentation. Removal of seed pulp mechanically or by treatment using pectinolytic enzymes can make fermentation more efficient (Wood, 1985; Schwan & Wheals, 2004), improving the final product quality. However, adoption of any of these two methods in a large-scale operation is costly and time consuming, and requires further development (Schwan & Wheals, 2004). As an alternative, the use of defined inoculation with pectinolytic yeasts, leading to an increased volume of drained liquids ('sweatings'), under small-scale conditions has been investigated (Sanchez *et al.*, 1985).

The hybrid yeast strain MMIII-41 was originally selected for improved inulase activity (Laguna, 1986), but also presented an enhanced pectinolytic activity, corroborating similar results described by Schwan *et al.* (1997) for *K. marxianus* mutants, with analogous increased activities for both enzymes. The estimated pectinolytic activity of the MMIII-41 hybrid described here was superior to yeast strains obtained from fermenting fruits (da Silva *et al.*, 2005), and for a *K. marxianus* lineage with elevated pectinolytic activity isolated from cacao seed fermentation (Schwan *et al.*, 1997).

The effect of the introduction of this hybrid *K. marxianus* strain with increased pectinolytic activity was evaluated in bean quality on a commercial scale, confirming previous results from micro-fermentation (Sanchez *et al.*, 1985). During the first hours of fermentation, liquids were drained from fermentation masses from both treatments at similar rates due to the effect of gravity (at 1 h; Table 1). The effect of yeast introduction on liquid drainage was noticed after 24 h, with an increase of one-third of drained volume in comparison with the traditional fermentation (1.05 from 3.15 L at 24 h; Table 1), as described previously for micro-fermentation with the introduction of pectinolytic yeasts, such as *S. cerevisiae* var. *chevalieri* and *K. fragilis* (Sanchez *et al.*, 1985; Buamah *et al.*, 1997). The increase in drained volume might be associated with the pectinolytic activity from the introduced yeast, causing more degradation of cell walls from the pulp parenchymatous tissue (Buamah *et al.*, 1997).

Introduction of the hybrid *K. marxianus* strain with pectinolytic activity at a high inoculum rate caused changes in yeast population dynamics, leading to an overall improvement of quality attributes of the final product in

comparison with natural fermentation. Beans fermented with the introduction of *K. marxianus* presented more degradation of seed storage proteins (Fig. 4), reduced acidity, increased percentage of fermented seeds with brown-colored cotyledons (Table 2) and improved sensorial attributes of the produced chocolate in relation to the control (Table 3). The relationship between increased drainage of liquids during fermentation and quality improvement by increased aeration corroborated previous results for small-scale fermentation experiments (Sanchez *et al.*, 1985; Buamah *et al.*, 1997).

The yeasts *K. marxianus* and *S. cerevisiae* have been considered the most predominant species in cacao bean fermentations because they had been identified at a high frequency in microbiological isolation from natural fermentations (Schwan *et al.*, 1995; Ardhana & Fleet, 2003; Schwan & Wheals, 2004). In our control conventional fermentations, these two species were detected based on DGGE profiles during the first 48 h (Fig. 2); *S. cerevisiae* had been detected in fermentations conducted in West Africa (Nielsen *et al.*, 2005). Here, we used a PCR-nested approach to amplify the D1 domain of the 26S ribosomal gene (Masoud *et al.*, 2004), instead of the one-step approach used by Nielsen *et al.* (2005), as our sample derived from a smaller amount of fermenting pulp material, producing less microbial DNA to amplify visible products in one PCR reaction. According to the amplified fragment profiles from a single culture of *C. pseudotropicalis*, *C. tropicalis* and *Pichia membranefaciens*, it was possible to detect that *Candida* species were predominant after the aerobic phase of fermentation started, whereas *P. membranefaciens* was favored toward the end of the fermentation inoculated with *K. marxianus*. Introduction of *K. marxianus* apparently did not stimulate the occurrence of novel yeast species in comparison with the control, altering only the yeast species succession and dynamics during the sampling periods. The clustering analyses of the yeast population DGGE profiles during fermentation (Fig. 3) formed arbitrary groups related to the two natural fermentation phases: aerobic or anaerobic (Schwan & Wheals, 2004). The anaerobic phase of the fermentation was represented by the major group established from profiles derived from samples from the beginning of the fermentation (0 and 24 h), whereas a distinct major arbitrary group was formed from samples of the aerobic phase for both treatments (Fig. 3). This pattern of grouping indicated the large effect of aeration caused by liquid drainage on the succession of yeast species in cacao bean fermentations. In the fermentation inoculated with *K. marxianus*, there was more variation between sampling time, suggesting an effect of the increased aeration on yeast diversity (Fig. 3). Changes in yeast species occurrence, verified by DGGE profiles, were also described in fermentations conducted in West Africa, with a

similar large effect after fermentation mass turning (Nielsen *et al.*, 2005).

The SDS-PAGE analyses of total seed proteins from both treatments indicated that both fermentations were completed at the end of 120 h (Fig. 4), with a typical general degradation of proteins, while analyses of dried seeds demonstrated that proteolysis continued during the drying process. Protein degradation became detectable by SDS-PAGE after 72 h, coincident with the increase in temperature (Table 1), as also described by Amin *et al.* (1997). The occurrence of proteolysis in fermenting cacao seeds had been described as early as 48 h (Biehl *et al.*, 1982a), and these differences in timing might derive from variation in fermentation practices, such as the turning regime adopted, and/or prefermentation factors, such as time from harvesting, degree of maturity, among others (Biehl *et al.*, 1982a; Amin *et al.*, 1997). During fermentation, the cacao seed storage proteins between 40 and 55 kDa (Spencer & Hodge, 1992) were apparently degraded after 96 h (Fig. 4), while the albumin Kunitz-like 21 kDa trypsin-inhibitor (Spencer & Hodge, 1991) was more resistant to proteolysis (Amin *et al.*, 1997). However, a difference in the timing of degradation was noticeable between treatments (Fig. 4). Vicilins were first degraded in the fermentation inoculated with *K. marxianus*, largely disappearing after 72 h, whereas in the control conventional fermentation, total degradation occurred only after 96 h (Fig. 4a and b). The 21 kDa trypsin inhibitor was strongly degraded only after 96 h for the conventional fermentation (Fig. 4a), while it was degraded earlier in the inoculated treatment (Fig. 4b). An increase in aeration and the subsequent increase in temperature, caused by improved drainage, could have been responsible for more efficient and earlier fermentation. It is generally believed that an ideal and efficient proteolysis occurs when cacao bean fermentations reach temperatures between 45 and 48 °C as early as possible (Biehl *et al.*, 1982b; Amin *et al.*, 1997). Proteolysis was improved with an increase in temperature, and both treatments here differed for up to 2° after 48 h (Fig. 1).

In terms of quality attributes, inoculation with *K. marxianus* clearly reduced titrable acidity. Both fermentation treatments produced seeds with acidity below the maximum acceptable limits of 35 mEq 100 g<sup>-1</sup> (Holm *et al.*, 1993), but a significant reduction of 4.8 mEq 100 g<sup>-1</sup> occurred for fermentation with *K. marxianus* inoculation, suggesting an alteration in the dynamics of production and/or degradation of acetic acid in the aerobic phase. Another positive effect of *K. marxianus* inoculation was the improvement of percent of beans with appropriate color and fermentation rating (Table 2), with inoculation with *K. marxianus* increasing the percent of brown beans considered well fermented. Previously, chocolate produced from beans derived from conventional fermentation was more acceptable by a taster panel when compared with those from artificial

inoculation using a cocktail of microorganisms (Schwan, 1998). Introduction of *Saccharomyces* in fermentation has produced good-quality cacao beans (Samah *et al.*, 1992), a result corroborated by the present study with the introduction of a defined yeast inoculum under large-scale conditions improving the final bean quality by altering natural fermentation conditions. The beneficial effects of drainage caused by the yeast pectinolytic activity (Sanchez *et al.*, 1985) were confirmed under large-scale conditions and under natural competitive pressure.

The positive results indicated the robustness of the process and the endurance of the yeast, demonstrating a potential for large-scale application in controlled fermentation. The results were obtained under field conditions under Brazilian adverse circumstances, without reaching 40 °C in 48 h of fermentation (Fig. 1). Typically, beans produced in Brazil and Malaysia present incomplete fermentation, with low pH (below the recommended range of 5–5.5). Introduction of a *K. marxianus* hybrid strain improved pectinolytic activity, demonstrating comparative improvements in fermented cacao bean quality that affected the flavor and acceptability of the derived chocolate products. The potential of this yeast hybrid strain, which attacks the spongy parenchymatous cells of the seed pulp more efficiently, increasing liquid drainage and the consequent aeration of the seed mass for the first 24 h, was demonstrated. This work also showed that changes in aeration at the beginning of the fermentation seemed to be more relevant to improve bean quality than the local microorganism population, and that the introduction of a yeast species that favors aeration might be sufficient to produce beans with superior quality in comparison with natural fermentation, without the need to inoculate with a complex mixture of microorganisms.

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