Biotechnological starter potential for cocoa fermentation from cabruca systems

Potencial biotecnológico de leveduras starter na fermentação do cacau de sistema cabruca

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ABSTRACT
Cocoa is a fruit of great economic importance, being the main raw material in the manufacture of chocolate. Among the stages of pre-processing, the main and most important is the spontaneous fermentation of the cocoa pulp by microorganisms, especially the yeasts, which initiate the process and contribute to the death of the germ of the seed, releasing compounds that directly influence the quality of the final product (flavor and aroma). Poorly fermented almonds confer bitter and astringent taste on chocolate, so it is advantageous to select autochthonous yeasts with better performance in the fermentation (producing enzymes of interest in the process) to be used as inoculum starter when added in the spontaneous fermentation, where they can accelerate the fermentation and contribute to raising the quality of the product. Therefore, the objective of this work was to qualitatively determine the production of enzymes of biotechnological interest by yeasts for the fermentation of cocoa through the cup plate method, in order to select a candidate yeast inoculum and use molecular typing technique to evaluate the

diversity. Many promising yeasts were identified for use as inoculum among the diverse yeast groups found.

Keywords: Enzymes, fermentation, molecular typing, yeast

RESUMO

O cacau é uma fruta de grande importância econômica, sendo a principal matéria-prima na fabricação do chocolate. Dentre as etapas do pré-processamento, a principal e mais importante é a fermentação espontânea da polpa do cacau por microrganismos, principalmente leveduras, que iniciam o processo e contribuem para a morte do gérmen da semente, liberando compostos que influenciam diretamente a qualidade do produto final (sabor e aroma). Amêndoas mal fermentadas conferem sabor amargo e adstringente ao chocolate, por isso é vantajoso selecionar leveduras autóctones com melhor desempenho na fermentação (produzindo enzimas de interesse no processo) para serem utilizadas como iniciador do inóculo quando adicionadas na fermentação espontânea, onde podem acelerar a fermentação e contribuir para elevar a qualidade do produto. Portanto, o objetivo deste trabalho foi determinar qualitativamente a produção de enzimas de interesse biotecnológico por leveduras para a fermentação do cacau através do método da placa do copo, a fim de selecionar um inóculo de levedura candidato e utilizar a técnica de tipagem molecular para avaliar a diversidade. Muitas leveduras promissoras foram identificadas para uso como inóculo entre os diversos grupos de leveduras encontrados.

Palavras-chave: Enzimas, fermentação, tipagem molecular, leveduras

1 INTRODUCTION

Cacao is a tropical tree species that needs regular rainfall and fertile soils and originates in the tropical regions of the Americas (Almeida and Valle, 2007). In Brazil, it is native to the Amazon, and was later introduced in other regions (Almeida and Valle, 2007) such as in the State of Bahia, Brazil, were cacao is commonly cultivate after the removal of lower vegetation without altering the canopy in a system called cabruca (Sambuichi et al., 2012).

Cacao almonds are known worldwide and used in the manufacture of various products such as juices, jellies, ice creams, fine distillates and especially for chocolate, which has high nutrient content. Cocoa, the main raw material of chocolate, is a source of saturated fatty acids that has the advantage of not increasing the lipids in the blood plasma (Lottenberg, 2009) and has already been reported to possess substances with functional properties as an adjunctive therapeutic resource in Parkinson's diseases, in hepatopathies, cystitis, diabetes, asthma, among others (Franco et al., 2013).

Varieties Criolo (from Central American origin) and Forasteiro (from South America) originated the Trinitarian variety (Bennett, 2003; Wickramasuriya and

Dunwell, 2018). The Forasteiro variety has become the main variety to be cultivated because of the stronger trees, in addition to a higher fat content. Although the Criollo variety is well known for its superior flavor and is used for the production of fine chocolate, but it is less frequent due to its susceptibility to diseases and pests (Wickramasuriya and Dunwell, 2018).

In a simplified way, the steps to obtain the almonds are: 1) Choice of the raw material, where the cocoa is selected and the fruits that are with illnesses, too mature or green are removed; 2) Fruit breaking and pulp extraction; 3) Transport of pulp in plastic bags or baskets to the fermentation house, in which they are deposited in wooden troughs for spontaneous fermentation to occur; 4) Drying the almonds (in the sun, in greenhouses or ovens); 5) Storage of the dry almonds until shipment to the industries, which perform the milling to obtain the by-products that will be used in the manufacture of the chocolate.

The cocoa pulp is rich in glucose, fructose and sucrose (Schwan and Wheals, 2004; Jespersen et al., 2005; Nielsen et al., 2007) and this makes the environment favorable to the action of microorganisms that enter the process through the contamination from fruit breakage, fruit surface, transport and, or even waste from previous fermentations (Nielsen et al., 2007; Visintin et al., 2016). The fermentation stage is of extreme importance, because the essential transformations are generated by the yeasts and later by the action of other microorganisms. The fermentation process is initiated by yeasts responsible for alcoholic fermentation; and then there is the action of the acid-lactic bacteria that consume the sugar generating lactic acid and, finally, the acid-acetic bacteria that consume the alcohol, transforming it into acetic acid (Schwan and Wheals, 2004). Yeasts are closely linked to the flavor, taste and color of chocolate, thus, it is necessary to study the yeast population in the fermentation, and select those that have the most desired metabolic activity to use as inoculum (Schwan and Wheals, 2004; Leal et al., 2008; Menezes et al., 2016; Sandhya et al., 2016; Santos et al., 2020).

Fermentation is directly related to obtaining quality almonds; in which elevate temperature (up to 50°C), together with the acetic acid present (which has the capacity to penetrate the seed), causes the death of the seed germ. This death results in the release of enzymes, hydrolysis of sugars and proteins and diffusion of phenolic compounds that are important for the formation of chocolate flavor and aroma (Efraim et al., 2010). As a form of quality control, the cut test is the most common used to evaluate the internal coloration of the almonds (Sant'Ana et al., 2020). Batches are cut from 100 to 300 almonds, where characteristics such as color and odor are analyzed. The commonly observed results are

brown coloring, which indicates good fermentation; violet color indicating insufficient fermentation and slate coloration, indicating that the almond has not undergone fermentation. The yeast diversity is directly related to the geographical location, the environment and the type of fermentative method. Thus, there are several studies on the population of yeasts in different localities that produce the almonds in the world where the most frequent genera in fermentations are: *Hanseniaspora*, *Saccharomyces* and *Pichia* (Illeghems et al., 2012; Magalhães da Veiga Moreira et al., 2017), where *Saccharomyces* is the most reported in cocoa fermentation (Magalhães da Veiga Moreira et al., 2017).

Given the relevance of the efficiency of fermentation process, with high quality, many researchers have tried to develop initial cultures for controlled fermentation of cocoa beans (Schwan, 1998; Meersman et al., 2015; Batista et al., 2016; Papalexandratou and Nielsen, 2016; Visintin et al., 2017; Santos et al., 2020). It is advantageous for fermentation that yeasts strains involved in the process produce specific enzymes, which may have biotechnological applicability not only for fermentative cocoa processes, but also for other industrial processes. Specifically for cocoa fermentation, we have: Amylase, which reduces the viscosity of the starch and increases the concentration of reducing sugars due to hydrolysis; pectinase, which dissolves the pectin and facilitates the breakdown of the cell walls, thus also releasing the anthocyanins; protease, which promotes the hydrolysis of pulp proteins, improving the concentration of free amino acids and invertase, which promotes the hydrolysis of sucrose.

The microbial identification associated with food is usually done by morphological and biochemical techniques. However, these tests involve time-consuming processes. In addition, the spontaneous fermentation technique that may introduce variations of microorganisms between fermentations (Schwan and Wheals, 2004). Then, alternatively, molecular methods have been developed for typing microorganisms such as $(GTG)_5$ allowing the differentiation inter- and intra-specific based on the amplification of regions between GTG and CAC microsatellites in a fast, safe and relatively easy way (da Silva-Filho et al., 2005; Illeghems et al., 2012; Englezos et al., 2015). It is based on the amplification of regions of high polymorphism for intraspecific characterization (Lieckfeldt et al., 1993), showing a great potential for the grouping of yeasts in processes of industrial dynamics of populations such as in wine productions (Englezos et al., 2015), sugar cane (da Silva-Filho et al., 2005; Basílio et al., 2008; Nova et al., 2009) and cocoa fermentation (Crafack et al., 2013; Papalexandratou

et al., 2013; Pereira et al., 2017) also being used in environmental yeasts (Silva-Bedoya et al., 2014). Thus, the objective of this work was to evaluate yeasts from four different fermentation collections, in addition to qualitatively determine the production of enzymes of biotechnological interest by these yeasts for the fermentation of cacao and to evaluate the diversity of these isolates.

2 MATERIAL AND METHODS

2.1 ORIGIN OF YEASTS USED IN THE STUDY

The yeasts used in this study are stored in the collection of the laboratories of Biotecnologia microbiana of the State University of Santa Cruz, and were isolated in previous unpublished studies. Two collections were obtained from two distinct fermentations of cacao from the Forasteiro type and the other two banks from Forasteiro and Scavina type cacao totaling four collections of yeast banks. To simplify and have a better understanding of the results, banks have received the acronyms CI, CII, CIII and CIV, respectively.

2.2 REACTIVATION AND MAINTENANCE OF YEASTS

All yeasts were reactivated by inoculation in liquid medium followed by plating with Drigalski loop. The medium used was Saboraud broth for inoculation in liquid medium. An aliquot of the stock was inoculated and incubated at 28 °C until growth was observed, with a maximum time of 120 hours. After growth in liquid medium, an aliquot was plated on Sabouraud Agar. All media used were supplemented with 0.05 g/L of chloramphenicol. The yeasts were incubated at 28 °C until growth was visualized by turbidity of the medium. The maintenance of the microorganisms was carried out through a medium-term method, where the microorganisms were preserved in GYMP (2% glucose, 0.5% yeast extract, 1% malt extract, 0.2% NaH_2PO_4) agar slant with mineral oil under refrigeration at 4-10 °C.

2.3 SCREENING FOR ENZYMATIC ACTIVITIES

After reactivation, yeasts were used for screening for enzymatic activities. For these tests the substrates sucrose, skin milk, starch and tributyrin were used to investigate the enzymes invertase, protease, amylase and lipase/esterase, respectively. Plates containing YNB medium and the specific substrate were prepared in a concentration of 1% of substrate for enzymatic assay. The yeasts were then inoculated on the surface of

the solid medium and were held for 5 days incubated at 30 °C for further observation of the formation of hydrolysis halos for a positive test. A 0.2% lugol solution was used to observe the results of the starch hydrolysis tests (amylase production). The invertase test was detected by observing microbial growth in medium containing sucrose as the sole source of carbon. In the others, the halo did not need to be revealed with any compound.

For the pectinase screening test, plates were prepared containing Czapeck medium (potassium chloride 0.5 g/L, sodium nitrate 3 g/L, magnesium sulphate 0.5 g/L, potassium phosphate bibasic 1.0 g/L, 1% citrus pectin and 1% agar-agar). Yeast were inoculated onto the solid medium in triplicate and left at 28 °C for about 18 hours. 1M NaCl and 5M NaCl were used for washes the Petri dishes and 1% congo red to reveal the halos.

2.4 DNA EXTRACTION OF YEASTS AND POLYMERASE CHAIN REACTION (PCR)

The isolates were inoculated in Saboraud broth and incubated for 3 days at 28°C for the DNA extraction. Yeast DNA extraction was performed according a phenol-chloroform protocol (da Silva-Filho et al., 2005; Basílio et al., 2008).

PCR for $(GTG)_5$ primers was also based on previously studies (da Silva-Filho et al., 2005; Basílio et al., 2008). The primer (5' -GTG GTG GTG GTG GTG -3') was used with the following reaction conditions: one denaturation cycle at 94°C/5 min, followed by 40 cycles at 94°C/30s, 55°C/45s and 72°C/1.5 min, and a final extension at 72°C/6 min. The reaction products were separated on 1.7% (w/v) agarose gels in 1X TAX buffer at 60 v for 180 minutes. Gels were stained with GelRed for visualization of the bands with UV light and a 1 Kb molecular weight (Invitrogen, São Paulo, Brazil) for comparison of band size.

2.5 SIMILARITY ANALYSIS BY CLUSTERS AND PRINCIPAL COMPONENT ANALYSIS (PCA)

Similarity analysis and PCA were performed based on the binary matrix of presence and absence of bands from the $(GTG)_5$ profile gels, using PAST 3.0 software (https://folk.uio.no/ohammer/past/). Similarity analysis were performed using Jaccard coefficient index.

2.6 YEAST IDENTIFICATION

The identification of the isolates was done using the sequencing method by Sanger technique. For this, the extracted DNA was required to undergo PCR with the yeast universal primers NL1 and NL4 that amplify the D1/D2 domain of 28S rDNA. PCR was done with NL1 (5 '-GCA TAT TAA GCG GAG GAA AAG-3') and NL4 (5 '-GGT CCG TGT TTC AAG ACG G-3') (da Silva-Filho et al., 2005) primers. This reaction started at 94 °C for 3 min followed by 35 cycles of 94 °C for1 min, 52 °C for1 min and 72 °C for1 min extension, and a final extension at 72 °C for 7 min. Sequencing was made by the service provider ACTGene analysis moleculares (Alvorada/RS, Brazil). DNA was sequenced in the ABI-PRISM 3500 Genetic Analyzer (Applied Biosystems) automated sequencer using the protocols of the service provider.

3 RESULTS

3.1 SCREENING FOR ENZYMATIC ACTIVITY

The enzymatic tests carried out with 185 yeasts aimed to prospect of yeasts with biotechnological potential for use as inoculum starter for cocoa fermentation processes. In all four collection, all yeasts presented positive activities for invertase and pectinase activities (Fig. S1). In the CI, all the yeast produces lipase. However, only four samples were positive for protease and no sample was positive for amylase. In CII, all the samples were also positive for lipase activity (Fig. S2). A total of seven positives activity were found for protease and only two for amylase. In CIII collection, four positive samples for the production of protease enzyme (Fig. S3) were detected and no sample was positive for amylase enzyme production. However, with the exception of samples S74a, S255b and S373, yeasts showed a positive result for lipase activity. Finally, in the CIV collection, all samples were positive for the production of lipase. Differently from the other samples, no positive samples were found for protease, whereas for amylase, seven samples were positive. The relationship of these results can be seen in table 1.

Table 1 Relationship of the yeasts isolated of the cocoa fermentation and enzymatic activity detected. Note that invertase and pectinase were not included as all yeasts (100%) presented those activities.

Collection	Cacao variety	Enzymatic activity screening		
		Amylase	Protease	Lipase
CI	Forasteiro	0	4 (7%)	56 (100%)
CII	Forasteiro	2 (10%)	7 (37%)	19 (100%)
CIII	Forasteiro	0	4 (10%)	36 (92%)
CIV	Scavina	7 (10%)	0	71 (100%)
TOTAL		9 (5%)	15 (8%)	182 (98%)

3.2 YEAST IDENTIFICATION

The selected yeasts according to enzymatic activity were submitted to DNA sequencing and compared to the database. From CI, four yeast samples were identified. 108b and 184a was similar to *Schwanniomyces etchellsii*, 122b and 184b identified as *Candida parapsilosis*. These were the only yeasts from this collection that proved to be positive for protease, invertase, pectinase and lipase. In CII, eight yeast samples were identified, 45a was similar to *Yarrowia lipolytica*, which was positive for all enzymes with the exception of protease and 87b was similar to *Pichia manshurica*, which was positive for all the enzymes tested. The others were 107c as *Zygoascus hellenicus*; 107d, 107a and 116b as *Schwanniomyces etchellsii*; and 116c and 98b as *Pichia manshurica* which were positive for all enzymes except for amylase. From the CIII bank the strains S354b, S357 and S230a that showed similarity with *Pichia manshurica*, and yeast S156b was identified as *Rhodotorula mucilaginosa*. The samples identified from this collection were positive for invertase, pectinase, lipase and protease. Seven samples were identified from the collection of yeasts from Scavina cacao (CIV), the only ones being indicated as positive for the amylase test, invertase, pectinase and lipase. They are T0h 12 as *Wickerhamomyces anomalus*; T 24h 81 and T122h 70 as *Torulaspora delbrueckii*; T36h 30, T36h 87, T108h 34 and T122h 40 as *Pichia kudriavzevii*. A summary can be seen in Table 2. No samples at this collection were positive for protease under the assay conditions. It is important to note that small differences were observed in the $(GTG)_5$ of many of the isolates identify as the same species.

Table 2 –Yeasts isolated from cocoa fermentation identified by D1/D2 sequencing

Yeast	Identification	Accession	Simil.	Inv	Pec	Pro	Amy	Lip
S156b	*Rhodotorula mucilaginosa*	MH000318	100%	+	+	+	-	+
S354b	*Pichia manshurica*	MK034750	100%	+	+	+	-	+
S357	*Pichia manshurica*	MK034750	100%	+	+	+	-	+
S230a	*Pichia manshurica*	MK034750	100%	+	+	+	-	+
CI 108b	*Schwanniomyces etchellsii*	NG_042636	99,9%	+	+	+	-	+
CI 122b	*Candida parapsilosis*	MK026351	99,7%	+	+	+	-	+
CI 184[a]	*Schwanniomyces etchellsii*	NG_042636	100%	+	+	+	-	+
CI 184b	*Candida parapsilosis*	MK026351	100%	+	+	+	-	+
CII 45a	*Yarrowia lipolytica*	NG_055393	100%	+	+	-	+	+
CII 87b	*Pichia manshurica*	MK101213	99,5%	+	+	+	+	+
CII 107c	*Zygoascus hellenicus*	NG_055323	100%	+	+	+	-	+
CII 107d	*Schwanniomyces etchellsii*	NG_042636	99,9%	+	+	+	-	+
CII 116c	*Pichia manshurica strain RHTD19*	MK034750	100%	+	+	+	-	+
CII 98b	*Pichia manshurica*	MK034750	100%	+	+	+	-	+
CII 107a	*Schwanniomyces etchellsii*	NG_042636	100%	+	+	+	-	+
CII 116b	*Schwanniomyces etchellsii*	NG_042636	99,9%	+	+	+	-	+
T0h 12	*Wickerhamomyces anomalus*	MH483547	100%	+	+	-	+	+

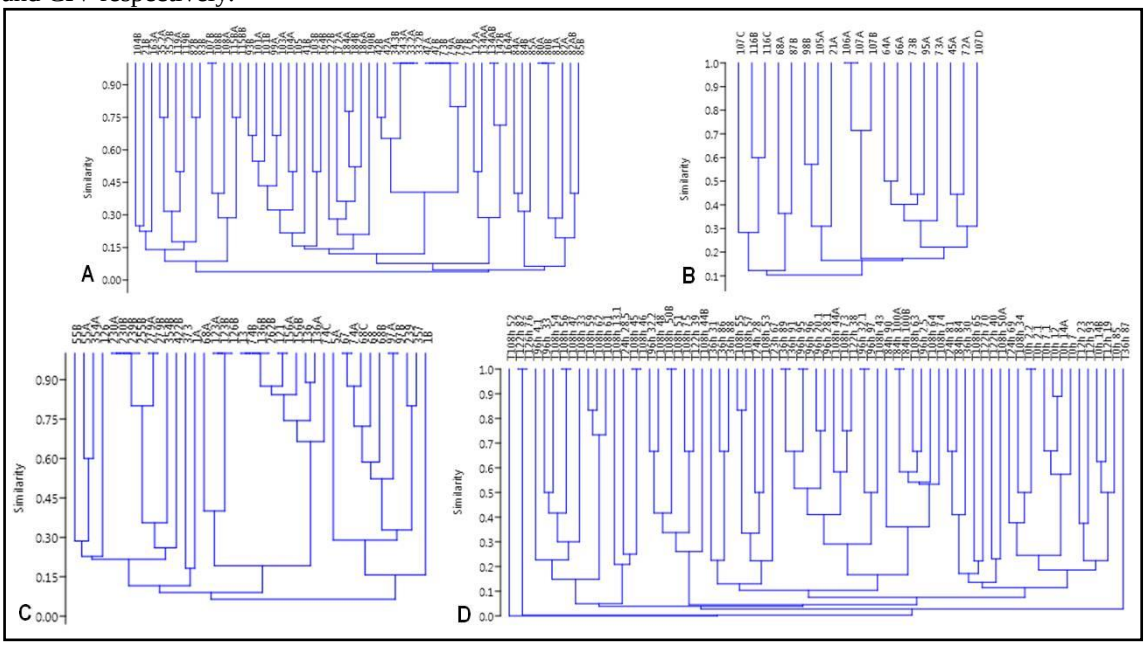
T24h 81	*Torulaspora delbrueckii*	NG_058413	100%	+	+	-	+	+
T36h 30	*Pichia kudriavzevii*	MK101219	100%	+	+	-	+	+
T36h 87	*Pichia kudriavzevii*	MK101219	100%	+	+	-	+	+
T108h 34	*Pichia kudriavzevii*	MK101219	100%	+	+	-	+	+
T122h 40	*Pichia kudriavzevii*	MK101219	100%	+	+	-	+	+
T122h 70	*Torulaspora delbrueckii*	NG_058413	100%	+	+	-	+	+

Simil. – similarity with the specified accession sequence from GenBank; Inv – Invertase activity; Pec – pectinase activity; Pro – protease activity; Amy – amylase activity; Lip – lipase activity

3.3 INTRASPECIFIC COMMUNITY ANALYSIS

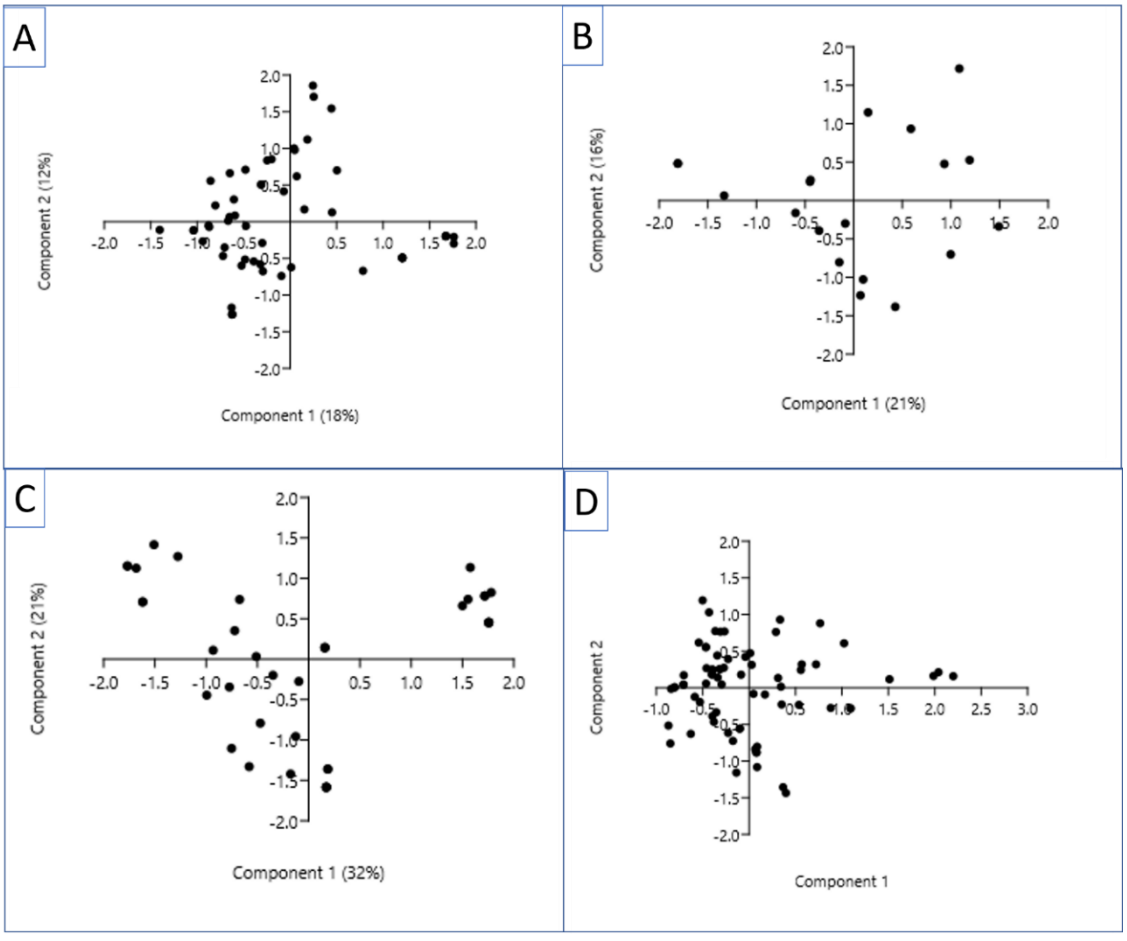
The $(GTG)_5$ was carried out aiming to analyze the molecular diversity and similarity of the yeasts isolates from each collection. Similar levels of similarity can be observed in dendrograms ranging from 100% to values close to 0% similarity between the individual yeast's profiles (Fig. 1). This large variation in the profiles shows that a great diversity of yeasts is present in the cocoa fermentation process, not only intraspecific diversity, but considering the low values of similarity, it is also possible to infer that there is a great inter-specific diversity. CI (Fig. 1A) and CIV (Fig. 1D) showed more clades with low similarities than CII and CIII (Fig. 1B-C; however, also presented a greater amount of isolates.

Figure 1. Similarity dendrogram based on the molecular typing of yeasts $(GTG)_5$. Dendrograms were generated using Jaccard´s similarity coefficient. A, B, C and D are related with the collection CI, CII, CIII and CIV respectively.



Principal component analysis was performed based on the presence and absence data matrix of bands made from the $(GTG)_5$ profiles of yeast. The purpose of the analysis is exploratory where the distribution of the clusters / overlaps of the identified isolates can be evaluated (Fig. 2). The yeasts of the CI collections (Fig. 2A) and CIV (Fig. 2D) presented a higher concentration in the first and third quadrant with some yeasts distributed without a visible cluster in the other quadrants. Due to the registered information of the isolation time during the fermentation in the CIV collection, it is possible to observe that the yeasts that were distributed farther from the group are yeasts present at the beginning of the fermentation (0h and 12h), however, others yeasts from the beginning of fermentation was detected in the other group. In CII (Fig. 2B), it is not possible to observe clusters as it was observed in the other collections, possibly related to the low number of isolates. And in CIII (Fig. 2C), the yeast distribution presented two well characteristic clusters in the first and second quadrant.

Figure 2. Principal component analysis based on the molecular typing of yeasts $(GTG)_5$. A, B, C and D are related with the collection CI, CII, CIII and CIV respectively.



4 DISCUSSION

Yeasts are essential in the cocoa fermentative process and storage of yeasts isolates is common practice in diversity studies associated with the fermentation process as it can be used to try to associate beneficial effects with the final product. Thus, in previous studies of our group yeasts were isolated (Ferreira, 2007; Santos, 2012; Diaz, 2016) and we evaluate aspects of the yeast activity that are essential yeast-related activity during cocoa fermentation, therefore, can be used as potential inoculum of the cocoa fermentation. These yeasts were submitted to the enzymatic tests and all yeasts were positive for the production of pectinase and invertase. The detection of all yeast producing pectinase is surprising as other fermentative process showed lower percentages (Haile and Kang, 2019). Pectinase activity may be attributed by the culture conditions (composition of the medium, carbon source, pH, temperature, etc.) that was favorable to pectinase production by those yeasts. Therefore, although the tests were not made with the composition of the fermentation process of cocoa, we can't confirm that none of those yeasts are producing those enzymes during fermentation, there is a high availability of these substrates in the process in a way that favors the production of pectinase (and invertase, lipase and protease) by most microorganisms. In addition, the invertase is an enzyme that cleavage sucrose into fructose and glucose, when sucrose is present in large amounts, the invertases are able to synthesize fructooligosaccharides through fructotransferase. The fructooligosaccharides are associated to improve human health (Veana et al., 2018), thus, invertase production can be consider a good attribute for a possible starter culture.

Within the lipase/esterases, 98.3% presented such activity and several identified genus are cited with ability to secrete lipase (Treichel et al., 2010). The genus *Candida*, a well-known lipase producer, is commonly found in cocoa fermentations (Schwan and Wheals, 2004; Lagunes Gálvez et al., 2007), including the present work. Furthermore, our research group had used *Candida parapsilosis* as starter in cocoa fermentation, and it showed a positive influence on the physical-chemical changes of the process (Santos et al., 2020). Regarding protease, a smaller fraction of yeasts was positive for proteolytic activity and none of them were isolated from Scavina type; however, the recovery amount (from stock) of 60% of yeasts isolated from Scavina type, the yeast protease importance for cocoa fermentation and the low percentage of protease producer in other variety may indicate that non-recovery yeasts may be associated with protease activity. Also, the composition of the growth medium, temperature, pH as well as the type of assay directly

influence yeast extracellular protease production (Nelson and Young, 1987). Furthermore, it indicates that protease activity identify may be higher among all yeasts if it was evaluated in other conditions.

The enzyme that presented the least number of positive samples was amylase, detected only in CII and CIV. The results suggest that yeasts from cocoa fermentation have little ability to degrade starch, that is known to range 4-7% of cocoa bean (Schmiederand and Keeney, 1980). Similar results were reported when investigate the amylase production in others yeast-related fermentative process such as wine, where 3.7% were positive (Strauss et al., 2001). Starch level was reported to not change significantly during fermentation and drying (de Brito et al., 2001), but a higher activity of amylase may improve the amount of reducing sugars, one of flavors precursor.

In general, the enzymes secreted by yeasts collected from the fermentation with Forasteiro had no differences between the collections (CI, CII and CIII) showing a similar production pattern. Their difference compared to the yeasts collected from the fermentation with Scavina type (CIV) is due to the absence of protease enzyme production. However, the collection that presented the highest number of different genera associated with enzymatic activities was the CII, where *Yarrowia*, *Pichia*, *Zygoascus* and *Schwanniomyces* was identified.

The molecular typing with the primer $(GTG)_5$ showed the difference in yeasts profiles between each collection and it detected intra-specific variation. Therefore, they present distinct genetic characteristics that lead to variation of the profile and, may present a potential for different inoculum, as observed in eight isolates of *Pichia manshurica* (Fig. S4); whereas intraspecific variation can be used to monitor the inoculum during controlled fermentations as shown in sugar cane fermentation for ethanol production with *Saccharomyces cerevisiae* (da Silva-Filho et al., 2005). Curiously, we did not identify any *Saccharomyces* with potential to be used as inoculum, yet, it is a common yeast associated with cocoa fermentation (Illeghems et al., 2012; Magalhães da Veiga Moreira et al., 2017). Previously study corroborate our findings regarding high variation in $(GTG)_5$ profiles in *Pichia* species (Pereira et al., 2017) showing a potential to monitor *Pichia* inoculum during cocoa fermentation. Despite the non-specificity for any phenotypic characteristic, intraspecific variations in $(GTG)_5$ profiles may be associated with genetic variations that may be important in the cocoa fermentation process. Although it is not possible to associated specific activity with the $(GTG)_5$ profiles, the variation of the amylolytic activity observed among *Pichia manshurica* lineages corroborates this

assertion as CII 87b presented such activity that is not detected in others. It evidence that some isolates were identified as the same species, but presented genetic differences (as shown in the dendrograms) that potentially led to variations in the detected activity and shown a high yeast intraspecific diversity in the cocoa fermentation.

The diversity is even more evident in the PCA analysis that showed a differential pattern distribution for each collection. This is a strong indicative of wild yeast fermentative process and may be responsible for differences in the chocolate quality. An inoculum may improve the reproducibility of fermentation and provide a standard or even improve the chocolate quality (Schwan, 1998; Schwan and Wheals, 2004; Batista et al., 2016; Santos et al., 2020). The analyzed enzymes represent important activities for cocoa fermentation that may be applied in the production of different products beside cocoa, such as cocoa honey, cocoa wine and cocoa liquor.

Although they are qualitative trials, the yeast *Pichia manshurica* (CII 87b) shown a potential for starter, as it was positive for all enzymatic tests followed by another *Pichia manshurica* (S357), as it was positive for most of the qualitative enzymatic tests (except for amylase), in addition to being reported as yeast with killer activity (Diaz, 2016). *Pichia manshurica* has great potential to be used as inoculum as it was related to be aroma-enhancing (Zhang et al., 2017) in fermentative process and it was reported to inhibit the grown of potential contaminants of cocoa fermentation (Romanens et al., 2019). Nevertheless, caution should be taken as strains of theses yeasts were also related with spoilage (Saez et al., 2011; Franco and Pérez-Díaz, 2012) of fermentative process. Furthermore, as demonstrated in our previous study (Santos et al., 2020), a potentially good inoculum did not require all enzymatic activities, thus, all 23 identified yeasts has a potential to be used as inoculum.

In fermentation, these yeasts, when inoculated alone or in consortium, can optimize and directly influence and standardized the quality of the final product, either in flavor or aroma characteristics, since this have already been reported in the production of the chocolate fermented with inoculum from the genus *Pichia* (Batista et al., 2016), *Torulaspora, Rhodotorula, Candida* and *Issatchenkia* (Santos et al., 2020). The characteristics of pectinase, invertase, lipase, protease and amylase in the same microorganism are promising, especially when they are associated with the capacity to produce toxins for another yeast (killer activity), which makes it even more promising, since this quality can be applied as a control of contaminants during the fermentation process.

5 CONCLUSION

A great diversity of yeast was observed in the cocoa fermentation process with intraspecific variations detected both at the DNA level with the use of molecular typing and at the level of enzymatic activity. Based on the enzymatic activity, it was possible to identify yeasts that potentially contribute to the process of cocoa fermentation and identify potential starter from spontaneous fermentations to be evaluated in future works. Therefore, good candidates for inoculum starter with good indications of applicability in cocoa fermentation were obtained.

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