

Assessment of alcoholic native yeasts isolated from the fermentation of wet processed coffee (*Coffea arabica*)

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ABSTRACT

Biofuels produced from waste biomass are the best alternative to fossil fuels; however, considering that bioethanol production occurs due to fermentation, the yield should be improved during this stage by incorporating microorganisms that can increase yields in the conversion of carbohydrates present in the residue to ethanol. Therefore, native yeasts were isolated and identified from natural fermentation of coffee cherries (*Coffea arabica*) from Chiapas, Mexico, and then, evaluated the yield of ethanol. First, a morphological study was conducted in which yeast-like microorganisms were isolated from plates with a YPDA culture medium, taking into account their texture, surface, margin, elevation, and color. In total, 15 isolates with macroscopic and microscopic characteristics typical of yeasts were obtained. Subsequently, biochemical tests were conducted to evaluate the fermentative capacity of the strains; simple sugars such as glucose, fructose, sucrose, lactose, and xylose were tested in a liquid medium composed of 0.3% yeast extract, 0.5% peptone, and 2% simple sugar. The mixture was incubated for 48 h at room temperature. The strains fermented with the aforementioned sugars were subjected to a qualitative iodoform test to confirm the presence of ethanol in the medium, identifying the best M2A and M2B strains with yields of 3.7 and 18 g/L, respectively. The M2A strain corresponded to *Wickerhamomyces anomalus*, and the M2B strain corresponded to *Pichia kudriavzevii*. To summarize, native yeasts represent a biological alternative to the production of bioethanol, which guarantees the conversion of the sugars present in the substrate to ethanol.

Key words: Bioethanol; fermentation of coffee cherry; native yeasts; non-Saccharomyces; residue.

1 INTRODUCTION

Biofuels (renewable fuels) are a promising alternative to minimizing the use of fossil fuels. Biofuels can be classified as first, second, third, or fourth-generation, depending on the origin of the biomass used in the production of bioethanol (Beschkov, 2017). Most studies have investigated the use of second-generation biomass, such as agricultural residues, for the following reasons. These residues generally do not compete with food products, such as sucrose and starch obtained from sugarcane and corn, respectively. The use of lignocellulosic biomass for producing ethanol is an alternative with the best growth prospects due to its low cost. It is also a renewable organic material that is widely distributed and abundant in the biosphere (Solarte-Toro et al., 2019; Lamichhane et al., 2021; Zhao et al., 2022).

Through a series of pretreatments, the main structure of the lignocellulosic biomass can be broken down, increasing the bioavailability of simple sugars, such as pentoses and hexoses, which can later be used as substrates by various microbial fermenters to convert them into ethanol (Offei et al., 2018; Kassim et al., 2022; Mekunye; Makinde, 2024). The total consumption of the substrate is a prerequisite, all the main sugars found there should theoretically be converted into ethanol. To achieve this, new sources need to be identified that can provide strains of microorganisms to perform this conversion efficiently. As most fermenting yeasts have a high capacity to ferment

hexoses (glucose, mannose, and galactose), resulting from the hydrolysis of cellulose and hemicellulose (Rastogi; Shrivastava, 2017; Zhang et al., 2017; Cunha et al., 2020).

The worldwide biomass production is around 146 billion metric tons per year. The major contributors of biomass include forest residues, crops, urban solid waste, and agro-industrial waste. Coffee residues are formed by wet processing; Chiapas ranks first in the production of organic and conventional coffee at the national level, with a production of 339,361 tons per year (ELMS, 2019).

In this study alcoholic native yeasts present in the natural fermentation wet processed of coffee cherries from the municipality of San Fernando, Chiapas, Mexico were isolated and identified.

2 MATERIAL AND METHODS

2.1 Sampling

Initially, 100kg of ripe organic arabica Coffee cherries (*Coffea arabica* L.), certified by Certimex, Naturland, JAS, Bio Suisse, Dakks, and USDA Organic, were collected from the “El Bosque” plot, located in the community of San Jorge, municipality of San Fernando, Chiapas, Mexico. This coffee grows at 880 m above sea level and temperatures between 17 and 23 °C. Immediately after the cut, the coffee cherries were placed in a 1.2m x 1m x 1m fermentation tank.

2.2 Obtaining the fermented coffee

Once the ripe coffee cherries were obtained, pulping was performed using a mechanical mill (BONASA N.2). In contrast, the natural fermentation of the pulped coffee was performed for 20 h at 25 °C during the day at 8:00h and 19 °C at night at 22h; samples of the ferment were collected in triplicate in sterile 50 mL Falcon tubes every 5 h. The fermented coffee juice samples were transported in coolers at 10 °C to the facilities of the research laboratory of the Universidad Politécnica de Chiapas (Silva et al., 2013).

2.3 Isolation and purification of yeast microorganisms

Yeasts were isolated by serially diluting sterile saline-peptone solution from a 1:10 dilution, for which 5 mL samples of fermented coffee juice were used. Decimal dilutions were made up to a 10^{-3} dilution mL. Next, 100 µL of the dilutions were seeded in extension plates in triplicate in a YPD agar culture medium, followed by the addition of chloramphenicol and oxytetracycline to inhibit bacterial growth, which favored the growth of yeasts. The plates were observed every 24 h for five days, and using a colony counter, colonies with different morphological characteristics were isolated on new plates containing YPD agar medium (López et al., 2004).

The isolates were named according to the time point in which the sample was collected during the coffee fermentation process; M1 indicates that the sample was taken at hour 5, M2 at hour 10, M3 at hour 15, and M4 at hour 20 of fermentation. To purify the strain, replanting was performed repeatedly until only strains with unique morphological characteristics were present in each culture medium (De Melo et al., 2014; Mukherjee et al., 2017).

2.4 Morphological characterization of yeasts

Pure cultures were inoculated in YPD agar media to observe the macroscopic characteristics (texture, surface, margin, elevation, and color) of the colonies of different isolates along with their microscopic characteristics (shape and size of the cells). This procedure was performed with each isolation to group similar samples (Méndez et al., 2001).

2.5 Identification of simple sugar-fermenting yeasts by CO₂ production

Fermentation of different carbon sources (glucose, fructose, sucrose, lactose and xylose) was performed for all isolated yeasts. Inoculation was performed by removing a small amount of the strain directly from the medium with a sterile handle and dispersing it in 16 mL of medium with the respective simple sugars contained in dilution tubes, which contained inverted Durham tubes, to detect CO₂ production. The fermentation medium was prepared according to the

methodology described by Brooks (2008) and contains (w/v) 2.0% monosaccharide, 0.3% yeast extract, and 0.5% peptone. Strains that produced no or very little CO₂ were considered non-fermentative, and those that produced CO₂ were evaluated based on the accumulation of CO₂ gas inside the Durham hood. As these hoods were similar in size (6 × 50 mm), they were marked at 16.6 mm, which represented 30% of the total length, and at 25 mm, which represented 50% of the total length; the percentage of displacement of CO₂ inside the Durham hood was reported based on this threshold. Using this method, fermenters that produced 50% or more CO₂ were considered to be good fermenters.

2.6 Growth in lysine medium

All isolated strains were inoculated on lysine hydrochloride lysine agar media (Sigma) and added to Yeast Carbon Base media (Difco Laboratories) for isolating non-*S. cerevisiae* species, according to the methods of Clavijo et al. (2010).

2.7 Iodoform test

This qualitative discriminatory test was performed only on the isolated strains that produced CO₂ during the fermentation of simple sugars. To conduct the test, 2 mL of fermented sample was taken in a dilution tube, to which 2 mL of Lugol's solution and 1 mL of sodium hydroxide were added at 20%. Those that produced a yellow precipitate were considered to be ethanol producers. These results indicated that the alcohol produced by oxidation can form methyl ketones (Yarrow, 1998).

2.8 Evaluation of growth kinetics

The different isolates considered to be highly fermentative were reseeded by depletion again on PDA agar media supplemented with antibiotics under microaerophilic conditions at 37 °C for 36 h. Following this, pre-inoculated samples were prepared, which consisted of a small sample of each strain from the YPD agar medium. These samples were then grown in YPG liquid medium supplemented with antibiotics (5% glucose, 0.5% yeast extract, 2% casein peptone, 0.02 g/L magnesium sulfate, 0.2 g/L ammonium sulfate, and 100 mg/L chloramphenicol) for 24 h at 120 rpm, after which the pre-inoculated samples were used for fermentation.

Fermentation was conducted in a 500 mL Erlenmeyer flask with 250 mL of previously sterilized medium, which was inoculated at a concentration of 1×10^7 cells/mL pre-inoculated in liquid YPG medium (5% glucose, 0.5% yeast extract, 2% casein peptone, 0.02 g/L magnesium sulfate, 0.2 g/L ammonium sulfate, and 100 mg/L chloramphenicol). The same procedure was performed in YPF medium (5% fructose, 0.5% yeast extract, 2% casein peptone, 0.02 g/L magnesium sulfate, 0.2 g/L ammonium sulfate, and 100 mg/L chloramphenicol).

The cultures were incubated at 37 °C under controlled shaking at 120 rpm for 48 h. All experiments were evaluated in triplicate. Next, samples (3 mL) were taken in Eppendorf tubes to determine glucose and ethanol. Additionally, 10 µL samples were used to determine biomass using a Neubauer chamber. Cell counting was performed to assess cell viability, for which methylene blue staining was performed, followed by an assessment of the stained cells under a microscope with a 40X objective (Wondimagegn; Aber, 2012).

2.9 Determination of glucose and fructose contents

Glucose and fructose levels were determined using the 3,5-dinitrosalicylic acid (DNS) method for reducing sugars. For this, a standard curve was developed in which the absorbance of known glucose concentrations was measured at a wavelength of 540 nm to detect the presence of free carbonic groups (C=O) of reducing sugars through a redox reaction, on a UNICO brand SQ2802 UV spectrophotometer (González et al., 2022).

2.10 Quantification of ethanol production by the dichromate technique

First, 10 mL of the previously fermented sample were centrifuged at 3500 rpm for 20 min, and the supernatant (Solution a) was collected for further analysis. The ethanol produced during fermentation was quantified by the colorimetric method of potassium dichromate described by Tello-Hinojosa et al. (2005). Briefly, 4 mL of an oxidizing solution (a mixture formed by 4,165 g of potassium dichromate and 250 mL of sulfuric acid, graded with distilled water to 1000 mL) was placed in test tubes; then, 2 mL of saturated potassium carbonate solution was added dropwise until the bubbling ended (Solution b). Next, 2 mL of Solution a was mixed with 1 mL of Solution b by vortexing at 1500 rpm for 10 s; then, the mixture was heated in a bath at 80–85 °C for 30 min. The mixture was cooled to room temperature, and the absorbance was recorded at 440 nm using a UNICO brand SQ2802 UV spectrophotometer. Fermentation was monitored every 24 h for three days in triplicate. A blank control and ethanol standards were used to obtain a standard curve for standardizing the method.

2.11 Molecular identification of yeasts

For molecular identification of yeasts, pure isolates were initially used to avoid cross-contamination due to the presence of more than one species in the same sample. The strain was cultivated for 48 h in a YPDA medium to isolate the DNA from new cells. After adding 1 mL of lysis buffer to the plate, samples were collected with a spatula, and 500 mL of buffer mixed with yeasts was subsequently recovered in an Eppendorf tube and mixed by vortexing. The tube was incubated at 65 °C for 40 min, and then, centrifuged at

12,000 rpm for 10 min. The supernatant was discarded, and the mixture was extracted once with an equal volume of chloroform/isoamyl alcohol (24:1). Next, the aqueous phase was transferred to a new Eppendorf tube, and the DNA was precipitated by adding 0.5 mL of 7.5 M ammonium acetate and one volume of cold isopropanol. The tube was mixed by inverting it gently three times, followed by centrifugation at 12,000 rpm for 15 min. The supernatant was discarded, and the precipitate was washed with 300 mL of cold 70% ethanol. This mixture was centrifuged at 12,000 rpm for 15 min. Finally, the ethanol was removed, and the DNA precipitate was dried at 37 °C, dissolved in 100 mL of sterile Milli-Q water, and stored at –20 °C until use. The samples were processed in the sequencing laboratory of the Potosi Institute for Scientific and Technological Research - IPICYT of San Luis Potosí, México.

The samples were sequenced using the labeled dideoxynucleotide method in a 3130 Genetic Analyzer sequencer (Applied Biosystems). The DNA sequences of the ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) amplification products were determined; a part of the ribosomal 5.8S gene, which is a highly conserved region between species and can be used to reliably identify yeasts, was used as the reference sequence. Two yeast strains isolated from the fermentative stage of organic coffee in Chiapas were analyzed. The *in silico* analysis of the amplified ITS sequences was subsequently performed, which involved searching for similarities by comparing DNA sequences found in the isolated yeast strain with those reported in the GenBank. (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The similarities were searched by comparing the problem sequence with those of the gene bank. The taxonomy, physiology, and relationship with the environment of previous isolates were searched in the Global Catalog of Microorganisms (<http://gcm.wfcc.info>). Finally, the proposal of the species that obtained 99% similarity was accepted (De Melo et al., 2014).

2.12 Preservation of strains

After identifying the microorganisms, the strains were preserved by adding an isolated colony to each tube containing 10% glycerol. The suspension was made using a McFarland scale, which is ideal for standardizing the number of bacteria and yeast; the McFarland standard unit was maintained between 3 and 4. This procedure was performed in duplicate. The suspension was subsequently stored in Eppendorf tubes, with 1.5 mL of suspension added to each tube. The microorganisms were allowed to acclimate, initially, to 4 °C for 1 h and finally, to –20 °C. The viability of the microorganisms was determined after 48 h (Kurtzman et al., 2011).

2.13 Statistic análisis

To determine significant differences between the results of biochemical tests performed on the isolated strains, the one

way ANOVA test was carried out at $p < 0.05$ level of significance; the statistical software used was the STATGRAPHICS PLUS 1999 version for windows. Prior to statistical analysis, data were assessed three replicate experiments, for equality of variance and normality. A Tukey test was carried out following to compare means of treatments.

3 RESULTS

3.1 Variations in pH and temperature during the natural fermentation of coffee

Coffee fermentation samples were obtained under standard atmospheric conditions every 5 h during 20 h of fermentation. The average pH and temperature inside the fermentation tank were determined. The pH gradually decreased from 6.3 to 4.4 during 20 h of fermentation (Figures 1A-B). On the other hand, the temperature in the fermentation tank ranged between 17 and 20 °C at all times.

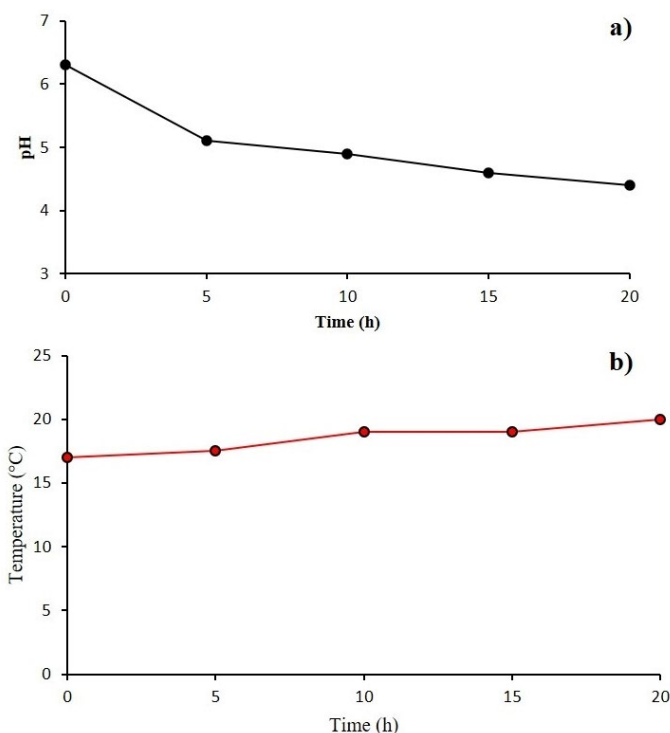


Figure 1: Graph of the behavior of the pH (a) and the temperature (b) of the humid fermentation tank from the ferment of cherries of coffee.

3.2 Identification of yeasts resulting from isolation and purification

After the isolation and purification of microorganisms from the different times of the sampling process (Figure 2), 15 strains with mostly yeast-like characteristics were identified (Table 1). Regarding the macroscopic characteristics of the

isolated yeasts, 80% had a viscous texture, 53.33% had a rough surface, 40% of the edges of the colonies were eroded with high growth, and 46.66% of the strains presented white and cream colors (Table 1).

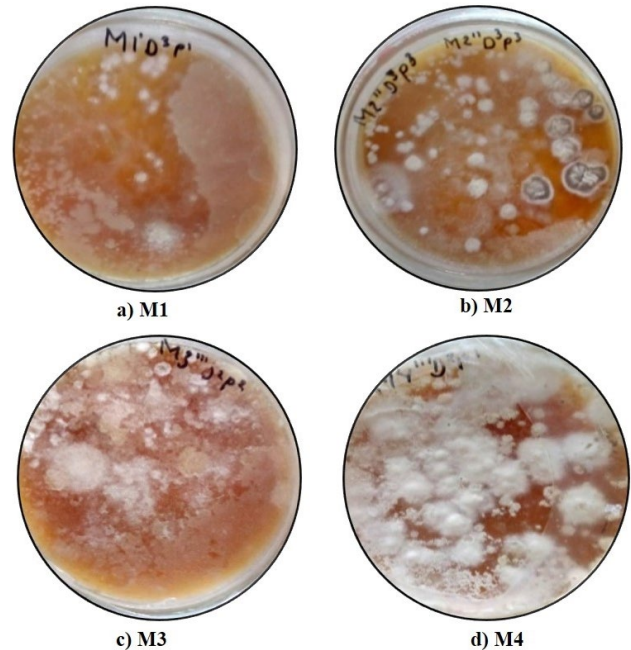


Figure 2: Seeding of microorganisms present in the coffee cherry ferment, corresponding to the hours in the sample taking: M1 (5h of fermentation); M2 (10h of fermentation); M3 (15h of fermentation); M4 (20h of fermentation).

3.3 Simple sugar fermenting yeasts

The results of the simple sugar fermentation strains are presented in Table 2. The results were considered to be positive when CO_2 was produced in the fermentation medium (Figure 3). Two strains (M2A and M2B) were the best fermenters of reducing sugars. The above, since it was shown that like *S. cerevisiae*, the M2A and M2B strains demonstrated high fermentation, since the production of CO_2 occupied more than 50% of the capacity of the Durham hood, a characteristic that is directly related to the consumption of sugars present in the medium (Table 2).

In the evaluated sweep, the M2A strain highly fermented hexose monosaccharides such as glucose and fructose, as well as, disaccharides such as sucrose and lactose. The M2B strain also fermented monosaccharides such as fructose and glucose; however, among disaccharides, it only fermented sucrose (Table 2 and Figure 3).

3.4 Growth in lysine medium

The results of the microorganisms that were grown in the lysine differential media are shown in table 3, to identify the non-*Saccharomyces* strains. This selective medium allowed us to observe the yeasts that belonged to the genus

Saccharomyces by not growing in this culture medium. The yeasts are listed based on their fermentative power in Table 3. From the 15 isolated strains, nine strains capable

of fermenting some of the sugars listed in Table 3 were selected; only the strain identified as M2C corresponded to the *Saccharomyces* genus.

Table 1: Morphological characterization of 15 strains isolated in YPD medium from the ferment of wet coffee milling.

Strain	Clue	Texture	Surface	Margin	Elevation	Color
1	M3A	Mucoid	Smooth and shiny	Border of the colony is entire	High growth	Translucent cream
2	M4B	Viscose	Rough	Eroded	Flat growth	White
3	M4H	Viscose	Rough	Eroded	Tall and convex growth	White
4	M2A	Viscose	Smooth and shiny	Whole	Conical	Cream
5	M1B	Viscose	Rough	Eroded	High growth	White
6	M4C	Viscose	Rough	Eroded	Conical	White
7	M2B	Viscose	Rough	Eroded	High growth	White
8	M3G	Viscose	folded	Curly	Depressed in the center	Cream
9	M4D	Membranous	Rough	Curly	Conical	Cream
10	M4I	Viscose	Rough	Curly	High growth	Cream
11	MID	Viscose	Smooth and shiny	Whole	Flat growth	Orange
12	M3C	Membranous	Rough	Eroded	High growth	Cream
13	M2C	Viscose	Smooth and shiny	Whole	tall and convex growth	White
14	M2E	Viscose	Shiny	Curly	flat growth	Cream
15	M1A	Membranous	Cottony	bordered with hyphae	High growth	White
Control	<i>Saccharomyces cerevisiae</i>	Viscose	Smooth	Whole	Conical	Cream

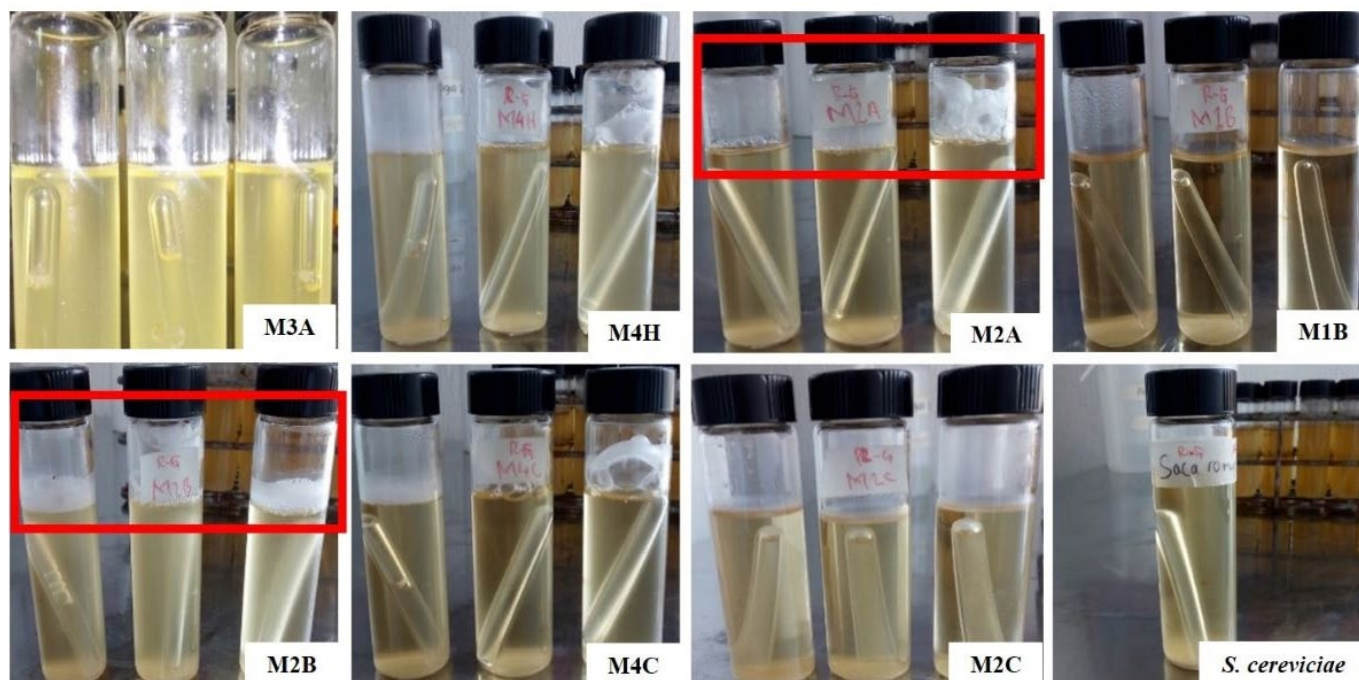


Figure 3: Evaluation of the fermentation potential of yeasts isolated from CO₂ production.

Table 2: Results of the simple carbohydrate fermentation test through CO₂ production of the isolated strains.

Code Proof	M4H	M4C	M2B	M2A	M3A	M3G	M1B	M2C	M4B	M4D	M4I	M1D	M3C	M2E	M1A	S. cerevisiae
Fermentation																
Glucose	+++	+++	+++	+++	++	-	+	++	-	-	-	-	-	-	-	+++
Fructose	+++	+++	+++	+++	++	++	+	-	+	-	-	-	-	-	-	+++
Saccharose	++	++	+++	+++	++	+	-	-	-	-	-	-	-	-	-	+++
Lactose	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total, Fermented sugars	3	3	3	4	4	3	2	1	1	-	-	-	-	-	-	3

+ = Fermentative (<30% CO₂ gas in Durham tubes), ++ = Moderately fermentative (<50% CO₂ gas in Durham tubes), +++ = Highly fermentative (≥ 50% CO₂ gas in Durham tubes), - = Non-fermentative.

Table 3: Growth in lysine medium of isolated strains to differentiate strains of the genus *Saccharomyces* from non-*Saccharomyces*.

Code	Genus non- <i>Saccharomyces</i>	Genus <i>Saccharomyces</i>
M3A	X	
M4B	X	
M4H	X	
M2A	X	
M1B	X	
M4C	X	
M2B	X	
M3G	X	
M2C		X
<i>S. cerevisiae</i>		X

3.5 Iodoform test

The results of the iodoform test (Table 4) showed that the isolated strains with positive results in the presence of ethanol produced the most amount of CO₂ in the simple sugar fermentation test (Figure 4). The best results were obtained for the M2A and M2B strains, which produced ethanol using substrates such as glucose, fructose, and sucrose, indicating a similar behavior to that of *S. cerevisiae*.

3.6 Growth kinetics

For growth kinetics, only the two strains that showed the best results in the simple sugar fermentation test and the iodoform test were considered, which included M2B and M2A. Figure 5 shows the results of the kinetics analysis of *S. cerevisiae* used as a reference strain. The dichromate test was also performed to assess ethanol production, and the results of the kinetic parameters obtained during fermentation in the YPF medium for 48 h are shown in Table 5.

During the evaluation of growth kinetics, it is observed that the exponential stage began at 3 hours of fermentation of the coffee cherries, but, the production of alcohol began between 8 and 20 hours for each group of yeasts evaluated, however, the high production of this metabolite were generated at 48 hours of fermentation (Figure 5 and Table 5).

According to the growth kinetics of *S. cerevisiae*, the maximum biomass reported was 2.5 x 10⁸ Cel/mL, reaching eight logarithmic cycles in 22 h. However, the highest ethanol production was recorded at 48 h at a concentration of 20 g/L. On the other hand, the results of the growth kinetics of the M2B strain were obtained after fermentation in the YPF medium for 48 h. For the M2B strain, maximum biomass accumulation was recorded at 18h, with a growth rate of 0.34 μ/h and the maximum alcohol production (18 g/L) was recorded at 48 days. Finally, the behavior of the M2A strain recorded its maximum production of biomass at 28h with eight logarithmic cycles and a growth rate of 0.05 μ/h, resulting in the greatest accumulation of alcohol (3.7 g/L) at 48 days (Table 5 and Figure 5).

Furthermore, in the present analysis it was observed that high ethanol production occurred between 36 and 48 hours and not at the beginning of the exponential stage, considering that alcohol is a primary metabolite (Table 5 and Figure 5).

According to the above, it was observed that the M2B strain reached the highest biomass production (3.72E+08) in a shorter time (18 days), demonstrating significant statistical difference between the M2A strain and *S. cerevisiae* with 6.53E+07 and 2.172E+08, respectively. Also, the M2B strain, like *S. cerevisiae* demonstrated the production of 18 and 20 g/L of ethanol, respectively, at 48 days, without significant statistical difference (Table 5).

3.7 Molecular identification of yeasts

The molecular identification of yeasts was performed by the amplification of the internal transcribed spacer (ITS)

Table 4: Results of the ethanol production test, through the qualitative analysis of iodoform, after 48 h of fermentation on 5 different substrates.

Codes Media	M4H	M4C	M2B	M2A	M3A	M3G	M1B	M2C	M4B	M4D	M4I	M1D	M3C	M2E	M1A	S. c.
YPG Medium	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-	+
YPF Medium	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+
YPS Medium	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+
YPL Medium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YPX Medium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(+) = presence of ethanol in the medium; (-) = absence of ethanol in the medium; Medium (YP)= yeast extract, peptone and (G)= glucose, or (F)= fructose, or (X)= xylose, or (L)= lactose.

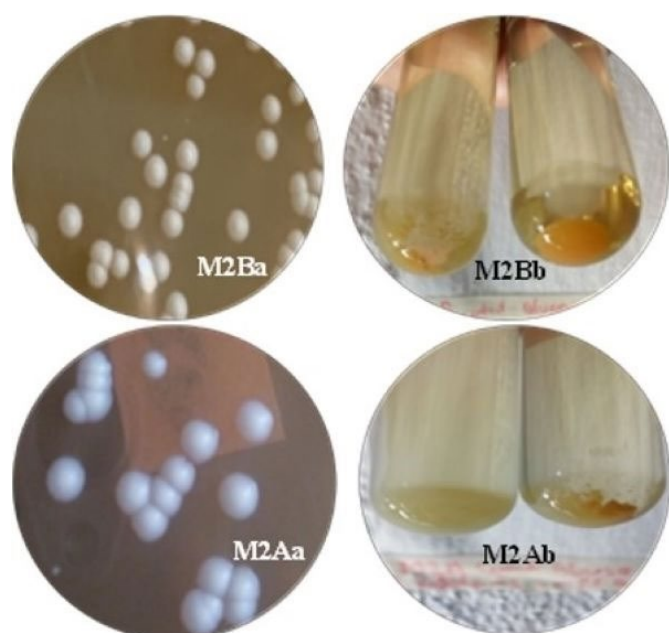


Figure 4: Strains that tested positive for iodoform (M2Bb and M2Ab) in ethanol production, higher CO₂ production, and consumption of hexoses as a carbon source. M2Ba and M2Aa: isolated strains.

of the 5.8S ribosomal gene, as it has been shown that two large groups of yeasts, i.e., ascomycetes and basidiomycetes, can be identified. ITS1 and ITS4 were sequenced to search for similarities using BLAST. The sequences of three yeast strains isolated from organic coffee in Chiapas are shown in Table 6. The M2A strain yielded ITS1 and ITS4 sequences of 589 and 585 bases, respectively. The M2B strain yielded ITS1 and ITS4 sequences of 475 and 476 bases, respectively. The resulting ITS were of different sizes, which made them more interesting when conducting the identification or matching of sequences as they represent greater possibilities of reducing comparison errors.

According to molecular identification, the M2A strain corresponded to *Wickerhamomyces anomalus*. The new sequence obtained was deposited in GenBank under accession number OP499849.1. The M2B strain corresponded to *Pichia*

kudriavzevii. The new sequence obtained was deposited in GenBank under accession number OQ592168.

3.8 BLAST sequence analysis

The FASTA formats of each sequence were analyzed separately to find similarities (~100%) and determine the BLAST proposal in relation to the ITS1 and ITS4 sequences of yeasts isolated from organic coffee from Chiapas. The BLAST search results showed that *Wickerhamomyces anomalus* was the dominant microorganism in the different isolated strains reported in the gene bank (Table 6).

4 DISCUSSION

4.1 Variations in pH and temperature during the natural fermentation of coffee

The evaluation of the temperature and pH during fermentation revealed variation and decrease, respectively, in these two parameters (Figures 1A-B), which occurred partly due to the temperature variation in the region and an increase in the metabolic activity of microorganisms over time (Chen et al., 2017).

The variation in pH during fermentation was largely due to the increase in the production of organic acids produced by other microorganisms, including bacteria. These variations in pH favored the growth of yeasts and fungi that were more tolerant to acidic media, as reported by Nasanit and Satyawut (2015). After the wet fermentation process, four samples of residual fermented juice were obtained in triplicate, following sampling every 5 h (Figure 1C).

4.2 Identification of yeasts resulting from isolation and purification

During the isolation of microorganisms from the coffee ferment, 15 different strains were identified, all of which presented yeast-like characteristics according to the macroscopic morphological characterization of Yarrow (1998) (Table 1). These results were similar to those of Silva et al.

(2013), who identified the following genera of microorganisms: *Enterobacter*, *Staphylococcus*, *Serratia*, *Candida*, *Torulopsis*, *Rhodotorula*, *Escherichia*, *Citrobacter*, *Hafnia*, *Streptomyces*, *Bacillus*, *Aspergillus*, *Penicillium*, and *Saccharomyces*, the last one being the most abundant, owing to the physicochemical characteristics of fermented coffee, which contained 76.7% moisture and 12.4% reducing sugars.

Therefore, according to the identified structures, viscous textures and rough surfaces predominated; similar results were found by De Melo Pereira et al. (2014), who reported that the characteristics of the cell structure of this eukaryotic type are dominated by viscous textures and rough surfaces of white and cream color (Méndez et al., 2001).

4.3 Simple sugar fermentation of yeasts

Alcoholic fermentation is a biological process in the complete absence of air (oxygen- O_2), originated by the activity of some microorganisms that process carbohydrates, such as glucose, fructose, saccharose and starch, to obtain as final products an alcohol in the form of ethanol (CH_3-CH_2-OH), carbon dioxide (CO_2) in the form of gas and some ATP molecules that the microorganisms themselves consume in their energy metabolism, which is why the detection of high CO_2 production is an important indicator that guarantees the fermentation of carbohydrates, which is why the present work demonstrated that the strains with the greatest degree of reducing sugar fermentation were

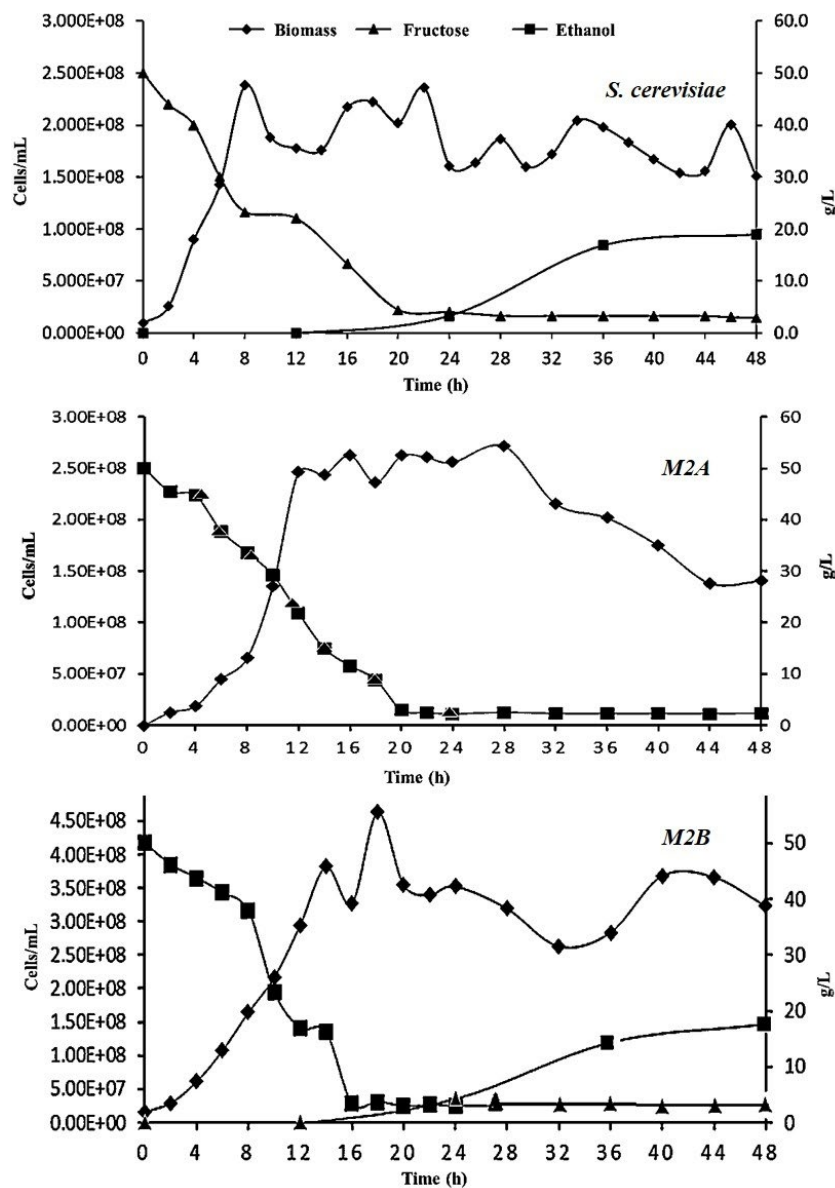


Figure 5: Growth kinetics in YPF medium of yeasts isolated from coffee ferment compared with *Saccharomyces cerevisiae* as control strain. The values are the result of the average of the triplicate of the measurements, their respective standard deviation (+) and their and its statistical analysis at $p < 0.05$.

M2A and M2B (Table 2). The most abundant fungal genus identified was *Saccharomyces*, and these strains have a high metabolic capacity to transform monosaccharides and disaccharides (generally sugars such as glucose, fructose, and sucrose). However, the assimilation capacity depends on the presence of esterase and glucosidase, which catalyze hexose hydrolysis (Clavijo et al., 2010; Wondimagegn Damtew; Aber, 2012).

4.4 Growth in lysine medium

During this analysis, the strains with the greatest fermentative potential corresponded to the genera other than

Saccharomyces (Table 3). There are other groups of yeasts with great potential under fermentation conditions. These groups are important alternatives to potentiate the use of reducing sugars in fermentation, which leads to the production of metabolites such as ethanol. As the regulation of factors in the fermentation medium is a useful way to control the balance between respiratory and fermentative activities, it can be used to determine the synthesis of compounds such as alcohol and other compounds of sensory importance in the production of non-traditional fermented beverages. These compounds are produced by genera other than *Saccharomyces* (Úbeda et al., 2014; Grellet et al., 2022).

Table 5: Kinetic parameters from fermentation in YPF medium of yeasts isolated from coffee ferment compared with *Saccharomyces cerevisiae* as control strain. The values are the result of the average of the triplicate of the measurements and their respective standard deviation ($\pm$); Different letters between columns of the same kinetic parameter denote a statistically significant difference at $p < 0.05^*$

Time (h)	Biomass [Cel / mL]			Growth rate [μ (h ⁻¹)]			Doubling time [td (h)]			Ethanol (g/L)		
	<i>S. cerevisiae</i>	M2A	M2B	<i>S. cerevisiae</i>	M2A	M2B	<i>S. cerevisiae</i>	M2A	M2B	<i>S. cerevisiae</i>	M2A	M2B
0	1.000E+07 ^b	1.00E+07 ^b	1.57E+07 ^{a*}	0.94 $\pm$ 0.02 ^a	0.23 $\pm$ 0.04 ^b	0.62 $\pm$ 0.18 ^a	0.73 $\pm$ 0.00 ^c	2.93 $\pm$ 0.32 ^a	1.10 $\pm$ 0.17 ^b	0 ^a	0 ^a	0 ^a
4	2.560E+07 ^b	1.27E+07 ^c	2.93E+07 ^a	1.25 $\pm$ 0.11 ^a	0.38 $\pm$ 0.06 ^c	0.73 $\pm$ 0.11 ^b	0.55 $\pm$ 0.23 ^b	1.78 $\pm$ 0.24 ^a	0.94 $\pm$ 0.00 ^b	0 ^a	0 ^a	0 ^a
8	9.024E+07 ^a	1.87E+07 ^c	6.10E+07 ^b	0.45 $\pm$ 0.02 ^b	0.87 $\pm$ 0.02 ^a	0.56 $\pm$ 0.19 ^b	1.51 $\pm$ 0.26 ^a	0.78 $\pm$ 0.11 ^b	1.22 $\pm$ 0.05 ^a	0 ^a	0 ^a	0 ^a
12	1.425E+08 ^a	4.50E+07 ^b	1.07E+08 ^a	0.51 $\pm$ 0.13 ^a	0.37 $\pm$ 0.00 ^b	0.42 $\pm$ 0.12 ^a	1.34 $\pm$ 0.21 ^b	1.85 $\pm$ 0.07 ^a	1.62 $\pm$ 0.09 ^a	0.7 ^b	0.5 ^c	2 ^a
18	2.172E+08 ^b	6.53E+07 ^c	3.72E+08 ^a	0.02 $\pm$ 0.02 ^c	0.72 $\pm$ 0.02 ^a	0.34 $\pm$ 0.11 ^b	28.56 $\pm$ 0.17 ^a	0.95 $\pm$ 0.01 ^c	2.52 $\pm$ 0.27 ^b	1 ^b	0.8 ^c	3 ^a
22	2.525E+08 ^b	1.35E+08 ^c	3.56E+08 ^a	0.17 $\pm$ 0.07 ^c	0.60 $\pm$ 0.13 ^a	0.30 $\pm$ 0.00 ^b	4.50 $\pm$ 0.11 ^a	1.15 $\pm$ 0.06 ^c	2.25 $\pm$ 0.23 ^b	2 ^b	1.5 ^c	5 ^a
28	1.606E+08 ^c	2.64E+08 ^b	3.64E+08 ^a	0.01 $\pm$ 0.04 ^c	0.05 $\pm$ 0.17 ^b	0.25 $\pm$ 0.16 ^a	37.62 $\pm$ 0.09 ^a	9.24 $\pm$ 0.15 ^b	2.66 $\pm$ 0.16 ^c	5 ^b	2 ^c	10 ^a
36	1.719E+08 ^c	2.36E+08 ^b	2.70E+08 ^a	0.17 $\pm$ 0.00 ^c	0.10 $\pm$ 0.13 ^b	0.34 $\pm$ 0.18 ^a	3.99 $\pm$ 0.02 ^b	6.64 $\pm$ 0.22 ^a	1.99 $\pm$ 0.14 ^c	18 ^a	2.5 ^c	15 ^b
48	1.557E+08 ^c	2.56E+08 ^b	3.50E+08 ^a	0.25 $\pm$ 0.21 ^a	0.05 $\pm$ 0.11 ^b	0.03 $\pm$ 0.10 ^c	2.74 $\pm$ 0.07 ^c	11.66 $\pm$ 0.31 ^b	18.94 $\pm$ 0.18 ^a	20 ^a	3.7 ^b	18 ^a

Table 6: Molecular identification through the amplification of ITS (Internal Transcribed Spacer) of the 5.8S ribosomal gene of yeasts isolated from coffee ferment by means of the BLAST tool for similarity and its registration in the GenBank.

Isolated strains	ITS1	Yeast identity	ITS4	Yeast identity	Registration in the GenBank
					<i>Wickerhamomyces anomalus</i> OP499849.1
M2A	589 b	<i>Wickerhamomyces anomalus</i> 93.89%	585 b	<i>Wickerhamomyces anomalus</i> 99.83%	
					<i>Pichia kudriavzevii</i> OQ592168
M2B	475 b	<i>Pichia kudriavzevii</i> 98.03%	476 b	<i>Pichia kudriavzevii</i> 97.68%	

*b: bases.

The non-Saccharomyces strains have low tolerance to low oxygen concentrations, especially compared to *S. cerevisiae*. When oxygen levels are low, fermentation is stimulated, which results in the conversion in ethanol (Ruyters et al., 2015; Escribano et al., 2017; Morata et al., 2022).

During the early stages of fermentation, the microbial population increases in size and competes for the substrate, displacing Saccharomyces yeasts. This favors the spread of native non-Saccharomyces yeasts in the wet coffee processing plant, since the process is generally carried out in open containers under aerobic conditions at low pH and high concentrations of reducing sugars (Grellet et al., 2022; Ruyters et al., 2015; Escribano et al., 2017; Morata et al., 2022).

4.5 Iodoform test

In more detail, during ethylic fermentation inside yeasts, the glycolysis pathway produces pyruvate that is finally converted into ethanol, as the main metabolite in an alcoholic fermentation, so its identification in fermenting yeasts is important (Rastogi; Shrivastava, 2017; Offei et al., 2018). According to the above, as yeast-like strains were present, this test was performed to evaluate the fermentative capacities, as they are influenced by different factors, such as the concentration and types of sugars that influence microbial growth. However, as Clavijo et al. (2010) reported, yeasts have greater hydrolytic metabolic activity in the presence of monosaccharides and disaccharides, since the activities of β -D-xylosidase and β -glucosidase are widely distributed in the *Saccharomyces* and non-*Saccharomyces* genera and also in most species of yeasts isolated in various environments (De Melo Pereira et al., 2014; Morata et al., 2022; Mekunye and Makinde, 2024); therefore, the best results in this study were recorded for the M2A and M2B strains (Table 4).

4.6 Growth kinetics

The growth kinetics data (Figure 5) showed that the M2B strain presented better results both in the production of biomass and in the accumulation of ethanol, with up to 18 g/L in 48 days (Table 5).

The kinetic behavior of these microorganisms was influenced by yeasts, which are facultative anaerobes and sensitive to variations in the oxygen concentration in the medium. However, under aerobic conditions, fermentation products such as acetate and ethanol are produced at relatively low concentrations because certain strains present low activity of acetyl-CoA synthetase, the enzyme that contributes to the complete oxidation of pyruvate in the Krebs cycle. Glycerol is produced to maintain the intracellular redox balance by converting excess NADH_2 into NAD^+ generated during glycolysis and in response to osmotic stress in the environment. Alcoholic fermentation occurs under anaerobic or semi-

aerobic conditions, which leads to the production of ethanol and a series of chemical compounds of sensory importance. Acetate accumulates due to the insufficient activity of the acetyl-CoA synthetase required for its complete oxidation. Studies on yeasts have revealed various strains with different abilities to consume sugars and produce alcohol (Clavijo et al., 2010; Wondimagegn Damtew; Aber, 2012; Nasanit; Satyawut, 2015).

Furthermore, in the present analysis it was observed that high ethanol production occurred between 36 and 48 hours of the fermentation of coffee cherries (Table 5 and figure 5), due to what was mentioned by Cunha et al. (2020), who found that during this conversion, glucose, ethyl alcohol, carbon dioxide and, to a lesser extent, glycerol and some organic acids are obtained as byproducts. Some of the enzymes that participate in fermentation are diastase, invertase or hydrolase and zymase, the latter being responsible for directing the biochemical reaction that converts glucose into ethanol. It is important to highlight that along with the production of ethanol, carbon dioxide, yeast biomass and, to a lesser extent, glycerol, higher alcohols, aldehydes and ketones are obtained. These reactions can usually occur in the range of 45 to 60 hours (Solarte-Toro et al., 2019; Kassim et al., 2022; Zhao et al., 2022), data similar to those reported in this research work.

It was also observed in Figure 5 that the highest ethanol production was 20 g/L, since ethanol increases in concentration during the fermentation process and because it is a toxic compound, when its concentration reaches approximately 12% by volume, the yeasts tend to die. This is one of the main reasons why alcoholic beverages (not distilled) do not reach values higher than 20% ethanol concentration (Solarte-Toro et al., 2019; Lamichhane et al., 2021; Grellet et al., 2022; Kassim et al., 2022).

4.7 Molecular identification of yeasts

After molecular evaluation, the two morphotypes with the best metabolic qualities corresponded to *W. anomalus* (MA2) and *P. kudriavzevii* (M2B) (Table 6). The latter demonstrated the best metabolic capacity for the production of ethanol since oxygen limitation activated the enzymes of the fermentative pathway. Pyruvate decarboxylase (PDC) activity was considerably higher (73%) than alcohol dehydrogenase (ADH) activity (51%). Hence, large quantities of ethanol and glycerol were produced, and the specific rate of glucose uptake increased substantially compared to the rate of glucose uptake under aerobic conditions. Under these conditions, the oxidation of glucose-6-phosphate by the pentose phosphate pathway was reduced to only 10%, and the Krebs cycle functioned via a branched pathway (Clavijo et al., 2010). The metabolic flow under these conditions was similar to the fermentative metabolism of *S. cerevisiae*; therefore, the kinetic behavior was similar (Dhaliwal et al., 2011; Yuangsaard et al.,

2013; Yuan; Guo; Hwang, 2017). The fungi identified in this study were part of the non-*Saccharomyces* group of yeasts, which are non-conventional yeasts with high tolerance to stress (high temperature, pH, and oxygen levels) and are relevant for the production of bioethanol. They exhibit high fermentation efficiency on par with or even better than that of *S. cerevisiae* under certain types of stress (Yuan; Guo; Hwang, 2017).

4.8 BLAST sequence analysis

The genus and species proposals for the highest identities are shown in Table 6. The yeasts *W. anomalus* and *P. kudriavzevii* have been reported in the Global Catalog of Microorganisms (<http://gcm.wfcc.info>). Isolates have been obtained from wastewater, soil, plants (flowers and fruits), fermented products such as beer, and products fermented with rice. This study allows promoting standardization fermentation processes that guarantee specific sensory features. Various aspects of biotechnological relevance, such as corn preservation, antimicrobial activity, and bioethanol production under oxygen-limiting conditions, have been described for *W. anomalus* and *P. kudriavzevii*, although their application in biofuel production has not been determined (Morata et al., 2022). Some studies have shown that these species can ferment various organic compounds such as simple sugars, breakdown cellobiose, and ferment xylose.

5 CONCLUSIONS

In this study, 15 yeast strains were isolated from coffee fermentation, 93.4% of which corresponded to non-*Saccharomyces* genus. After molecular analysis, these yeasts corresponded to *W. anomalus* and *P. kudriavzevii* which could ferment reducing sugars such as fructose and produced ethanol 3.7 and 18 g/L, respectively. The importance of native yeasts isolated from agro-industrial residues with the ability to convert sugars from other residues into ethanol was highlighted, which guarantees satisfactory yield due to the high capacity of yeasts to metabolize certain sugars. This specificity helps establish efficient fermentation processes.

6 AUTHORS' CONTRIBUTIONS

Conceptual Idea: Saldaña, S.; Methodology design: Pérez, Y.D.C; Data collection: Canseco, M. A.; Berrones R; Data analysis and interpretation: Toalá, C.V.; and Writing and editing: Sánchez, Y.

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