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Research article

Bioprospecting *Saccharomyces cerevisiae* in fruits from Amazonian region for beer brewing

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Abstract: This research aimed to isolate *Saccharomyces cerevisiae* strains from Amazonian fruits for potential utilization in beer production. Yeast strains were derived from the spontaneous fermentation of Arazá (*Eugenia stipitata* MacVaught), cocoa (*Theobroma cacao* L.), and cupuassu (*Theobroma grandiflorum* Wild. Ex Spreng. Schum) fruits. Identification of the isolated strains was achieved through biochemical assays and ITS rDNA (Internal Transcribed Spacer ribosomal DNA), sequencing, with emphasis on determining their affiliation to *S. cerevisiae* and assessing phylogenetic ties. Out of the 76 colonies isolated from the fruit fermentations, seven were distinctly identified as *S. cerevisiae*. Phylogenetic assessments unveiled significant parallels between regional isolates and commercial strains. Notably, beer brewed with the *S. cerevisiae* AR 03 isolate exhibits physical-chemical attributes

characteristics similar to those found in American ale commercial beers. These findings underscore the untapped potential of leveraging Amazonian yeasts in brewing, a step forward for the region's burgeoning bioeconomy.

Keywords: fermentation; diversity; beer; Amazonian fruits; brewer's yeast

1. Introduction

Beer, as the world's predominant alcoholic beverage, has witnessed significant diversification in terms of flavors, styles, and brewing techniques [1,2]. With the industry constantly evolving, one of its most intricate components, fermentation, has emerged as a focal point [2]. Although fermentation is pivotal in beer production, there remains a gap in the comprehensive exploration of yeast's aromatic potential [3,4].

Historically, beers have been categorized based on the type of yeast and fermentation process used, with ales and lagers representing the primary classifications [5]. Ales are brewed using *Saccharomyces cerevisiae* strains fermenting at the top, while lagers employ strains of *Saccharomyces pastorianus* that are hybrids of *S. cerevisiae* and *S. eubayanus* [6]. *Saccharomyces pastorianus* are yeasts that ferment at the bottom and are optimized for temperatures between 4 and 13 °C [1,5]. The *Saccharomyces* genus, particularly *S. cerevisiae*, is recognized for its dominance in spontaneous fermentations. These strains have evolutionary advantages, such as high ethanol tolerance and the absence of toxin production, which may have evolved as an adaptation to fruit-bearing plants' emergence [1,3,5–8].

Despite the extensive documentation on yeast's role in beer production [3–5,8–11], there is a noticeable absence of studies exploring tropical forests as potential sources of yeast strains for brewing [1,7]. While fruits, particularly those from biodiversity-rich regions like the Amazon, provide a natural habitat for *S. cerevisiae*, the literature remains silent on leveraging these unique strains for brewing.

The yeast biodiversity of the Amazon has the potential to be investigated by the brewing industry, introducing novel flavors and aromas, thereby meeting the ever-increasing consumer demand for diverse beer profiles. Moreover, by addressing this gap, we can promote sustainable brewing practices, harnessing nature's bounty in a region rich in biodiversity [12].

This study aims to explore several scientific inquiries related to yeast isolation from Amazonian fruits undergoing spontaneous fermentation. Specifically, it seeks to accomplish the following: (1) quantify the yeast isolated from these fruits, (2) identify whether the isolates include *Saccharomyces cerevisiae* by employing biochemical methods (such as sugar assimilation and fermentation tests) alongside ITS rDNA (internal transcribed spacer ribosomal DNA) sequencing for species-level identification, and (3) evaluate the feasibility of producing cream ale beer using the regional isolates. The yeast strains under investigation originate from the spontaneous fermentation of three specific fruits: Arazá (*eugenia stipitata* McVaugh), cocoa (*Theobroma cacao* L.), and cupuassu (*Theobroma grandiflorum* Wild. Ex Spreng. Schum).

2. Materials and methods

2.1. Reference microorganisms

Industrial strains of *Saccharomyces cerevisiae* were obtained from commercial sources. From White Labs (San Diego, California-USA), the strains include California ale WLP001, German ale WLP029, Belgian ale WLP550, and Cry havoc WLP862, the commercial yeasts Safale S-04 and Safale US-05, both produced by Fermentis, a division of Lesaffre in Marcq-en-Barœul, France. These strains are recommended in brewing recipes from four schools: North American, German, Belgian, and English. The preserved microorganisms were reactivated in YMA (yeast, malt and agar) medium with yeast extract 0.3% (w/v), malt extract 0.3% (w/v), peptone 0.5% (w/v), glucose 1% (w/v), and agar 2% (w/v).

2.2. Fruits of the Amazon region

Three fruit species native to the Amazon region in Brazil were selected: Arazá (*Eugenia stipitata* MacVaught), cocoa (*Theobroma cacao* L.), and cupuassu (*Theobroma grandiflorum* Wild. Ex Spreng. Schum.). These fruits were collected in the campus I grove of the national Amazon research institute (INPA) (−3.096369° latitude and −59.985893° longitude), Manaus-Amazonas tree-ripe fruits were collected from each of these species, and they were fully mature, ready for human consumption, with no visible bruises or signs of pathogens. The fruits were placed in sterile bags and transported to the mycology laboratory.

2.3. Materials feedstock

Pale ale malt (Cooperativa Agrária, Paraná-Brazil) was used, with the following specifications: color between 5.5 and 7 EBC (European Brewery Convention); minimum friability of 83%; maximum concentration β-glucans of 160 g/L; maximum viscosity 1.60 mPa/s; proteins from 10 to 11.5%; diastatic power 200 Wk.

The hops added to the boil were Hallertau Magnum (Hopteiner, Germany) with 11 to 16% alpha acid, beta acid from 5 to 7%, and 100 g total oil between 1.6 and 2.6 mL.

The water (Santa Cláudia, Manaus-AM) used in the brazing process contained the following concentrations: 0.54 mg/L calcium; 1.57 mg /L magnesium; 0.88 mg/L sodium; 0.07 mg/L sulphate; 2.67 mg/L nitrate; 1.07 mg/L chloride, having a pH of 5.8 at 25 °C.

2.4. Isolation of wild yeast

The fruits (10 g) had their pulp cut into pieces of approximately 0.5 cm^3 and were inserted into 50 mL falcon tubes containing 30 mL of malt extract, and initial density was adjusted to 1040 g/cm^3. Every five days, samples were taken for analysis of reducing sugars, colony forming units (CFUs), soluble solids, and yeast isolation.

Yeast colonies were isolated in petri dishes containing YMA [13]. The surface of the culture medium was inoculated with 100 µL of the fermented. The colonies selected to be isolated were chosen by observing the morphotypes. From each plate, tree colonies of the each morphotypes were isolated. The obtained colonies were subjected to striae sowing to obtain pure cultures. This procedure was

repeated three times. After obtaining the pure colonies they were picked in 5 YMA tubes, incubated for 48 hours, and stored at 5 °C.

The yeast strains have been preserved using long-term methods (cryopreservation, lyophilization) at the collection of microorganisms of medical interest at INPA (national institute of Amazonian research). This research was registered in the national system for the management of genetic heritage and associated traditional knowledge (SISGEN-A9A738C).

2.5. Identification of yeast isolates

2.5.1. Biochemical tests for identification of isolates

The isolates underwent fermentation and sugar assimilation tests [12]. The yeasts were grown in plates containing YMA medium for 48h at room temperature, then transferred to test tubes containing 5 mL of sterile water. The suspension was adjusted from the McFarland 0.5 scale, this provides a standard yeast suspension of 5×10^6 cells/mL.

Assimilation test occurred in plates containing nitrogen base medium added with 2.0% agar, the suspensions were inoculated by pour plate and the sugars distributed at equidistant points on the medium, sucrose, galactose, lactose, maltose, fructose, glucose, and raffinose.

Fermentation test of carbon sources 6.7 g of nitrogen base (Difco ™) medium was diluted in 100 mL of distilled water and 5 g of carbon source (maltose and lactose) were added. After homogenization 5 mL of the medium was transferred to test tubes containing duham tubes, then autoclaved at 120 °C for 20 min. In a microbiological safety cabinet, inoculum of 0.5 mL of microbial suspensions were performed. Incubation occurred at room temperature and the experiment was observed for 30 days.

2.5.2. Molecular identification of isolates

In the present study the isolates obtained in the previous stage were submitted to identification by the polymerase chain reaction (PCR) technique of the transcribed internal space (ITS) region of rDNA, with its subsequent sequencing. Genomic DNA extraction from yeasts was performed by the phenol: chloroform: isoamyl alcohol method [14].

PCR was performed by mixing 22.5 μL Milli-Q water solutions, 5.0 μL 10X PCR buffer, 2.0 μL $MgCl_2$ (50 mM), 5.0 μL dNTPs (Deoxyribonucleoside triphosphates) (2 mM), 5.0 μL from each ITS1 primer (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (10 μM) [15], 0.5 μL Taq polymerase Platinum (Invitrogen, Carlsbad, CA, USA) (5 U/μL) and 5.0 μL fungal DNA (20–100 ng), in which each reaction had the final volume of 50 μL. Reactions were performed in a thermal cycler (super cycler ™ SC-200, Kyratec) with programming starting with denaturation for 5 min at 94 °C plus 40 cycles, consisting of a denaturation step (30 seconds at 94 °C), a pairing (30 seconds at 53 °C) and one stretching step (60 seconds at 72 °C) and finally a final extension step (10 min at 72 °C) [16]. Amplification results were evaluated with 8 μL aliquots of amplicons by 1.5% agarose gel electrophoresis in 1x TAE buffer [40 mM Tris-Acetate, 1 mM EDTA (Ethylenediaminetetraacetic acid), pH 8.0)], being stained with Syber Green SYBR® Safe (Invitrogen, Carlsbad, CA, USA) for visualization of an approximately 1000 bp fragment. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Sciences Inc., Valencia CA) according to the manufacturer's instructions.

Sequencing was performed using the Big Dye® kit 3.11 (Thermo Fisher Scientific Inc., Waltham, MA, USA) and according to the manufacturer's instructions. After the sequencing reaction, the samples were precipitated with ethyl alcohol and EDTA to remove excess unincorporated reagents. Then, the reaction product was submitted to the automatic DNA analyzer (ABI 3130xL, Thermo Fisher Scientific Inc., Waltham, MA, USA). These steps were performed following the manufacturer's recommendations.

The nucleotide sequences of ITS generated from the yeast isolates were checked, compiled and edited using the BioEdit 7.0.9.0 program. Genomic sequence analyzes provided by the sequencer were performed with the aid of the BLASTn (nucleotide basic local alignment search tool) program using the national center for biotechnology information (NCBI) database, available at (http://blast.ncbi.nlm.nih.gov/Blast. CGI) (02.22.2024). The identification criterion adopted in this project was with similarity results above 96%.

DNA sequences from related species retrieved from the national center for biotechnology information (NCBI) database using BLASTn were compared to those obtained considering values above 97% similarity. The genetic distance tree was generated using the neighbor joining (NJ) method with the aid of the MEGA X (molecular evolutionary genetics analysis X) program. The percentages of replicate trees in which associated taxa clustered in the bootstrap test (1000 replicates) were also determined.

The nucleotide sequences of ITS were deposited at NCBI GenBank under (accession numbers in the Table 1).

Table 1. Sequence codes deposited in the NCBI database of the yeast species evaluated in this study.

Isolate code*	Species	NCBI number
AR-3	*Saccharomyces cerevisiae*	PP410285
AR-4	*Saccharomyces cerevisiae*	PP412074
AR-9	*Saccharomyces cerevisiae*	PP416828
AR-12	*Saccharomyces cerevisiae*	PP416829
AR-13	*Saccharomyces cerevisiae*	PP416830
AR-15	*Saccharomyces cerevisiae*	PP416831
AR-14	*Lachancea fermentati*	PP414195
AR-16	*Lachancea fermentati*	PP414196
CC4	*Wickerhamomyces anomalus*	PP414198
CP10	*Wickerhamomyces anomalus*	PP414199
CP13	*Wickerhamomyces anomalus*	PP414200
CP15	*Wickerhamomyces anomalus*	PP414201
CP21	*Wickerhamomyces anomalus*	PP414202
CP25	*Wickerhamomyces anomalus*	PP414203
CC15	*Torulaspora delbrueckii*	PP414221
CP18	*Pichia kudriavzevii*	PP414222
CC6	*Candida blattae*	PP420031
CC14	*Candida blattae*	PP420032
CC30	*Candida blattae*	PP420033
CC19	*Candida intermedia*	PP431057
CC29	*Candida intermedia*	PP431058
CC8	*Candida intermedia*	PP431059
CC10	*Candida intermedia*	PP431060

*The isolates obtained from the fruiting bodies were encoded using the following codes: AR for isolates from arazá, CC for isolates from cacao, and CP for isolates from cupuassu. This coding system was adopted to facilitate the identification and classification of isolates based on their source of origin, allowing for a more precise and organized analysis of the collected data.

2.5.3. PCR-RAPD (random amplified polymorfic DNA): M13 minisatellite

Genomic DNA samples from both *Saccharomyces cerevisiae* strains and commercial yeast strains were standardized to a concentration of 20 ng/μL. We utilized the RAPD fingerprinting method with the M13 primer (sequence 5'-GAGGGTGGCGGTTCT-3') to generate a genetic fingerprint of these strains [17]. The RAPD technique amplifies random segments of genomic DNA using a single primer, in this case, the M13 primer, which is particularly useful for generating a diverse array of DNA fragments for comparison.

The reaction mixture for the PCR included the following reagents in a total volume of 60 μL: 39.1 μL of Milli-Q water, 6 μL of 10X PCR buffer, 5 μL of the standardized DNA (20 ng/μL), 1 μL of the M13 primer (10 μM), 2.4 μL of $MgCl_2$ (50 mM), 6 μL of dNTPs (2 mM), and 0.5 μL of Taq Polymerase (5 U/μL) Platinum (Invitrogen, Carlsbad, CA, USA) (5 U/μL). The PCR cycling conditions were set on a Super Cycler™ SC-200 (Kyratec, Brisbane, QLD, Australia) as follows: initial denaturation at 94 °C for 2 min, followed by 36 cycles of denaturation at 94 °C for 45 seconds, annealing at 50 °C for 60 seconds, and extension at 72 °C for 60 seconds, concluding with a final extension at 72 °C for 6 min.

Amplicons (10 μL) were analyzed by 2% (w/v) agarose gel electrophoresis in 1X TAE buffer, with migration at 90 V by 45 min. The gels were stained with Syber Green SYBR® Safe (Invitrogen, Carlsbad, CA, USA) for viewing under UV light and digital image capture was performed with a semi-professional camera.

The genetic relationships between *S. cerevisiae* strains from the dendrogram constructed by the GelJ program [17]. The similarity between the fingerprints of each lineage was calculated from the Ochai correlation coefficient, establishing a tolerance of 1%, and the clustering analysis was performed by the unweighted pair group method with arithmetic mean (UPGMA).

2.6. Evaluate the feasibility of producing cream ale beer using the regional yeast (Amazon ale)

We decided to produce the beer in triplicate using the yeast isolate that demonstrated the highest gas production during preliminary fermentation tests. Alongside this selected isolate, we employed a control yeast strain, the American ale US-05 from Fermentis, for comparative analysis. The fermentation process was monitored closely, with evaluations conducted every 24 hours. The parameters measured included: density, alcohol by volume (ABV), reducing sugars, Brix, and pH. These assessments allowed us to quantify fermentation progress and efficiency, providing insights into the fermentative capabilities of the selected yeast isolate compared to the control strain.

Propagating for beer production: strains were reactivated on YMA-containing petri dishes and incubated for 2 days at room temperature. Cell biomass from the dishes was transferred to 50 mL Falcon-type tubes with 10 mL of DME (dry malt extract) 2% broth and incubated for 48 hours. This culture was then moved to 250 mL erlenmeyer with 100 mL of DME 2% broth, incubated with orbital shaking at 120 rpm for 48 hours. The final propagation step involved inoculating the culture in 2000 mL erlenmeyer containing 1000 mL of DME 2% broth, aerated using a 0.5 μm diffuser system attached to a 0.22 μm filter (Millipore Merck Millipore, USA) and an aquarium air vent, and incubated for 24 hours.

In the brewing process, the clarified must was first brought to a boil and 20 g of hops were added, boiling for 60 min, followed by a whirlpool and 15 min of trub decantation before cooling using a 40-plate chiller. Then, the fermentation phase began with 4×10^{10} cells/mL of propagated yeast cells inoculated

into fermenters, targeting a final yeast concentration of 0.5×10^6 cells/mL of must. This phase was carefully monitored at 19 °C for five days, tracking density, soluble solids, and pH levels. After achieving a steady state in fermentation, the temperature was increased to 20 °C for two days, followed by cold clarification at 0 °C for seven days to sediment the cells. Finally, for bottling and carbonation, beers were transferred to bottles and primed with a solution containing invert sugar at 7 g/L for yeast reactivation and CO_2 production over seven days at 19 °C.

2.7. Analytical methods

2.7.1. Cell count

Cell enumeration was performed utilizing a Neubauer improved counting chamber (L. W. Hawksley Ltd., United Kingdom). Sample preparation involved serial dilutions ranging from 10^{-4} to 10^{-8} of the fermentative broth. Methylene blue dye (0.025% w/v, Sigma-Aldrich, St. Louis, MO, USA, Cat. No. M9140) was incorporated at a 1: 10 v/v ratio for viability staining. A 10 µL aliquot of each prepared sample was then carefully loaded onto the counting chamber. Observations were conducted under an optical microscope (Brand: Zeiss, Model: Axioscope 2, with a 40 × objective), focusing quantify number on live cells per mL of fermentation must.

2.7.2. Concentration of reducing sugars

The concentration of reducing sugars was determined by the 3.5 dinitrosalicylic acid (DNS) method [18], in which 0.02 mL aliquots of the samples were previously diluted in 0.98 mL of distilled water and transferred to test tubes containing 1 mL of the solution. of the DNS reagent. After heating in a water bath at boiling temperature for 5 min and subsequent cooling in running water, the absorbance reading was taken at 540 nm of each sample in a 600S digital spectrophotometer (FEMTON-SP-Brazil). The reaction blank used to calibrate the apparatus was obtained using only 1mL distilled water. The observed absorbance was correlated in concentration using a standard glucose curve, obtained from a stock solution containing 0.33 g of glucose in 100 mL of distilled and conveniently diluted water.

2.7.3. Soluble solids

Soluble solids values were obtained by analog refractometer, measured with distilled water for total soluble solids equal to 0 ºBrix. The sample values visualizations occurred positioning the refractometer against the light, samples with temperatures between 20 and 25 °C.

2.7.4. Density

Densities were measured using a 0.980–1.100 scale densimeter (Incoterm, São Paulo, Brazil). Fermentation samples were transferred to 100 mL beakers, then the densimeter was submerged and after stabilization the scale was visualized.

2.7.5 pH

The pH of the samples was measured by pH meter (Chimis model Q400MT). After calibration of the instrument in pH 4.01 and 9.18 buffer solutions.

2.7.6. Ethanol

Fermented media samples were steam-distilled in a micro alcohol still (Tecnal, Te-012 micro) before the determination of the ethanol concentration. The ethanol concentration was determined using a spectrophotometer (600 plus, Fenton, São Paulo) at 600 nm using the potassium dichromate method. Ethanol yield was calculated as amount of ethanol produced divided by the theoretical amount (calculated based on the quantity of sugar in the must) and expressed as a percentage w/w.

2.8. Statistical analysis

All assays were conducted in triplicate. Data are presented as mean ± standard deviation (SD). Differences between means were assessed using the ANOVA followed by the t-test for pairwise comparisons. A significance level of 95% confidence was employed in all analyses.

3. Results

To isolate yeasts involved in the fermentation of Amazonian fruits, we submerged the selected fruits in a malt extract solution, allowing the native fruit biota to initiate spontaneous fermentation. Figure 1 illustrates data from spontaneous fermentations of *Eugenia stipitata* MacVaught, *Theobroma grandiflorum* Wild. Ex Spreng. Schum, and *Theobroma cacao* L. fruits, focusing on yeast colony-forming units (CFU), soluble solids (ºBrix), and reducing sugars (g/L).

During the fermentation of *T. grandiflorum*, *T. cacao*, and *E. stipitata* fruits over a period of 30 days, the quantification of yeast colony-forming units (CFU/mL) and the number of isolates were determined. For *T. grandiflorum*, the (CFU/mL) values increased from 6.10×10^6 on day 5 to 4.70×10^8 on day 20. In the case of *T. cacao*, (CFU/mL values ranged from 2.00×10^4 to 1.80×10^8. *E. stipitata* exhibited (CFU/mL) values from 5.80×10^9 to 3.50×10^7.

Under our experimental conditions, we isolated a total of 76 yeast colonies: 26 yeast colonies from *T. grandiflorum*, 32 colonies from *T. cacao*, and 18 colonies from *E. stipitata*.

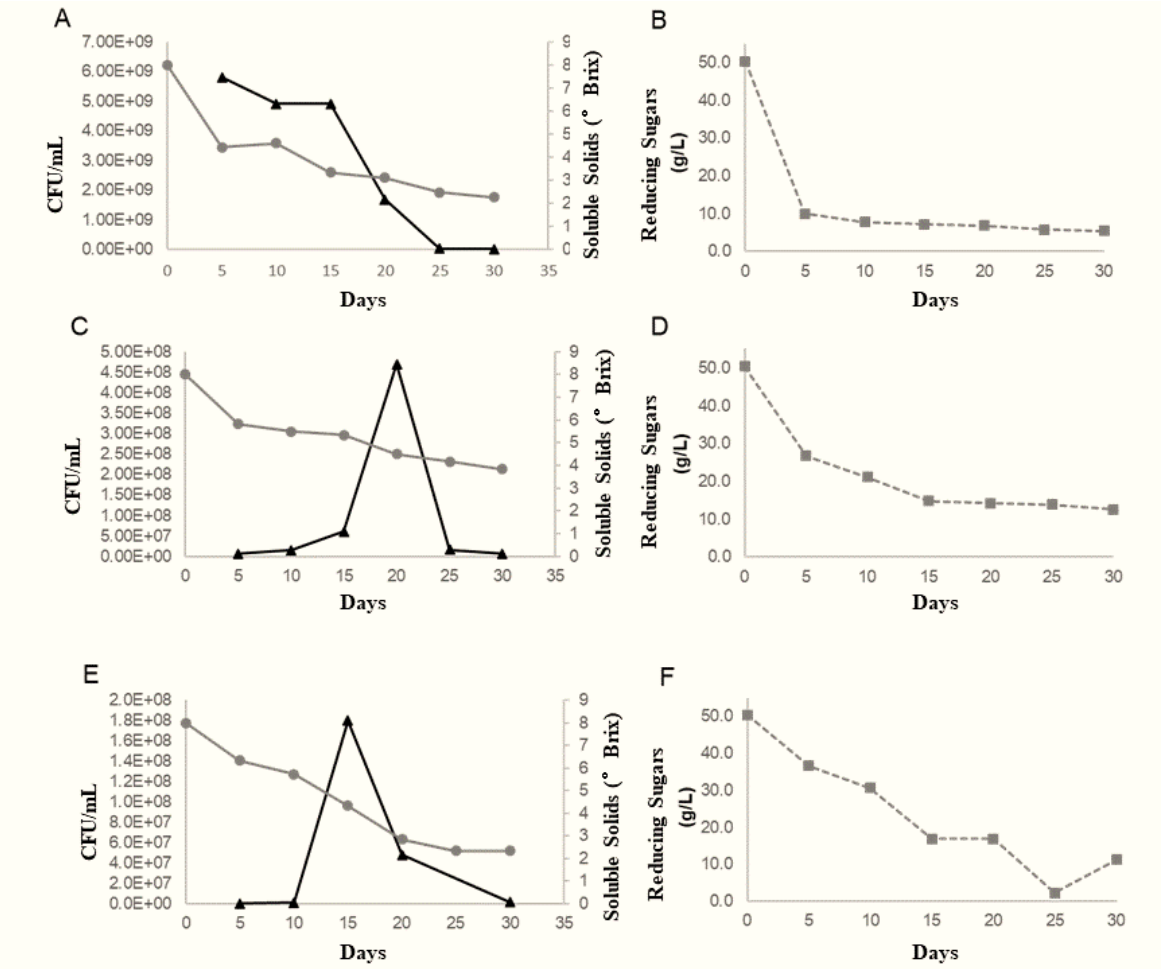

Figure 1. Monitoring spontaneous fermentations of Arazá (*Eugenia stipitata* MacVaught), cupuassu (*Theobroma grandiflorum* Wild. Ex Spreng. Schum), and cocoa (*Theobroma cacao* L.) fruits. Subfigures A and B represent the follow-up of Arazá fermentations, while C and D depict cupuassu, and E and F illustrate cocoa. The analysis includes, in the black lines, the colony forming units (CFU/mL) and in the gray lines the soluble solids (°Brix) in panels A, C, and E, and reducing sugars (g/L) in panels B, D, and F.

To identify *S. cerevisiae* isolates, all 76 underwent sugar assimilation, sugar fermentation, and ascospore production tests. Maltose fermentation was the initial screening step, with only 25 isolates fermenting maltose. Isolate AR 03 notably displayed maltose fermentation within 24 hours. These 25 isolates underwent further biochemical testing (Table 2).

Table 2. List of auxanogram, zymogram and ascospore results of isolated yeasts isolated from spontaneous fermentations of Arazá (*Eugenia stipitata* MacVaught), cupuassu (*Theobroma grandiflorum* Wild. Ex Spreng. Schum), and cocoa (*Theobroma cacao* L.) fruits.

Lineage	Sugar assimilation						Fermentation		Reproduction
	Sac	Raf	Mal	Glyc	Gal	Lac	Lac	Mal	Ascospore
AR 01	+	+	+	+	+	−	−	+	+
AR 03	+	+	+	+	+	−	−	+	+
AR 04	+	+	+	+	+	−	−	+	+
AR 09	+	+	+	+	+	−	−	+	+
AR 12	+	+	+	+	+	−	−	+	+
AR 13	+	+	+	+	+	−	−	+	+
AR 14	+	+	+	+	-	−	−	+	−
AR 15	+	+	+	+	+	−	−	+	+
AR 16	+	+	+	+	-	−	−	+	−
CC 04	+	+	+	+	+	−	−	+	−
CC 06	+	+	+	+	+	−	−	+	−
CC 08	+	+	+	+	+	−	−	+	−
CC 10	+	+	+	+	+	+	−	+	−
CC 14	+	+	+	+	−	−	−	+	−
CC 15	+	+	+	+	−	−	−	+	−
CC 19	+	+	+	+	−	−	−	+	−
CC 23	+	+	+	+	+	+	−	+	−
CC 29	+	+	+	+	+	+	−	+	−
CC 30	+	+	+	+	+	−	−	+	−
CP 10	+	+	+	+	+	−	−	+	−
CP 13	+	+	+	+	−	−	−	+	−
CP 15	+	+	+	+	−	−	−	+	−
CP 18	−	−	−	+	−	−	−	+	−
CP 21	+	+	+	+	−	−	−	+	−
CP 25	+	+	+	+	+	−	−	+	−
WLP029 German Ale	+	+	+	+	−	−	−	+	−
WLP862 Cry Havoc	+	+	+	+	+	−	−	+	−
WLP550 Belgian Ale	+	+	+	+	+	−	−	+	−
WLP001 California Ale	+	+	+	+	+	−	−	+	+
SafAleS-04 English Ale	+	+	+	+	−	−	−	+	−
SafAleS-05 American Ale	+	+	+	+	+	−	−	+	+

AR: isolates of Arazá, CC: cocoa isolates, CP: cupuassu isolates, Glyc: glucose, Gal: galactose, Fru: fructose, Raf: raffinose, Mal: maltose, Sac: sucrose and Lac: lactose.

The 25 maltose-fermenting yeasts were molecularly identified through ITS rDNA sequencing (Figure 2). Seven yeast species were recognized, with all *S. cerevisiae* isolates (n = 7) from *E. stipitata* spontaneous fermentations.

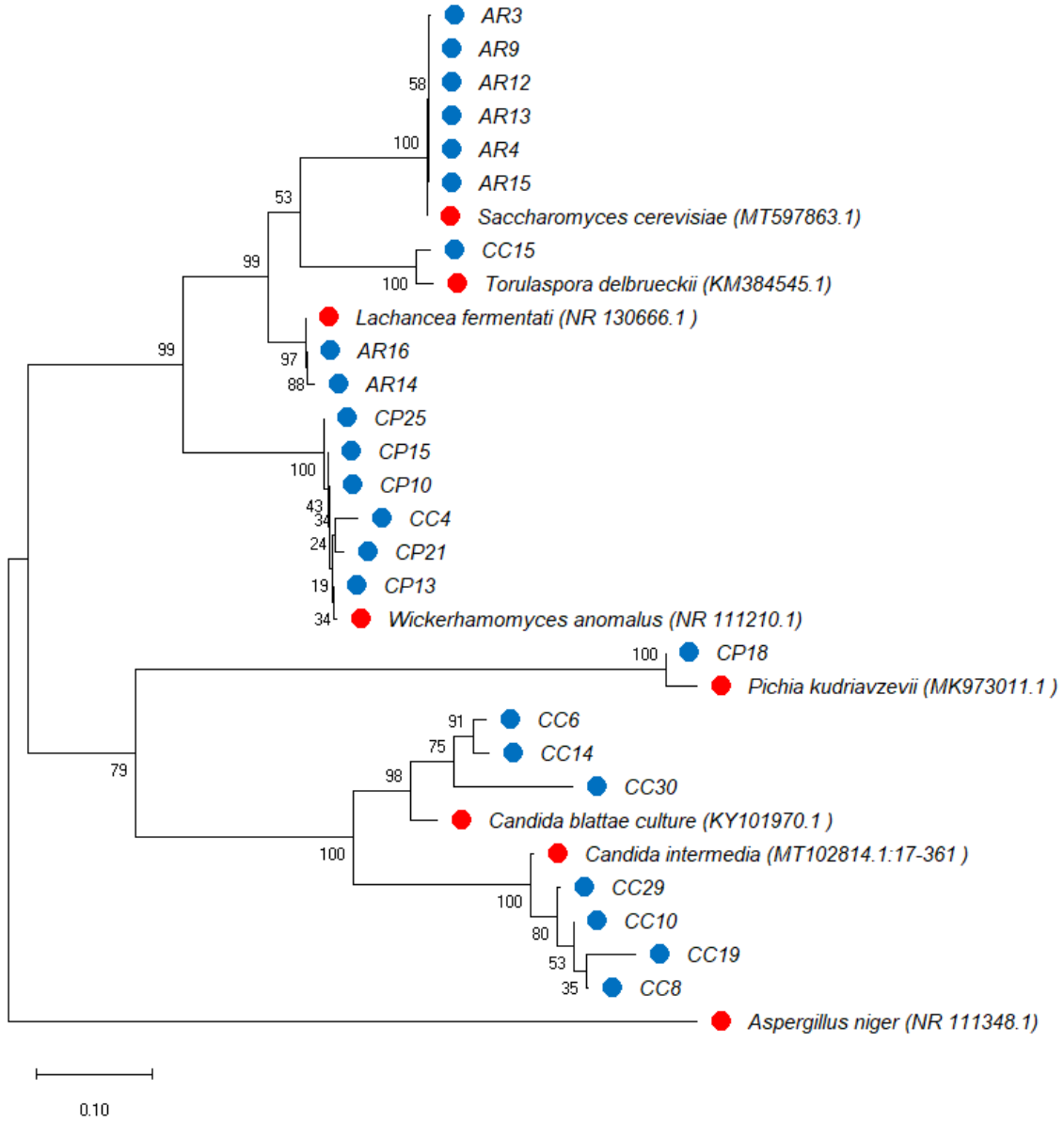

Figure 2. Tree generated by the neighbor-joining (NJ) method constructed for the internal transcript spacer (ITS) molecular marker in the MEGA X (molecular evolutionary genetics analysis) software. This phylogenetic tree displays the relationships among yeast strains using the neighbor-joining method in MEGA X software. Red dots represent 25 wild yeast isolates obtained from spontaneous fermentations of Arazá, Cupuassu, and Cocoa fruits. Blue dots indicate sequencing types from the NCBI database, providing a comparative overview of genetic diversity. Aspergillus niger (NR 111348.1) is included as an outgroup for reference.

Phylogenetic relationships were also assessed through RAPD-PCR fingerprinting (M13) polymorphism analyses, resulting in a dendrogram with six main groups (Figure 3). Isolates from this study clustered into two separate groups (C and D).

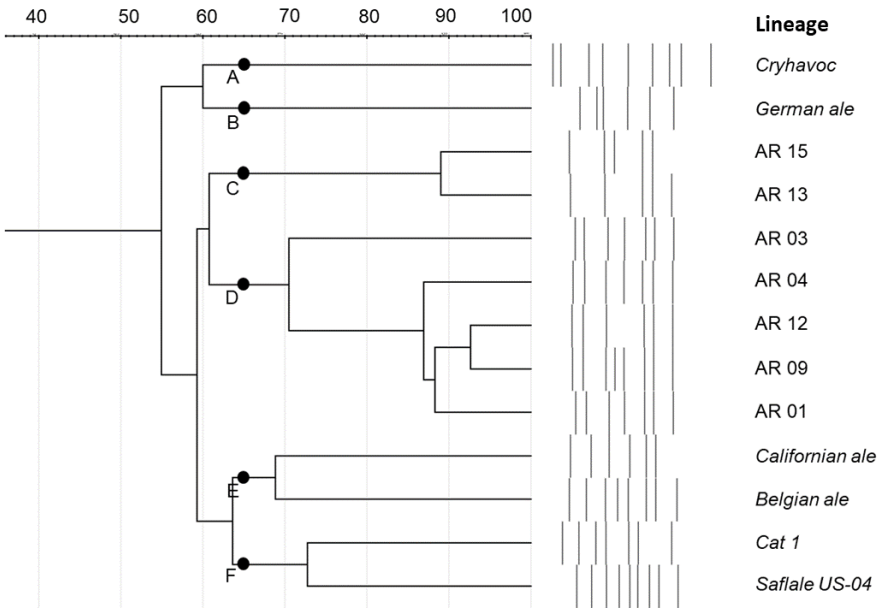

Figure 3. "Dendrogram of RAPD M13-PCR Fingerprints in *S. cerevisiae* Strains". "Cluster analysis dendrogram" (Unweighted Pair Group Method with Arithmetic mean-UPGMA). showing the genetic relationships among seven *S. cerevisiae* strains, both isolated and commercial. Strains include: WLP862 Cry Havoc, WLP029 German Ale, WLP001 California Ale, WLP550 Belgian Ale, Cat 1 SafAle S-05 American Ale, and SafAle S-04 English Ale. Isolates from fruiting bodies are coded as AR (arazá), CC (cacao), and CP (cupuassu).

Due to its rapid fermentation capabilities, *S. cerevisiae* isolate AR 03 was selected for beer production trials, compared with *S. cerevisiae* strain American ale Safale US-05. Sugar depletion from the fermentation mash was comparable for both yeasts (Figure 4).

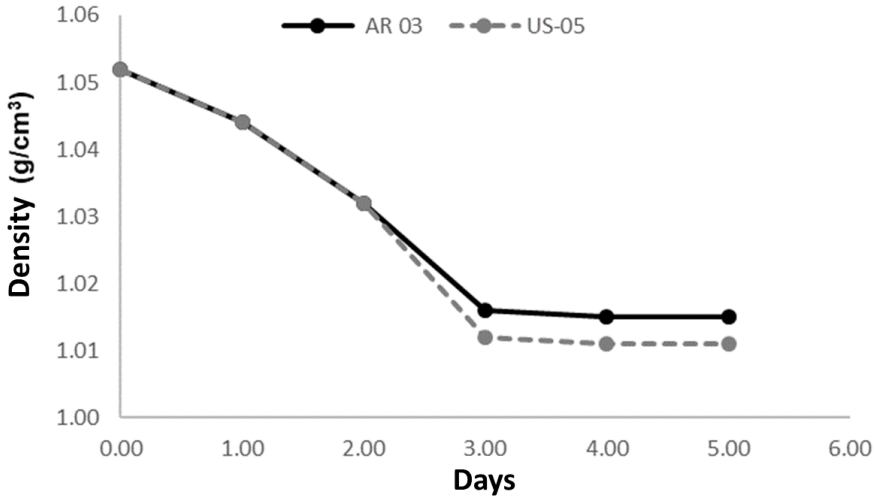

Figure 4. Density variation along fermentation of brewer wort between *S. cerevisiae* AR 03 isolate and *S. cerevisiae* strain American ale Safale S-05.

Table 3 delineates comparative characteristics of beer produced using *S. cerevisiae* AR 03 and the strain *S. cerevisiae* American ale Safale US-05.

Table 3. Characteristics of beers produced by the strain AR 03 and Safale US-05.

Analytics	*S. cerevisiae* AR 03		*Safale US-05*	
	Initial	Final	Initial	Final
Soluble Solids (ºBrix)	13.0 ± 0.1	7.6 ± 0.1	13.0 ± 0.1	7.0 ± 0.1
Density (g/cm^3)	1.052 ± 0.1	1.015 ± 0.001	1.052 ± 0.001	1.011 ± 0.001
Ethanol (ABV%)	-	4.9 ± 0.1	-	5.4 ± 0.1
pH	5.2 ± 0.1	4.4 ± 0.2	5.2 ± 0.1	4.3 ± 0.2

The fermentation of beer utilizing yeast AR 03 demonstrated an attenuation of 71%, characterized by low flocculation, resulting in a slightly cloudy final product. In comparison, the commercial strain exhibited a more translucent visual profile and an attenuation of 78%.

4. Discussion

Our recent research investigated fruit fermentations, aiming to isolate cultivable strains from the genus *Saccharomyces*. Our focus primarily centered on the isolation and recognition of *Saccharomyces cerevisiae* in fermentations. Notably, we found this organism in the spontaneous fermentations from *Eugenia stipitata* fruit. This discovery supports our hypothesis that fruit fermentations harbor various yeast strains, holding significant implications for industrial applications.

Our investigation revealed a diverse microbial community in spontaneous fermentations. Alongside *Saccharomyces cerevisiae*, we identified the following other yeast species: *Lachancea fermentati, Wickerhamomyces sydowiorum, Torulaspora delbrueckii, Candida blattae, Candida intermedia, Pichia kudriavzevii,* and *Wickerhamomyces anomalus* [5,19,20]. Previous studies have associated *Lachancea fermentati* and *Torulaspora delbrueckii* with considerable potential in beverage fermentations [19,20]. *Wickerhamomyces anomalus* stands out for its biocontrol properties against spoilage microorganisms, offering opportunities in food preservation [19]. *Saccharomyces cerevisiae* was accounted just for a fraction of the isolated yeasts, aligning with past findings that various species co-isolate with *S. cerevisiae* at frequencies of 1–10%. Our results reinforce this consensus, suggesting commonality with broader scientific observations [3,6,20,21].

Sequencing of the ITS region allowed the grouping isolates into the species *S. cerevisiae*. This genetic marker, ITS, has been capable of grouping and identifying isolates of this species in previous studies. Recent studies present the ITS region as an excellent region for identification of yeasts [22,23]. The isolates in the present study were also confirmed as belonging to the species *S. cerevisiae* in the ITS + 28S (LSU) concatenated analysis carried [24]. The *S. cerevisiae* isolates had unique M13 fingerprints, differentiating them from environmental and industrial strains. In experimental condition, M13 fingerprint allowed distinction to allow regional isolates to well-known industrial strains. This finding is significant because it's the one of first analysis comparing regional isolates to well-known industrial strains [22–24].

Seven yeast strains, identified as *S. cerevisiae* through ITS rDNA region and biochemical assays, fall under the "generally recognized as safe" (GRAS) category [25]. Originating from the Amazon, these strains offer direct potential for bioeconomic development in the region. They not only pave the

way for beverage production but also highlight the Amazon's microbial diversity for various biotechnological applications [5,25].

In beer production kinetics, the isolate *S. cerevisiae* AR 03 exhibited brewing behavior similar to the commercial American ale Safale US-05 strain. Phylogenetic assessments revealed significant parallels between regional isolates and commercial strains. Notably, the beer produced with the AR 03 isolate of *S. cerevisiae* presents characteristics of physicochemical attributes similar to those found in commercial American ale beers. More studies should be carried out to verify whether there are phylogenetic differences in other beer characteristics such as flavor. However, the parallels in attenuation, fermentation speed, and final product characteristics underline AR 03's potential in brewing applications. Identifying such a strain with comparable efficacy to a commercial strain from the Amazon holds promise for regional bioeconomic growth, particularly in the beverage industry.

In the present work, certain limitations warrant consideration. The absence of organoleptic testing in the beer represents a gap, precluding a direct sensory analysis of the final product's quality. Additionally, the comprehensive sequencing analysis of regional yeasts and the assessment of metabolites produced during fermentation using analytical methods such as GC/MS-MS were not conducted, which could offer a deeper understanding of the involved biochemical processes. Despite these limitations, it is crucial to highlight the importance of this study.

The utilization of strains isolated from the Amazon region have potential as commercial strains. The production of beer with the *S. cerevisiae* AR 03 strain exhibited physicochemical attributes comparable to those found in commercial American ale beers. These results highlight the possibility of utilizing Amazonian yeasts in brewing, marking a noteworthy progress for the potential emerging bioeconomy in the Amazon region.

5. Conclusions

In this study, yeast strains were isolated from spontaneous fermentations of araza-boi (*Eugenia stipitata* MacVaught), cocoa (*Theobroma cacao L.*), and cupuassu (*Theobroma grandiflorum* Wild. Ex Spreng. Schum) fruits in the Amazon region. During the tests, 76 colonies were isolated, of which seven were identified as *Saccharomyces cerevisiae* through biochemical tests and ITS rDNA sequencing. Phylogenetic assessments evaluated the parallels and differences between isolated *S. cerevisiae* strains and commercial strains. The isolated yeasts could be grouped into species by the ITS region and could be grouped regionally isolated by reaction with RAPD M13. Finally, the beer produced with the AR 03 isolate exhibited physicochemical attributes similar to those of the commercial strain American ale Safale US-05.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Authors' contributions

All authors contributed to the article conception and design. Flávia da Silva Fernandes, Jacqueline da Silva Batista e João Vicente Braga de Souza had the idea for the article. Flávia da Silva Fernandes, Abrames Francisco Ferreira Goes, Matheus Alberto Vasconcelos de Lima, Luan Reis Honorato da Silva performed the literature search and data analysis. Flávia da Silva Fernandes, wrote the first draft of the manuscript Érica Simplício de Souza, Lívia Melo Carneiro, João Paulo Alves Silva critically revised the work. Flávia da Silva Fernandes, Jacqueline da Silva Batista e João Vicente Braga de Souza wrote the later and final drafts, and all the authors read and approved the final manuscript.

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