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Research article

Isolation, genetic identification of Amazonian yeasts and analysis of thermotolerance and alcohol tolerance of *Saccharomyces cerevisiae* from *Theobroma grandiflorum* and *Eugenia stipitata*

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Abstract: Although yeasts of the *Saccharomyces cerevisiae* species are industrially significant, few studies have investigated their presence in environmental samples from the Amazon rainforest. This study aimed to isolate *S. cerevisiae* yeasts associated with trees of the Amazon Forest and investigate their thermotolerance, alcohol tolerance, and single nucleotide polymorphism (SNP) characteristics, along with those of regional strains from previous research and reference strains from the industry. We collected fruits, bark and decaying plant material from *Theobroma grandiflorum*, *Spondias mombin* L., *Mangifera indica* L., and *Eugenia stipitata*, and isolated yeasts using the culture media. To identify the yeasts, we conducted morphological and biochemical analyses, including sugar assimilation and fermentation, and sequencing analyses of the rDNA (ITS and LSU (D1 and D2)). We also performed fermentation tests to determine the optimum temperature, thermotolerance and ethanol tolerance. Finally, we subjected the selected strains to SNP analysis to study the reported genes that are important

for alcohol tolerance in *S. cerevisiae*: FPS1 (farnesyl diphosphate synthase1) and ASR1/YPR093 (alcohol sensitive RING/PHD finger1) genes. As a result, we isolated 53 yeasts, and 10 of which exhibited a sugar assimilation and fermentation profile that was similar to that of *S. cerevisiae*. These ten isolates were identified using sequencing of the ITS and LSU regions, which revealed the species to be *Wickerhamomyces anomalus* (n = 4), *Torulaspora pretoriensis* (n = 3), *Debaryomyces hansenni* (n = 1), and *Saccharomyces cerevisiae* (n = 2). Through the analysis of the ASR1 and FPS1 regions, we found an SNP at nucleotide 1552 A > G (FPS1), which was associated with ethanol tolerance under our experimental conditions. This work is significant because it is one of the first studies to focus specifically on the isolation of *S. cerevisiae* from samples in the Amazon region. Furthermore, the SNP analysis allowed us to differentiate isolates that showed greater tolerance to ethanol.

Keywords: Amazonian fruits; Saccharomyces cerevisiae; SNPs; ethanol tolerance; yeast; thermotolerance

1. Introduction

In 2022, global bioethanol production reached 15.4 million (US) gallons (≈58,200,000,000 liters) of ethanol. In the same year, Brazil achieved a record ethanol production of 7,420 million gallons (≈28,000,000,000 liters) [1].

Brazilian distilleries use a standardized inoculum of *S. cerevisiae* strains in order to achieve high fermentation efficiency. The *S. cerevisiae* strains used as inoculum are BG-1 (Copersucar São Paulo-SP), SA-1 (Copersucar São Paulo, SP), CAT-1 (Fermentec Piracicaba, SP), and PE-2 (Fermentec Piracicaba, SP). These strains have been used in approximately 70% of all Brazilian distilleries [2], which has resulted in an increase in ethanol productivity of around 3% [3]. Some of the requirements for these strains are rapid fermentative potential, improved flocculating ability, sufficient osmotolerance, ethanol tolerance, and thermotolerance [4,5]. Therefore, for these strains to exist, there is need to isolate or produce new strains, since the number of commercially available strains for this sector is quite small [6].

Previous studies have investigated the presence of yeasts in the Amazon, including from *Theobroma cacao* seeds [7–9], *Myrciaria dubia* [10]; larvae of coleoptera [11]; and cocoa bean fermentations [12]. Recently, yeasts of *Saccharomyces cerevisiae* (AR-1, AR-3, AR-4, AR-9, AR-12, AR-13) were isolated om *Eugenia stipitate* in our laboratory (Mycology Laboratory at INPA). Despite providing important information about the presence of *S. cerevisiae* in the Amazon, these studies e few in number and probably describe only a small fraction of the diversity of yeasts from the Amazon.

Thus, this study aimed to isolate *S. cerevisiae* yeasts associated with trees of the Amazon forest, investigate their thermotolerance, alcohol tolerance, and single nucleotide polymorphism (SNP) characteristics, along with those of regional strains from previous research and reference strains from the industry.

2. Materials and methods

2.1. Sampling and isolation

We investigated the presence of *S. cerevisiae* in samples of fruit, tree bark, decomposing plant material (litter) directly below the plant. The fruits trees found in the small forest of the National Institute of Amazonian Research (INPA) were *Theobroma grandiflorum* (−3.096520, −59,986494), *Spondias mombin* (−3.094211, −59,988771), *Mangifera indica* (−3,094902, −59,988660), *Eugenia stipitate* (−3.094279, −59,988138). Samples were collected in the period between January and April 2018 in self-sealing plastic bags using sterile forceps and were then transported to the Mycology Laboratory at INPA. This study was authorized by the Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado - SisGen (registration number: A9A738C).

2.2. Yeast strains

2.2.1. Standard strains

In this study, we employed a set of industrial yeasts as standards for comparative analysis, namely CAT-1, PE-2, ANGEL, and US-05. The industrial strains were characterized as follows: CAT-1 (Fermentec Piracicaba, SP), which is an ethanol-producing commercial strain, PE -2 (Fermentec Piracicaba,SP), which is another ethanol-producing commercial strain, ANGEL (Latin American LNF), another ethanol-producing commercial strain, US-05 (Fermentis, USA), which is a commercial strain used in beer production.

2.2.2. Regional strains

Six regional isolates (n = 6) of *S. cerevisiae* previously obtained from the Mycology laboratory at INPA were incorporated into the study. The regional isolates (AR-1, AR-3, AR-4, AR-9, AR-12, AR-13, all sourced from *E. stipitate* fruit at INPA) were included for their potential significance in this work.

2.3. Yeast isolation

Approximately 5 g of the samples (fruit, tree bark and vegetal material in decomposition under the tree) were transferred to Erlenmeyer flasks (125 mL) containing 45 mL of broth. We used two broths: RE medium (0.67% nitrogen-based [Difco Laboratories, Detroit, MI, USA], 1% raffinose, 8% (v/v) ethanol and 0.05% chloramphenicol) [13] and YPD medium (1% yeast extract, 2% peptone, 2% dextrose, 8% (v/v) ethanol and 0.05% chloramphenicol), which were incubated at two distinct temperatures (25 °C and 40 °C) for 48 h. When the medium showed turbidity, serial dilutions were performed (up to 10−6) and 0.1 mL of these dilutions were transferred to plates containing YPD medium (1% yeast extract, 2% peptone, 2% dextrose, 2% agar, 0.05% chloramphenicol, 8% (v/v) ethanol), then the plates were incubated at 30 °C for 48 hours. During incubation, the colonies were isolated and checked for culture purity.

2.4. Biochemical tests (fermentation and assimilation tests)

The phenotypic characterization of the isolated yeasts was based on the standard methods described by Kurtzman et al. [14] as follows:

Fermentation tests: A suspension of 10^6 cells/mL of the strains was inoculated into each test tube containing the fermentation basal medium (4.5 g/L of powdered yeast extract and 7.5 g/L of peptone) with different single carbon sources (glucose, sucrose, maltose) including a sugar-free control. Each tube (1.5 cm × 15 cm tubes with an inverted Durham tube (1 cm × 5 cm)) was incubated at 25−28 °C for up to 28 days. The tubes were shaken and inspected at frequent intervals for CO_2 accumulation in the Durham tube and whether the sugars had been consumed.

Sugar Assimilation tests. The ability of these strains to grow using different carbon sources was determined in plates of YNB (YNB; Difco Laboratories, Detroit, Mich) supplemented with 1% of each carbon source. The YNB medium was melted and cooled to between 40 and 45 °C and a suspension of 10^6 cells/mL of strains was added to a Petri dish. The suspension was pipetted into a Petri dish into which the agar medium was then poured, and the dish was then gently shaken to mix the contents. Four carbon sources (glucose, sucrose, maltose, lactose) were tested in each Petri dish. The growth assays were carried out at 30 °C for 2 to 6 days. The plates were examined for growth around the carbon source every 2 days for up to a week.

2.5. Molecular identification

Molecular identification was carried out by performing a sequencing analysis of the D1/D2 domain of the LSU ribosomal RNA gene and the internal transcribed spacer (ITS) 1 and 2 regions. DNA was extracted from the yeast cells using a protocol described by Sambrook et al. (1988) for DNA extraction using the method: phenol: chloroform: isoamyl alcohol [15].

The D1/D2 hypervariable area of the LSU ribosomal RNA gene was amplified using polymerase chain reaction (PCR) and the primer pairs NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') [16]. Amplification conditions consisted of initial denaturation at 94 °C for 5 min; 25 cycles of denaturation at 94 °C for 30 s, primer annealing at 50 °C for 30 s and extension at 72 °C for 30 s; and a final extension at 72 °C for 10 min. The ITS1 - 5.8S rRNA - ITS2 region was also amplified using PCR and the primer pairs ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [17]. Amplification conditions consisted of initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 1 min, primer annealing at 53 °C for 1 min and extension at 72 °C for 1 min; and a final extension at 72 °C for 7 min. The PCR products were analyzed via agarose gel electrophoresis and then purified with 20% polyethylene glycol (PEG8000) [18]. The nucleotide sequencing reaction was performed with the PCR product, purified using the BigDye Terminator v3.1 kit, following the manufacturer's recommendations, and precipitated with ethanol/EDTA. The samples were electro-injected in the automatic analyzer (ABI PRISM 3130xL, ThermoFisher).

The nucleotide sequences of D1/D2 and ITS generated from the yeast isolates were checked, compiled, and edited using the BioEdit 7.0.9.0 program [19] and deposited at NCBI GenBank (accession numbers in the supplementary material 1)

The DNA sequences from related species that were retrieved from the National Center for Biotechnology Information (NCBI) database using BLASTN [20] were compared to those obtained

and considered values above 97% similarity. The genetic distance tree was generated using the neighbor-joining (NJ) method with the aid of the MEGA X program [21]. The percentages of replicate trees in which associated taxa clustered in the bootstrap test (1,000 replicates) were also determined.

2.6. Amplification regions (SNPs) in Saccharomyces cerevisiae strains

We investigated the polymorphism in the sequences of genes related to stress tolerance ASR1//YPR093 (alcohol sensitive RING/PHD finger1) [22–25] and FPS1 (farnesyl diphosphate synthase1) [26–28] via analysis of the SNPs. Expression of the FPS1 gene contributes to the reduction of alcohol accumulation within the cell during the fermentation process. The ASR1/YPR093ASR1 gene encodes the ASR1 protein. Under alcoholic stress, this protein modifies its intracellular distribution in the cytoplasm and accumulates in the nucleus, transmitting an alcoholic stress signal from the plasma membrane to the nucleus. For the analysis of the SNPs, DNAs were amplified in 20 μL reactions consisting of 10–20 ng DNA, 200 μM of each dNTP, 2 μL of 10× PCR buffer minus Mg, 0.5 U Pfu DNA polymerase (Invitrogen), 4 mM $MgSO_4$, d 10 pmol of forward and reverse primers (Table 1). Amplification was performed as follows: 5 min at 94 °C, 34 cycles of min at 94 °C, 45 s at 56 °C (ASR1) or 58 °C (FPS1), 1 min at 72 °C) and 5 min at 72 °C. Amplification was confirmed by placing an aliquot of the PCR reaction product in 1.5% agarose gels. The PCR products were purified and both strands were sequenced at automatic sequencer (ABI 3130xl, Applied Biosystems). Only SNP-confirmed sequencing of both strands is reported. The presence of heterozygote DNAs for a certain SNP was confirmed by visually inspecting the superimposed presence of two peaks of different colors for that position in readings from both strands [29]. The sequences were aligned using the MUSCLE program in the Genious Prime software.

Table 1. Sequence of oligonucleotides used for the amplification of gene fragments related to alcohol tolerance and temperature in *Saccharomyces cerevisia*e strains.

Oligonucleotide	Genes	Direction	Sequence (5'-3')
ScASR1_F1	ASR1/YPRO0923C	F	ATGGAAGAGTGTCCTATTTGTTTG
ScASR1_R1		R	CTACTCATCATGACAGTAAATTAGGAG
ScFPS1_Fb	FPS1/YLLO43W	F	GCCACGTTGAAATTGTCCTGC
ScFPS1_Rb		R	CTTTCTGTCCAGTGAATCTGTTC
ScFPS1_Fc		F	GCAGGACAATTTCAACGTGGC
ScFPS1_Rc		R	GAGAAGTCACTCAGGTCCG

*Note: Antonangelo (2012), Unpublished data.

2.7. Determination of the optimum fermentation temperature of S. cerevisiae strains

The yeast isolates were inoculated into YPD medium at 25 °C for 24 h. An initial cell concentration of 1×10^6 cells/mL was inoculated into the ethanol production medium (2 g/L KH_2PO_4, 3 g/L of $(NH_4)_2SO_4$), 7 g/L of peptone, 3 g/L of yeast extract, 200 g/L of glucose) [30] in a 25 × 200 mm test tube, which was incubated at 25 °C, 30 °C, 35 °C, 40 °C, 45 °C for 48 h [31]. The yeast growth was determined by cell counting (cell/mL). Under the experimental conditions, the optimal temperature was considered the temperature that allowed the highest microbial growth rate, and the maximum growth temperature was considered the maximum temperature that still allowed fermentation.

2.8. Determination of optimal and maximum ethanol concentration for fermentation of S. cerevisiae strains

To screen for ethanol-tolerant yeasts, the yeast isolates were inoculated into a YPD medium and left for 24 h at 25 °C. An initial cell concentration of 1×10^6 cells/mL was inoculated in screw-capped tubes containing the ethanol production medium (2 g/L KH_2PO_4, 3 g/L of $(NH_4)_2SO_4$), 7 g/L of peptone, 3 g/L of yeast extract, d 200 g/L of glucose) [30] containing different ethanol concentrations of 0, 5, 10, 15, 20% (v/v). Subsequently, the cultures were incubated at 25 °C for 48 h [32]. The yeast growth was determined by cell counting (cell/mL). Under the experimental conditions, the maximum growth ethanol concentration was considered the maximum ethanol concentration that still allowed fermentation.

2.9. Analytical methods

Biomass (yeast growth): S. cerevisiae cell growth was determined by cell count using a Neubauer counting chamber (Neubauer, Germany) [33] and determined by measuring the optical density 600 nm using a spectrophotometer (600 plus, Fenton, São Paulo). Biomass (g/L) was determined using a calibration curve that related biomass (dry weight) to optical density.

Reducing sugars: The concentration of the reducing sugars (RS) was determined using the 3,5-dinitrosalicylic acid (DNS) method. Samples were centrifuged at 3,500 rpm for 5 min, diluted with distilled water, and 1 mL of the DNS reagent was added, which was then incubated in a boiling water bath for 5 min. After cooling to room temperature, we measured the absorbance of the supernatant at 540 nm in a spectrophotometer (600 plus, Fenton, São Paulo). The absorbance values for the substrate and enzyme blanks were subtracted from the absorbance value for the sample. The observed absorbance was correlated to the concentration of reducing sugar using a standard glucose curve [34].

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 [35]. 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.

3. Results

The isolation of the yeast from the natural samples (tree bark, litter, and fruit) was carried out to investigate the presence of the species *S. cerevisiae* in economically important plants (*Theobroma grandiflorum*, *Spondias mombin*, *Mangifera indica*, *Eugenia stipitate*) of the Amazon forest (Table 2). The total number of yeasts isolated was 53 and the number of yeasts isolated from each plant species ranged from 10 to 19. The plant species that provided the highest number of isolates was *Eugenia stipitate* (n = 19) and the temperature that resulted in the highest number of isolates was 25 °C (n = 31). The culture medium that allowed the highest number of isolates was YPD medium (supplemented with 8% ethanol) (n = 29) and the type of sample that resulted in the highest number of isolates was litter (n = 26).

Table 2. Investigation of yeasts from fruit, litter, and bark samples of *Spondias mombin, Theobroma grandiflorum, Mangifera indica and Eugenia stipitate* RE medium (8% ethanol) and YPD (8% ethanol) under different temperatures 25 °C and 40 °C.

Plant	Plant part	Culture medium	Temperature	Yeasts present in the sample	Isolate code
S. mombin L	Bark	YPD+ ET 8%	25	1	TCY-251
			40	1	TCY-401
		RE+ ET 8%	25	1	TCR-251
			40	1	TCR-401
	Fruit	YPD+ ET 8%	25	1	TFY-251
			40	1	TFY-401
		RE+ ET 8%	25	1	TFR-251
			40	1	TFR-401
	Litter	YPD+ ET 8%	25	1	TSY-251
			40	1	TSY-401
		RE+ ET 8%	25	1	TSR-251
			40	0	-
T. grandiflorum	Bark	YPD+ ET 8%	25	2	CCY-251 /CCY-252
			40	0	-
		RE+ ET 8%	25	2	CCR-251/ CCR-252
			40	1	CCR-401
	Fruit	YPD+ ET 8%	25	0	-
			40	0	-
		RE+ ET 8%	25	0	-
			40	0	-
	Litter	YPD+ ET 8%	25	1	CSY-251
			40	1	CSY-401
		RE+ ET 8%	25	2	CSR-251 /CSR-252
			40	1	CSR-401

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Plant	Plant part	Culture medium	Temperature	Yeasts present in the sample	Isolate code
M. indica	Bark	YPD+ ET 8%	25	1	MCY-251
			40	1	MCY-401
		RE+ ET 8%	25	1	MCR-251
			40	1	MCR-401
	Fruit	YPD+ ET 8%	25	0	-
			40	0	-
		RE+ ET 8%	25	1	MFR-251
			40	1	MFR-401
	Litter	YPD+ ET 8%	25	2	MSY-251 / MSY-252
			40	1	MSY-401
		RE+ ET 8%	25	2	MSR-251 / MSR-252
			40	2	MSR-401 /MSR-402
E. stipitate	Bark	YPD+ ET 8%	25	2	ACY-251/ ACY-252
			40	2	ACY-401/ ACY-402
		RE+ ET 8%	25	2	ACR-251 / ACR-252
			40	0	-
	Fruit	YPD+ ET 8%	25	1	AFY-251
			40	1	AFY-401
		RE+ ET 8%	25	0	-
			40	0	-
	Litter	YPD+ ET 8%	25	4	ASY-251/ ASY-252 / ASY-253 / ASY-254
			40	4	ASY-401/ ASY-402 / ASY-403 / ASY-404
		RE+ ET 8%	25	2	ASR-251/ ASR-252
			40	1	ASR-401

Isolated yeasts were investigated for their biochemical characteristics in regards to the fermentation test and sugar assimilation. The results regarding fermentable sugars and the assimilation of carbohydrates by the yeasts are shown in Table 3. The commercial yeasts CAT-2 and US-05 were used as reference strains. In the fermentation and sugar assimilation assay, we found 10 isolates that presented a biochemical profile that was similar to *S. cerevisiae.* All yeasts assimilated and fermented glucose during the tests (Table 3).

Table 3. Sugar fermentation and assimilation tests of yeasts isolated from samples of fruit, litter, bark of *Spondias mombin, Theobroma grandiflorum, Mangifera indica, Eugenia stipitate*. All yeasts assimilated and fermented glucose during the tests.

Isolates	Fermentation test		Assimilation test		
	Sucrose	Maltose	Sucrose	Maltose	Lactose
S. cerevisiae CAT-2	+	+	+	+	-
S. cerevisiae 0S-05	+	+	+	+	-
CCY-251	+	+	+	-	-
CSY-251	+	+	+	+	-
CSR-252	+	+	+	+	-
CSR-402	+	+	+	+	-
ACY-251	+	+	+	+	-
ACY-252	+	+	+	+	-
ACY-401	+	+	+	+	-
ACY-402	+	+	+	-	-
ACR-251	+	+	+	+	-
ACR-252	+	+	+	+	-
ASY-254	+	+	+	+	-
ASY-404	+	+	+	+	-
TCY-251	+	-	+	-	-
TCY-401	-	-	-	-	-
TCR-251	-	-	-	-	-
TCR-401	+	-	+	-	-
TFY-251	-	-	-	-	-
TFY-401	-	-	-	-	-
TFR-251	-	-	-	-	-
TFR-401	-	-	-	-	-
TSY-251	-	-	-	-	-
TSY-401	-	-	-	-	-
TSR-251	-	-	-	-	-

Continued on next page

Isolates	Fermentation test		Assimilation test		
	Sucrose	Maltose	Sucrose	Maltose	Lactose
CCY-252	-	-	-	-	-
CCR-251	-	-	-	-	-
CCR-252	+	-	+	-	-
CCR-401	+	-	+	-	-
CSY-401	+	-	+	-	-
CSR-251	+	-	+	-	-
AFY-251	-	-	-	-	-
AFY-401	-	-	-	-	-
ASY-251	-	-	+	-	-
ASY-252	-	-	-	-	-
ASY-253	-	-	-	-	-
ASY-401	-	-	-	-	-
ASY-402	-	-	-	-	-
ASY-403	-	-	-	-	-
ASR-251	-	-	-	-	-
ASR-252	-	-	-	-	-
ASR-401	-	-	-	-	-
MCY-251	-	-	+	-	-
MCY-401	+	-	+	-	-
MCR-251	-	-	-	-	-
MCR-401	-	-	+	-	-
MFR-251	-	-	+	-	-
MFR-401	-	-	+	-	-
MSY-251	+	-	+	-	-
MSY-252	+	-	+	-	-
MSY-401	+	-	+	-	-
MSR-251	+	-	+	-	-
MSR-252	-	-	-	-	-
MSR-401	-	-	-	-	-
MSR-402	-	-	-	-	-

We extracted the DNA from the 10 yeasts with a biochemical profile that was similar to that of *S. cerevisiae*, which was then submitted to sequencing of ITS and LSU (D1 and D2) regions for further taxonomical investigation. The isolates CSR252 and CSR-402 were confirmed as being from *S. cerevisiae.* The isolates ACY 401, ACY 251, CCY 25251, ACR252 were identified as being from *Wickerhamomyces anomalus.* The isolates ASY254, ACR-25251, ACY-252 were identified as being from *Torulaspora pretoriensis* and the isolates ASY- 404 were identified as being from *Debaryomyces hansenii* (Figure 1).

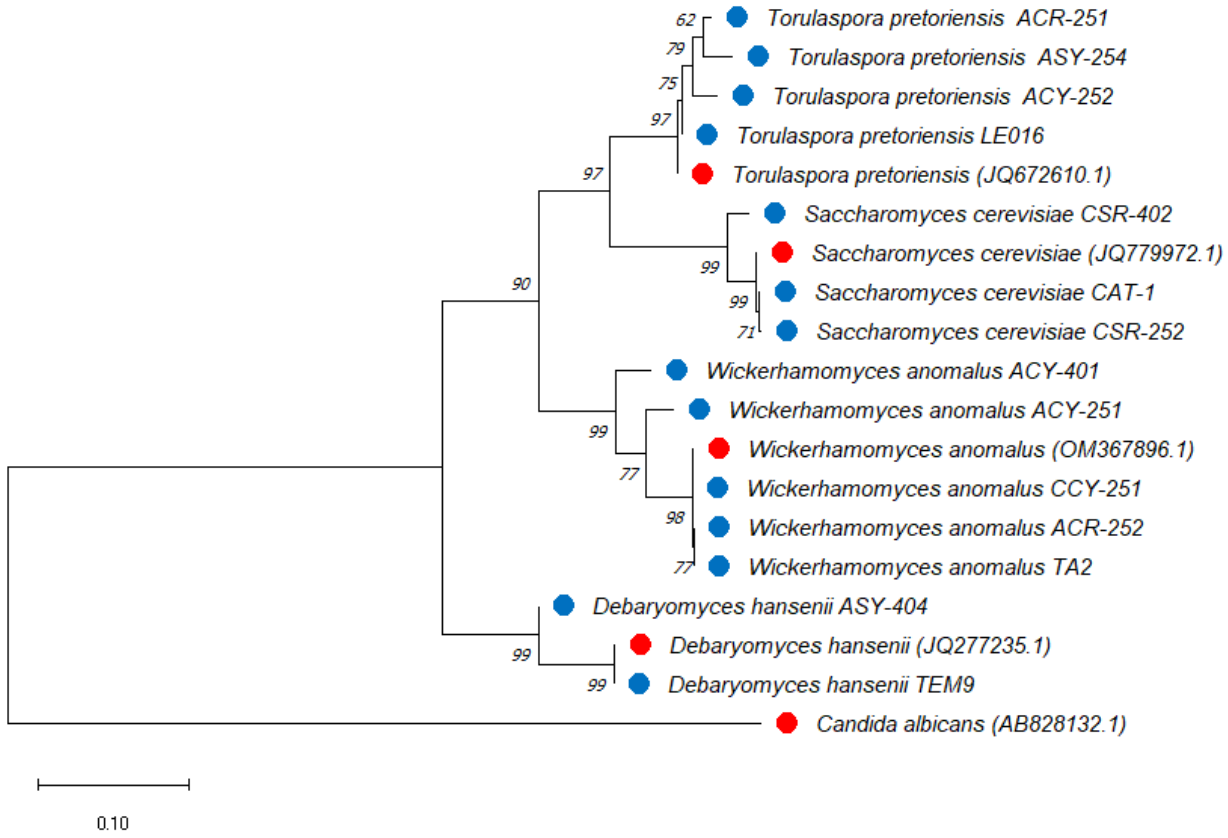

Figure 1. Tree generated by the neighbor-joining (NJ) method constructed for the internal transcribed spacer (ITS) and LSU (D1 and D2) molecular markers in the MEGA X (Molecular Evolutionary Genetics Analysis) software. The tree was constructed from the alignment of the sequences of isolated yeasts (CSR252, CSR-402, CCY-251, ACY 251, ACY 401, ACY 252, ACR251, ACR252, ASY254, ASY404). As the positive control (C+), the *Saccharomyces cerevisiae* CAT-1, *Wickerhamomyces anomalus* TA2, *Debaryomyces hansenii* TEM9, *Torulaspora pretoriensis* LE016 strains were used. *Candida albicans* (AB828132.1) was used as an outgroup. The sequences available in the NCBI database are in red and the sequences of the strains used in the study are in blue.

In order to investigate the industrial potential of the isolates from *S. cerevisiae*, the optimum temperature and alcohol tolerance were investigated. In the results, we observed that all the strains presented optimal growth at a temperature of 30 °C and the maximum temperature that allowed growth was 40 °C. The reference strains (CAT-1, PE-2, ANGEL, US-05) and the strains isolated from *Eugenia stipitate* (AR-1, AR-3, AR-4, AR-9, AR-1212, d AR-13) presented the highest ethanol tolerance (15%), while the isolates CSR 252 and CSR 402 presented an ethanol tolerance of 10% (supplementary materials 2 and 3).

The DNA sequence of ethanol tolerance-related genes (FPS1 and ASR1) in various *S. cerevisiae* strains was analyzed (Supplementary material 4 and 5). We identified five single nucleotide polymorphisms (SNPs), in the form of transitions in the FPS1 gene (2010 bp) at positions 255 (A > G), 594 (C > T), 1188 (A > G), 1347 (G > A), and 1552 (A > G), present in all

ten strains with high ethanol tolerance (CAT-1, PE-2, ANGEL, US-05, AR1, AR-3, AR-4, AR-9, AR-12, and AR-13). Upon analyzing the SNPs in the FPS1 gene sequence, we observed that among the five SNPs studied, a non-synonymous mutation occurs at position 1552 (A > G), while the remaining mutations are synonymous (Figure 2).

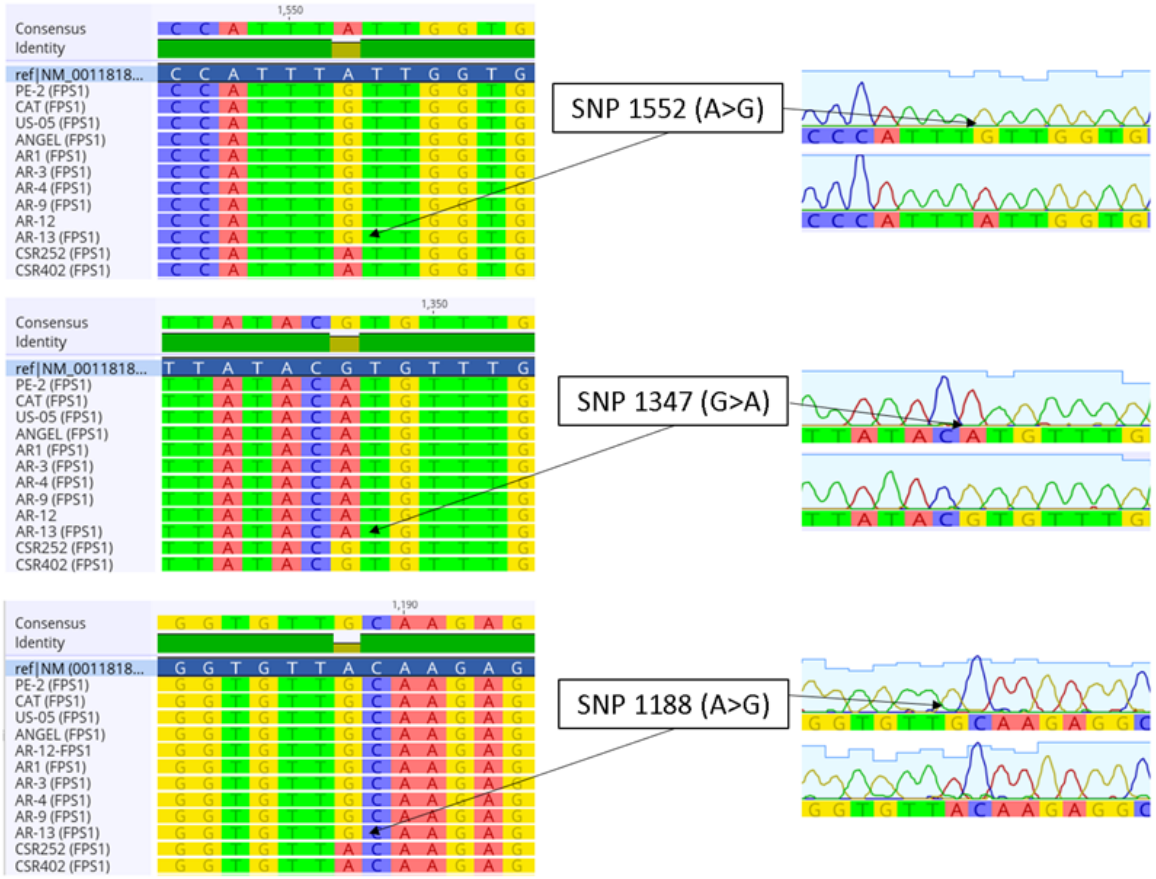

Figure 2. Multiple sequence alignment and sequencing analysis files showing SNPs at positions 1188, 1347 and 1552 of the FPS1 gene in *Saccharomyces cerevisiae* strains tolerant to concentrations of 15% (more tolerant strains than PE-2 and CAT-1) and 10% (less tolerant than CSR252 and CSR402) of ethanol in the medium. Alignment was performed using the MUSCLE program in Genious Prime® software.

Based on the DNA sequence of the ASR1 gene (867 bp), only the strains PE-2 and CAT-1 exhibited six transition SNPs at positions 90 (C > T), 102 (G > A), 150 (A > G), 213 (T > C), 291 (C > T)), 378 (T > C), along with two transversion SNPs at positions 70 (G > C) and 690 (C > G). In the DNA sequence of the ASR1 gene, all the identified mutations were found to be synonymous (Figure 3).

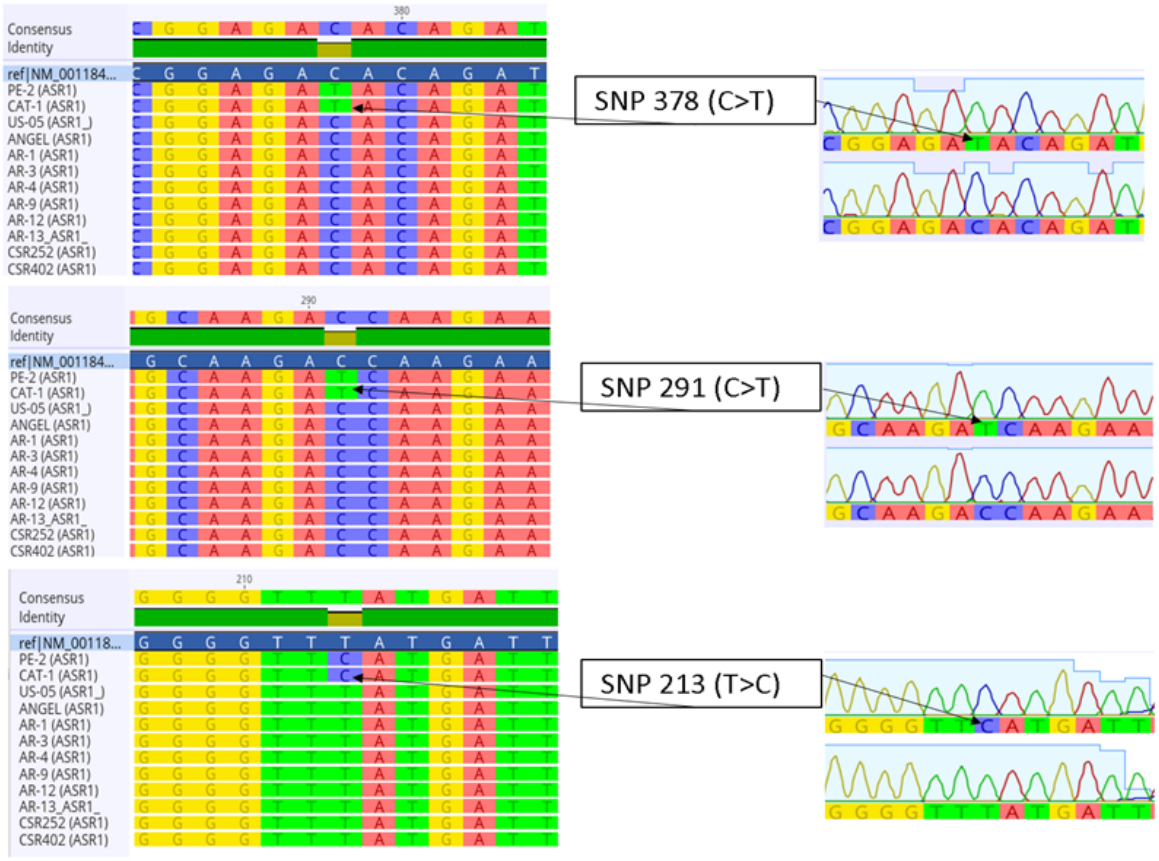

Figure 3. Multiple sequence alignment and sequencing analysis files showing SNPs at positions 291,378,690 of the ASR1 gene in in *Saccharomyces cerevisiae* strains tolerant to concentrations of 15% (more tolerant than PE-2 and CAT-1) and 10% (less tolerant than CSR252 and CSR402) of ethanol in the medium. Alignment was performed using the MUSCLE program in the Geneious Prime® software.

4. Discussion

In the present work, we found ten new yeast isolates with biotechnological potential, including two new isolates from *S. cerevisiae* (CSR252 and CSR402). Thermotolerance and ethanol tolerance of the isolated strains were investigated, as well as the SNPs related to these characteristics. The present work is important since the potential of the biodiversity Amazon is very high; however, few studies investigate the presence of isolates of *S. cerevisiae* with industrial potential in our region. The new isolates presented in this study can be applied in new biotechnological products and adapted for different fermentation conditions.

The isolated species in our study were *S. cerevisiae, W. anomalus, T. pretoriensis*, *D. hansenni*. The isolation of *Saccharomyces cerevisiae* from environmental sources results in low productivity [13,36,37] and the presence of other fermenting species, as observed in our study. Previous isolation works carried out with samples associated to plants found similar yeast species [13,31], [38–41], [42–46]. All the yeast species isolated in the present study were previously described as having biotechnological potential: a) *Wickerhamomyces anomalus* has been

investigated for biocontrol, food fermentation, production of biofuels and production of therapeutic molecules used in human medicine [47]; b) *Torulaspora pretoriensis* has a high potential for fermentation and resistance to freezing and thawing [48]; c) strains of *T. pretoriensis* have been used for winemaking [49]; and are found in sourdough [50]. *T. pretoriensis* is a producer of enzymes such as invertases [51] and α-glucosidases [48]. *Debaryomyces hansenii* is highly heterogeneous and, therefore, has differences between strains, such as variations in its ability to assimilate and ferment various carbon sources, the expression of different lipases and protease activities, and has high diversity under ideal growing conditions [52].

Under the experimental conditions, *S. cerevisiae* was isolated from litter directly below the individual of *T. grandiflorum*. Previous work carried out in the Amazon isolated this species from soil [10] and from *Theobroma cacao* [7].

The maximum growth of all regional and industrial strains, when tested with increments of 5 °C, was at 30 °C. The highest temperature that allowed yeast growth, when tested with increments of 5 °C, was 40 °C. These fermentation profiles are similar to what is shown in previous studies [53–57].

In the ethanol tolerance test, we observed that, under the conditions tested, the industrial strains CAT-1, US-05, PE-2, ANGEL and the regional strains AR-1, AR-3, AR-4, AR-9, AR-12, AR-13 showed growth in wort in up to 15% (v/v) of ethanol. The strains CSR-252 and CSR-402 showed growth in up to 10% v/v of ethanol. This result corroborates the literature which states that environmental isolates of *S. cerevisiae* tolerate ethanol concentrations of between 7 and 13% and industrial strains have higher ethanol tolerance [58–61].

We analyzed single nucleotide polymorphisms (SNPs) in the FPS1 gene and identified four distinct nucleotide transitions at positions 255 (A > G), 594 (C > T), 1347 (G > A), and 1552 (A > G). These transitions were consistently present in the yeast strains that exhibited a higher tolerance to ethanol, including CAT-1, US-05, PE-2, ANGEL, AR-1, AR-3, AR-4, AR-9, AR-12, AR-13. These specific SNPs were absent in environmental isolates of *T. grandiflorum* (CSR-252 and CSR-402). The present work corroborated with previous work that described the FPS1 gene as a target for identifying tolerance to ethanol. Similar SNPs have been previously observed in sequences in a monosporic isolate from *S. cerevisiae* (PE-2) [62]. Furthermore, these SNPs were also detected in sequences of *S. cerevisiae* strains isolated from soil (Sol7-1, Sol7-2) and grape juice from a winery in Kumeu River (T8) in New Zealand [63], and in strains isolated from coconut (YJM1383) and sugarcane (YJM1250) in the Philippines [64]. This highlights the potential functional importance of these genetic variations in conferring tolerance to ethanol.

In a comparative analysis of industrial and environmental fungal strains, *Saccharomyces cerevisiae* PE-2 and CAT-1 exhibited similar single nucleotide polymorphisms (SNPs) within the ASR1 gene. Contrarily, these genetic variations were absent in the environmental samples, including CSR-252, CSR-402 and AR-1 to AR-4, as well as AR-9, AR-12, AR-13. This observation aligns with previous research in which *S. cerevisiae* isolates from the beverage industry demonstrated analogous SNPs in the ASR1 gene [62,64]. The presence of such SNPs in industrial strains (CAT-1 and PE-2) may suggest potential adaptation to specific environmental conditions or selective pressures related to industrial processes.

This study opens up perspectives for other studies focused on a) induction and adaptation of regional isolates and b) application of regional isolates for producing value-added products. In particular, the limited number of strains tested is a limitation of our study. Therefore, an increase in the number of strains would be interesting so better validate our results. *S. cerevisiae* is the fungal strain

with the greatest biotechnological role and, therefore, the acquisition of strains of *S. cerevisiae* and other yeasts with biotechnological potential carried out in this work is strategically important for the development of the regional bioindustry/bioeconomy and helps to maintain the forest and its biodiversity.

5. Conclusions

A total of 53 yeasts were obtained from environmental samples found in the Amazon. *Saccharomyces cerevisiae* strains (n = 2) were isolated from the bark of *T. grandiflorum* and litter; in addition, we isolated other yeasts with biotechnological potential such as *Torulaspora pretoriensis* and *Debaryomyces hansenii.*

In the ethanol tolerance test, the industrial strains (CAT-1, PE-2, US-05, and ANGEL) d the regional strains (AR1, AR-3, AR-4, AR-9, AR-12, AR-1313) owed ethanol tolerance up to 15% v/v.

S. cerevisiae strains (CAT-1, PE-2, US-05, ANGEL, AR1, AR-3, AR-4, AR-9, AR-12, AR-13), which had high ethanol tolerance, had synonymous mutations located at sites 255 (A > G), 594 (C > T), 1347 (G > A), a non-synonymous mutation at position 1552 (A > G) of the FPS1 gene.

Use of AI tools declaration

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

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Conflict of interest

The authors declare no conflicts of interest

Author Contributions:

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

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