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Morphophysiological and molecular characterization of wild yeast isolates from industrial ethanol process

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Wild yeasts are commonly found during the fermentation process, displaying different survival and competition strategies that commonly enable their successful spread in the medium. Identifying these yeasts by biological and molecular monitoring, and knowing their traits is vital for the fermentation yield, and this can be done by simple methods such as differential mediaplating, growth rate evaluation and DNA sequencing. The aim of this work was to perform morphophysiological and molecular characterization of 14 yeast isolates from a bioethanol plant in the State of Sao Paulo, Brazil. This was done by employing different culture media to assess the growth and the morphophysiological characteristics of the isolates. The molecular characterization was also done in order to identify the samples in intra-specific levels, compared to the reference strains. The *Saccharomyces cerevisiae* CAT-1 and PE-2, Brazil's two main commercial strains, were used as reference. The results suggest that a single ethanol-producing unit may display a highly diversified microbiome, with the occurrence of distinctive wild yeast strains disclosing diverse morphophysiological traits, as observed in the differential media plating and growth rate assay results. The molecular characterization shows that these yeast isolates differ from the reference strains, as observed in interdelta-based PCR fingerprint banding patterns. These findings are a statement of the yeast diversity found in the fermentation process, and are of interest for the ethanol industry, being that many of the commercial strains were firstly isolated from the local biome.

Key words: Bioethanol, differential media, growth rate, molecular characterization, morphology, *Non-Saccharomyces*, *Saccharomyces cerevisiae.*

INTRODUCTION

Brazil is the second largest producer of bioethanol in the world (Lucena et al., 2010). Most Brazilian industrial processes utilize the Melle-Boinot method, where by yeast cells are recovered from the process and subjected to an acid wash treatment before starting a new fermentation cycle (Amorim et al., 2011). Most commercial *Saccharomyces cerevisiae* strains utilized in the Brazilian processes are not the result of induced

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Table 1. Identification of yeast isolates according to date of sampling.

Sampling date	Isolate	Sampling period of crop season
03 May 2013	A1 to A5	Beginning
28 May 2013	B1	Middle
29 June 2013	C1, C2, C4	Middle
10 October 2013	D1 to D5	End

genetic modifications. In fact, they come from selection processes of autochthonous yeast isolates that showed desirable characteristics to that particular fermentation process (Amorim et al., 2011). The commercial yeast strains mostly employed in several Brazilian ethanol plants are: CAT-1 (from Catanduva Mill); SA-1 (from Santa Adelia Ethanol Plant) and PE-2 (from Pedra Agroindustrial) (Basso et al., 2008).

Several factors may negatively affect the fermentative yield, but bacteria and wild yeasts spoilage are the most frequent causes (Basso et al., 2008; Amorim et al., 2011). Even under controlled process conditions, in which the domesticated yeasts are more prevalent, recent comparative genomic studies have demonstrated cases of spoilage in ethanol plants where native yeast strains were found to indeed overlap those inoculated ones. These wild yeasts can be found in different matrices, from wine making-related environments to tree barks, fruits and even seawater (Duina et al., 2014; Barbosa et al., 2016).

Nonetheless, several non-*Saccharomyces* strains have been isolated from bioethanol plants by using differential culture media, including specimens of the genera *Candida*, *Brettanomyces*, *Trichosporon*, *Pichia*, *Dekkera* and *Hansenula* (Ceccato-Antonini and Silva, 2000). This gives a brief indication about the diversity of the local microbiota commonly found in those fermentation units. Wild yeasts can be introduced into the fermentation process through the raw material and water, or even become adapted residents due to lack of good practices and sanitation. Yeast strains with undesirable characteristics, such as flocculation or excessive foam production, are considered potential spoilage agents, constituting a major problem to the bioethanol industry (Basso et al., 2008).

Wild yeasts may become dominant in the process, occasionally displaying desirable technological traits, and can be often isolated and used for subsequent fermentations. The identification of those emerging yeast strains through biological monitoring is vital for the fermentation yield, and such control can be performed from the simplest culture media plating methods to other more complex and costly ones (Priest and Campbell, 2003).

The origin of those wild yeasts and their natural habitat still remains unknown, even though recent studies have shown a relationship to the surroundings of the sugarcane fields, perhaps dispersed by birds or insects, exudates of trees, and water (Stefanini et al., 2012; Beato et al., 2016). High incidence of wild yeasts is usually associated with significant reduction of fermentation yield, increased processing time, and viscosity (Ceccato-Antonini, 2010).

Both wild and industrial yeast strains shows similar metabolism, making it difficult to control contamination (Amorim et al., 2011), and a solution to this problem would be to better understand the behavior of those wild microorganisms. Monitoring the permanence of selected yeasts and controlling the growth of wild strains are primary parameters for saving inputs used to control the negative effects of spoilage in the fermentation process (Amorim et al., 2011).

Therefore, the aim of this study was to evaluate the morphophysiological and molecular traits of yeast isolates in comparison to commercial strains, shedding light on the diversity of yeasts found in ethanol-producing unit environments.

MATERIALS AND METHODS

Sampling and yeast strains

Samples were directly collected from fermentation tanks during the 2013 to 2014 harvest season (Table 1), in a bioethanol plant in Piracicaba, State of São Paulo, Brazil. The samples were serially diluted in sterile 0.9% (wt/v) NaCl solution and aseptically inoculated on Petri dishes containing YEPD medium (10 $g.L^{-1}$ yeast extract; 10 $g.L^{-1}$ peptone; 20 $g.L^{-1}$ glucose; 18 $g.L^{-1}$ agar) plus chloramphenicol (100 $\mu g.L^{-1}$) and tetracycline (100 $\mu g.L^{-1}$). Plates were incubated at 30°C for 48 h. Fourteen distinctive morphotypes were selected for the subsequent assays.

Isolates were maintained in 16% (v/v) glycerol solution at -80°C. The commercial *S. cerevisiae* strains namely, CAT-1 and PE-2 (LNF Latino Americana, Bento Gonçalves, Brazil) were used as reference; these strains were chosen as they are the two most prominent amongst the commercial strains utilized in Brazil. The isolates and reference yeast strains will be named "samples".

Molecular characterization

Genomic DNA samples were obtained as follows: cells from YEPD medium were resuspended in 1 mL saline solution, and centrifuged at 2046 × *g* for 3 min. The pellet was resuspended in lysis buffer (Tris 500 mM, pH 8.0; *β*-mercaptoethanol 100 mM); followed by the addition of sterile glass beads (Sigma-Aldrich, Brasil LTDA) subsequently, it was homogenized by vortexing, incubated at 100°C for 15 min, and homogenized again. The supernatant containing the

DNA was obtained by centrifugation and stored at -20ºC for further analysis.

Identification of yeast isolates was carried out by deploying PCR amplification of the D1/D2 domain of 26S rDNA utilizing the Sanger sequencing method, as previously described by Kurtzman and Robnett (1998). The resultant PCR products (~600 bp) were purified with charge switch PCR clean-up kit (Invitrogen, USA), according to manufacturer's instruction, and sequenced in the Laboratory of Animal Biotechnology (College of Agriculture 'Luiz de Queiroz', Piracicaba, Brazil).

The chromatograms were analyzed with the software Chromas-Pro (version 1.49; Technelysium Pty Ltd, Australia), and the BLASTn search tool (National Library of Medicine, National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/blast)) for comparison of sequences included in the GenBank database [NCBI (http://www.ncbi.nlm.nih.gov/genbank)] for species determination.

Genetic fingerprinting of *S. cerevisiae* isolates was performed by simple PCR amplification based on the variable interdelta region, using the primers delta-12 (5'-TCAACAATGGAATCCCAAC-3') and delta-2 (5'-GTGGATTTTTATTCCAAC-3'). PCR conditions and analysis of results were realized according to Xufre et al. (2011).

Morphophysiological characterization

One yeast colony from YEPD growth medium was resuspended in saline solution and analyzed under microscopic optical microscope (Axioscopte 40, Zeiss, Germany). Three differential culture media were employed to discriminate morphological and physiological traits of yeast samples: 1) WLN (Wallerstein Laboratory, BD Difco™); 2) BiGGY (Bismuth-Sulfite-Glucose-Glycine-Yeast Extract, BD Difco™); 3) Nagai medium (20 $g.L^{-1}$ glucose; 1.5 $g.L^{-1}$ peptone; 1.5 $g.L^{-1}$ yeast extract; 1.5 $g.L^{-1}$ potassium sulfate; 1.5 $g.L^{-1}$ ammonium sulfate; 1.0 $g.L^{-1}$ magnesium sulfate; 12 $g.L^{-1}$ Agar).

The WLN and BiGGY media were prepared according to manufacturer's instructions, and Nagai medium according to Nagai (1963), using a mixture of dyes (15 $mg.L^{-1}$ trypan blue; 8 $mg.L^{-1}$ eosin). The isolates were punctually inoculated in the plates with a platinum needle, and incubated at 30°C for up to five days. All microbiological analyses were performed in triplicate.

Distinct morphological characteristics such as shape, color and texture were analyzed to classify the isolates cultivated in WL medium. Analyzes on BiGGY and Nagai media considered the color displayed by the colonies after growth, which were further numerically classified and statistically analyzed. Hidrogen sulphide (H_2S) production was detected by the color of the colonies, done through qualitative evaluation, assigning values on a continuous scale based on colony coloration on BiGGY medium, according to Neto and Mendes-Ferreira (2005), namely: 1) white; 2) Beige; 3) light brown; 4) dark brown; 5) black.

The presence of petite cells (brilliant purple colony stained) and normal yeast cells (grayish violet colony stained) indicated in the Nagai medium was also evaluated through the assignment of values on a continuous scale, namely: 1) no growth; 2) both normal and petite cells; 3) normal cells; 4) petite cells.

Growth rate of isolates in different media was determined by a microplate reader (Tecan Infinite M200, RChisto), with capacity for a 96 microwell plate. Each sample was analyzed in four different growth media namely YEPD (10 $g.L^{-1}$ yeast extract; 10 $g.L^{-1}$ peptone; 20 $g.L^{-1}$ glucose), YPSac2 (10 $g.L^{-1}$ yeast extract; 10 $g.L^{-1}$ peptone; 20 $g.L^{-1}$ sucrose), MCC5 (sterilized sugarcane juice at 50 $g.L^{-1}$ TSS) and MMel5 (clarified molasses at 50 $g.L^{-1}$ TSS). The analyses were conducted in triplicates.

Each microwell was inoculated with 50 µL of a yeast cell suspension, obtained from the dilution of a single colony previously grown in YEPD medium in saline solution. The optical density (OD) of the inoculum was monitored spectrophotometrically (Femto 700S) at 600 nm, in order to achieve 0.1 OD. Along with the inoculums, 50 µL of the growth media was added (2 x concentrations). A negative control consisting of 50 µL of growth media and 50 µL of saline solution (9g L^{-1}) was added to every plate. Cell growth was carried out at 30ºC for 24 h, with optical density readings (OD) at 600 nm measured every 2 h.

Statistical analysis

All of the experiments were performed in triplicates. Table data represents the mean values ± standard deviation (n = 3). The qualitative data referring to the morphophysiological analyses were transformed into quantitative ones through the assignment of values. The interaction between the growth rate results was evaluated by clustering according to similarity. The statistical analyses of the standardized values were performed using the SAS statistical program (Statistical Analysis System, version 9.3).

RESULTS

Molecular characterization

The 14 yeast isolates were identified as *S. cerevisiae* by analysis of 26S rDNA sequencing (intra-specific similarity > 99%) using GenBank database search. The interdelta-based PCR fingerprints from the samples are shown in Figure 1, disclosing the band patterns obtained for each sample strain.

Isolates A1, A2, A4 and A5, obtained at beginning of sugarcane harvest, presented a similar banding arrangement (main-pattern 1), which was distinct from the ones observed for A3, B1 and reference strains. Isolates B1, C1, C2 and C4 (mid harvest season) showed similar band patterns to those observed for D2-D5 and PE-2 (main-pattern 2). Isolates A3 and D1 in particular showed distinct interdelta amplification bands.

Morphophysiological characterization

First of all, the cell morphology of YEPD grown yeast samples was assessed. It was observed that the cell morphology was predominantly rounded or oval shaped, with the presence of budding cells. Only the isolate D1 differed by cell multilateral budding tendency, suggesting it has a flocculent pattern under the assayed conditions, as well as in fermentation systems.

The samples cultivated in differential media were characterized as to colony morphotypes. The data of growth in WLN, BIGGY and Nagai media are shown in Table 2. The growth of the sample strains in WLN medium provided colonies of varied sizes, and with different coloration, allowing differentiating variations of morphological aspect like texture, elevation and appearance of the surface of the colony. Isolates from early harvest season, namely A1 and A2 presented smooth colony morphology in WLN medium, however, the isolate A5 was not able to grow in this specific

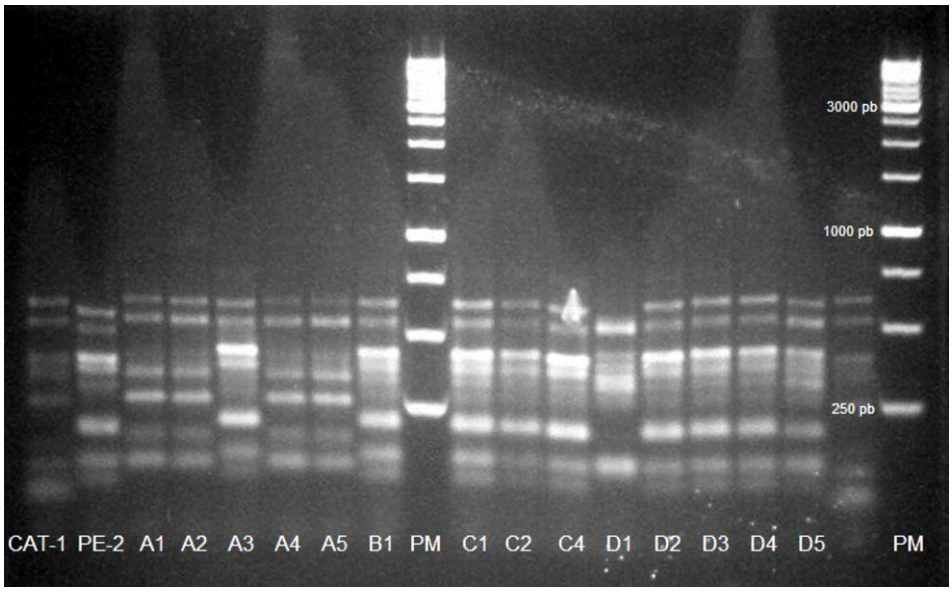

Figure 1. Interdelta banding patterns of yeast isolates and reference strains.

medium (Table 2).

Mid-harvest isolates, namely C1, C2 and C4 also presented smooth colony morphology, like the end of harvest isolates D2 and D4. Isolates A1, A2 and reference strains showed similar colony morphologies. Isolates A3, A4, B1, D1, D3 and D5 displayed rough edges with opaque texture in the assayed conditions. Isolates A3 and A4 also displayed rough edges, but are different in morphology from each other. The mid-season isolates namely C1, C2 and C4 are analogous morphotypes, but with divergent traits from the related isolate B1. End-season isolates, D1 and D5 are related in morphotype, while D2, D3 and D4 displayed a distinct profile (Table 2). Both colony and cellular morphology of indigenous and industrial strains can vary in response to environmental stimuli.

The utilization of BiGGY culture medium to identify production of H_2S in yeast strains was validated by studies that linked the dark colonies obtained directly to the strain's capability to produce H_2S (Zambonelli et al., 1964; Jiranek et al., 1995). The assayed isolates were mainly characterized as low hydrogen sulfide producers, with the exception of isolates A4 and A5, who were not able to grow in these conditions, and isolate C2, who was characterized as moderate hydrogen sulfide producer (Table 2).

The Nagai medium allows the detection of petite cells, with reduced respiratory capacity, leading to very small colonies (Nagai, 1963). The presence of those mutant cells in the medium can be perceived by smaller sizes and brilliant purple color, while the normal cells grow with grayish violet shades. Even distribution of normal and petite cells between the isolates and commercial strains was observed (Table 2).

The analysis of the appearance of isolates cultured in this medium through the assignment of continuous scale values showed that five of the isolates (A1, A2, A5, C4 and D3) presented respiratory deficient cells in the assayed conditions, five of the isolates (A3, A4, B1, C1, and D4) showed the presence of normal cells, and four isolates (C2, D1, D2 and D5) were characterized by the presence of both normal and deficient cells. Both reference strain, namely CAT-1 and PE-1 presented normal cells under assayed conditions (Table 2).

Growth rate evaluation

The samples growth rates were evaluated in four distinct media (Table 3). The mean maximum specific growth rate (μ_{max}) of the samples grown in YEPD medium was 0.409±0.054, superior to that observed for the PE-2 reference strain. Overall, early season samples had a better performance in this medium. In the YPSac medium, the mean μ_{max} of the samples was 0.484±0.047, while PE-2 was 0.440; highlighting the samples A3 (0.530), C2 (0.539) and D3 (0.538).

As for the MCC medium, the mean μ_{max} of the samples was 0.208±0.041 and PE-2 was 0.186; in general all the samples performed poorly in this medium. In the MMel medium, the mean μ_{max} of the samples was 0.443±0.212 and PE-2 was 0.338; in particular the mid-season samples presented mean μ_{max} of 0.650, highlighting the samples C1 (0.992), C2 (0.717) and D4 (0.688). It is noteworthy that the performance of sample D1 in all media utilized is being lower than all the samples and both reference strains. Although the sugarcane juice has in its composition sucrose and glucose, the least expressive results were obtained in MCC medium.

Table 2. Morphophysiological characteristics of yeast colonies in different media*.

Colony name	WLN medium						BiGGY medium		Nagai medium	
	Diameter (mm)	Texture	Color	Surface	Edge	Elevation	Diameter (mm)	Value [1]	Color[2]	Value [3]
A1	5	Shiny	Light green	Smooth	Smooth	Convex	6	2	Brilliant purple	3
A2	5	Shiny	Light green	Smooth	Smooth	Convex	5	2	Brilliant purple	3
A3	7	Opaque	Beige	Rough	Rough	Convex	8	2	Grayish violet	2
A4	6	Shiny	Beige	Rough	Rough	Convex	n/g	n/g	Grayish violet	2
A5	n/g	n/g	n/g	n/g	n/g	n/g	n/g	n/g	Brilliant purple	3
B1	7	Opaque	Beige	Rough	Rough	Convex	9	2	Grayish violet	2
C1	6	Opaque	Beige	Smooth	Rough	Convex	10	1	Grayish violet	2
C2	5	Opaque	Beige	Smooth	Rough	Convex	6	3	Brilliant purple	1
C4	5	Opaque	Beige	Smooth	Rough	Convex	4	2	Brilliant purple	3
D1	10	Opaque	Beige	Rough	Rough	Convex	4	2	Brilliant purple	1
D2	10	Opaque	Beige	Smooth	Rough	Convex	5	2	Brilliant purple	1
D3	6	Opaque	Beige	Rough	Rough	Convex	5	2	Brilliant purple	3
D4	7	Opaque	Beige	Smooth	Rough	Convex	5	2	Grayish violet	2
D5	10	Opaque	Beige	Rough	Rough	Convex	6	2	Grayish violet	1
CAT-1	8	Shiny	Beige	Smooth	Smooth	Convex	5	2	Grayish violet	2
PE-2	6	Shiny	Beige	Smooth	Smooth	Convex	5	2	Grayish violet	2

*Cultivation conditions: 30ºC for 48 h; (n/g) no growth; [1]Numerical value attributed to colony color after growth: (0) white, (1) beige, (2) light brown, (3) dark brown, (4) black; [2]Presence of petite cells (brilliant purple colony stained); normal yeast cells (grayish violet colony stained).[3] (0) no growth, (1) both normal and petite cells, (2) normal cells, (3) petite cells.

Table 3. Maximum growth rate and optical density of yeast isolates and reference strains grown in different media*.

	YEPD			YPSac			MCC			MMel		
Isolate	μ_{max} (h^{-1})	OD_{max}	time (h)	μ_{max} (h^{-1})	OD_{max}	time (h)	μ_{max} (h^{-1})	OD_{max}	Time (h)	μ_{max} (h^{-1})	OD_{max}	Time (h)
A1	0.440	0.442	4	0.472	0.748	10	0.272	0.320	12	0.347	0.680	14
A2	0.440	0.416	4	0.477	0.743	10	0.267	0.282	12	0.278	0.687	14
A3	0.461	0.449	4	0.530	0.860	8	0.212	0.350	12	0.371	0.720	14
A4	0.434	0.389	4	0.473	0.744	10	0.164	0.277	14	0.323	0.694	14
A5	0.401	0.398	4	0.455	0.718	10	0.198	0.299	12	0.312	0.696	14
B1	0.370	0.365	4	0.466	0.784	8	0.201	0.416	20	0.506	0.665	12
C1	0.382	0.349	6	0.469	0.787	10	0.246	0.374	20	0.992	0.612	14
C2	0.444	0.367	6	0.539	0.817	8	0.259	0.349	20	0.717	0.650	14
C4	0.415	0.358	4	0.523	0.795	8	0.194	0.433	20	0.390	0.672	14

Table 3. Contd.

D1	0.249	0.265	4	0.361	0.659	8	0.133	0.241	8	0.259	0.605	10
D2	0.385	0.358	4	0.467	0.731	8	0.167	0.300	14	0.318	0.584	14
D3	0.450	0.415	4	0.538	0.84	8	0.199	0.472	22	0.394	0.679	14
D4	0.439	0.401	4	0.528	0.839	8	0.215	0.470	20	0.688	0.750	14
D5	0.417	0.352	4	0.482	0.730	8	0.185	0.237	8	0.309	0.615	12
Mean	0.409	0.380	4.3	0.484	0.771	8.7	0.208	0.344	15.3	0.443	0.665	13.4
SD	0.054	0.047	0.73	0.047	0.056	0.99	0.041	0.079	4.9	0.212	0.047	1.2
CAT-1	0.391	0.471	6	0.372	0.810	10	0.306	0.257	12	0.304	0.619	16
PE-2	0.352	0.358	4	0.442	0.780	10	0.186	0.106	22	0.338	0.673	12

*Cultivation conditions: 30ºC for 24 h in microplates. μ_{max}, maximum specific growth rate; OD_{max}, maximum optical cell density; time (h) at which OD_{max} was reached.

Interaction between analyzed characteristics

The values of μ_{max}, OD_{max} and time (Table 3) were submitted to cluster analysis by similarity using origin software (OriginLab, 2017), which allowed to define 2 groups besides the isolated D1 and reference lines that were separated (Figure 2). The reference lines and the samples D1, C1 and C2 are separated from the rest of the samples forming a large group. This means that most of the samples were similar to each other but different from the reference strains.

DISCUSSION

The variety of yeast strains found during the industrial fermentation process is highly correlated to the conditions of which they are exposed (Silva-Filho et al., 2005), including the raw material quality and the ethanol concentration reached during the process, among other factors (Amorim et al., 2011).

Several studies aimed to characterize native *S. cerevisiae*, showing that most of those strains found in bioethanol plants are found in the surrounding environment, and are brought into the fermentation process along with the substrates (Beato et al., 2016; Sampaio and Gonçalves, 2008). Contrariwise, some isolates were found to derive from genetic modifications, a clonal differentiation that occurs during the fermentation process. This mainly occurs due to the unique large-scale fed-batch process, which utilizes acid cell recycling and submits the cells under a great deal of stress (Della-Bianca et al., 2013). Yeast isolates subjected to harsh conditions tend to develop genetic-induced physiological traits that make them more resistant, including the activation of genes responsible for cell wall integrity and oxidative stress response (Elsztein et al., 2011). Chromosomal rearrangements referred to as adaptive evolution were reported for the industrial PE-2 strain, under industrial-related environments and extended laboratory storage conditions (Argueso et al., 2009). This can be observed in the results obtained by the analysis of 26S rDNA sequencing (Figure 1), where isolates presented distinct banding patterns when compared to the reference strains. This can be caused by the emergence of a native strain in the process, or due to adaptive evolution, as mentioned by Argueso et al. (2009), Burke (2012) and Xufre et al. (2011). The results made it possible to identify groups of wild strains that are probably of a common origin, most likely like the local microbiota, as similarly observed by Kishkovskaia et al. (2017).

Adaptive evolution in yeast populations is driven by genetic accumulation (Burke, 2012). The dynamics involving mutations in populations depend on accumulation, natural selection, competition within the population itself and overall fitness (Lee and Marx, 2013; Bergström et al., 2014). Understanding native yeast population and evolution dynamics is important in order to comprehend how the stressing factors of the fermentation process impact the commercial yeast strains go through and how they interact with competitive invasive strains (Payen et al., 2014). Additionally, repetitive DNA sequences may display inter-specific patterns, punctual variations in position and number of such regions constitutes a genetic fingerprinting that permits to identify and differentiate yeast strains or clonal variants from a given local microbiota (Xufre et al., 2011).

One isolates namely D1, displayed flocculent patterns during cell morphology assays, which

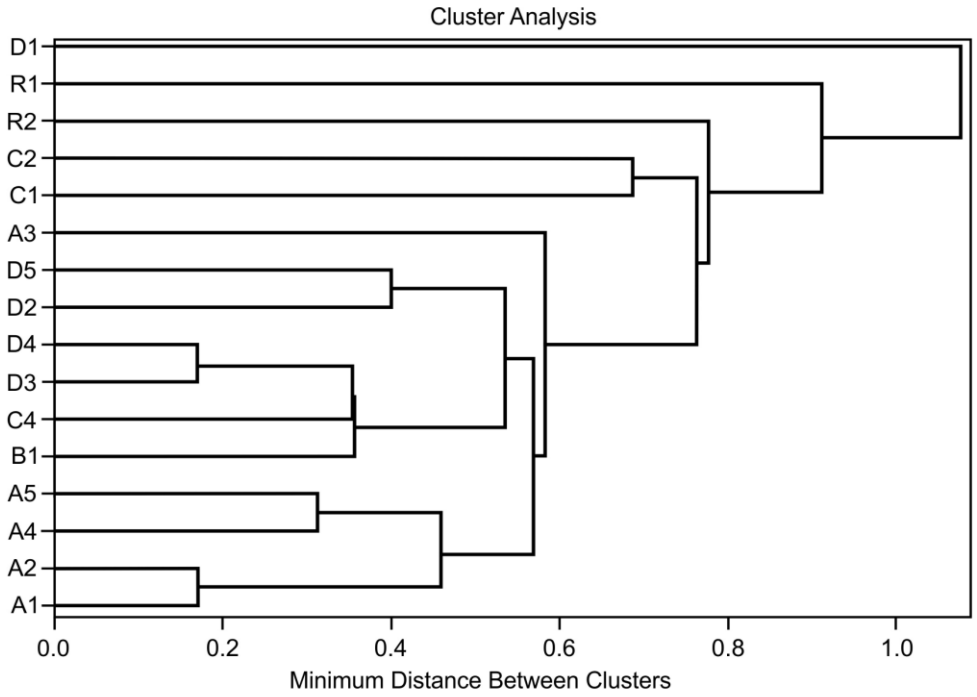

Figure 2. Cluster analysis from the values of μ_{max}, OD_{max} and time.

can be a sign of cell-cell adhesion, known as flocculation, which in turn can be an indicator of stress. It is used by the cell as a form of protection. This mechanism is activated by cell-surface adhesions; the flocculin (Flo) proteins are activated in response to stressing conditions (El-Kirat-Chatel et al., 2015). This response is mainly regulated by environmental conditions such as changes in pH and ethanol concentration or nitrogen and glucose depletion (Braus et al., 2003; Rossouw et al., 2015; Reis et al., 2016; Stewart, 2009). On the other hand, some *S. cerevisiae* strains can show sexual aggregation patterns, described as co-flocculation and cell chain formation, derived from the failure of the bud to separate itself from its mother cell, resulting in an aggregation of approximately 30 to 50 cells, unable to regroup after being mechanically dispersed (Soares, 2010). The results observed for isolate D1 point out in the direction of it being a native yeast strain that emerged in the industrial process; taking into consideration that this particular sample showed a difference banding pattern (Figure 1) and distinct growth rate performance (Table 3).

The response of *S. cerevisiae* strains to Brazil's unique fermentation process is yet poorly understood, but it is hypothesized that an enhanced stress response, and adaptive capacity play an important role in this. Transcriptional studies that investigated industrial strains such as CAT-1 and PE-2 showed genome-wide responses to environmental stress and acid wash treatment, as well as ethanol-induced stress. These adaptive characteristics are an insight of what to look for in native strains, so as to select them for industrial use (Brown et al., 2013).

The results obtained in this study revealed the diversity of morphophysiological traits of fermenting yeast isolates found in an ethanol-producing unit (Table 2), coexisting with the commercial strain pitched at the beginning of the process. These emerging wild yeasts can either overgrow, or act in consortium carrying out the fermentation with no damage to the production (Beato et al., 2016). In many cases, however, wild yeasts may display undesirable characteristics that can damage the ongoing production. The utilization of plating techniques to determine morphophysiological traits is a valuable and cost-effective tool to discriminate strains that might exhibit desirable characteristics for fermentation processes (Palmann, 2001).

Concerning the growth in different media, a study conducted by Casalone et al. (2005) described that, among a thousand colonies grown on YEPD medium, 2.5% exhibited the rough colony phenotype. *S. cerevisiae* strains that exhibit this trait are often associated with disturbances in the fermentation process (Andrietta et al., 2011).The morphological pattern of commercial strains grown in YEPD medium supplemented with rose Bengal (Moreira et al., 2015) shows similar results for the morphological patterns of CAT-1 and PE-2 strains, with shiny colonies, smooth surface and edges and convex elevation, as observed in Table 2.

However, the isolates, mainly early harvest and late harvest season, showed rough colony morphology,

displaying the presence of diverse *S. cerevisiae* in this particular fermentation process. Previous investigations show that the acid treatment utilized in the Brazilian industry can inhibit the growth of the “rough strains” to a certain degree, and can also be applied as a tool to avoid spoilage and minimize damages caused by rough strains, due to their peculiar metabolic profile (Reis et al, 2013). However, the so-called “rough strains” are usually variants of smooth colony *S. cerevisiae*.

The WLN medium is commonly employed to isolate and identify yeasts, molds and bacteria. This method was originally developed for monitoring yeast populations during brewing processes (Green and Gray, 1950), but shows reliable results in monitoring native flora fermentation (Palmann, 2001), as most yeast typically occurring in those systems can be distinguished by colony color and morphology (Jespersen and Jakobsen, 1996). The isolates displayed diverse morphotypes when grown in this medium, with the exception of isolate A5. Mid-season isolates displayed predominantly smooth colony surface, differing from the end-season isolates, which displayed more rough morphotypes (Table 2). This can be caused by adaptive evolution, caused by the cell recycling process, among other stressing factors (Bergström et al., 2014).

It was previously proposed that BiGGY agar should be used for the identification of species of the genus *Candida* spp., whilst simultaneously offering an indirect measurement of hydrogen sulfide production by fermenting yeasts. This compound is a common by-product of alcoholic fermentation, and once its production is a strain-dependent trait (Giudici et al., 1993), the utilization of this differential medium allows the characterization of wild yeast isolates in comparison to reference strains (Neto and Mendes-Ferreira, 2005). The sulfite reductase activity in commercial *S. cerevisiae* and non-*Saccharomyces* strains was documented by Mendes-Ferreira et al. (2002), showing the diversity of characteristics found in yeast strains. The results obtained for the isolates and reference strains showed predominantly low sulfite reductase activity (Table 2), which is a trait of interest for both the ethanol industry and beverages industry, since the interest in utilizing wild yeasts is growing constantly.

When analyzing the growth rate in different medium, the qualitative analysis of the isolates and reference strains growth on microplates indicates that they presented different performances, depending on the medium utilized. YEPD medium is considered good for cell growth and longevity, because of the yeast extract and peptone present in the composition (Table 3). Studies show that the nutrient composition in growth media directly impacts the growth rate and longevity of yeast cells: yeast grown in culture media with different ratios of nutrients presented diverse life spans (Wu et al., 2013); high nitrogen concentration is not mandatory to achieve maximum growth rate, however, it is shown to influence the fermentation performance due to higher biomass concentration (da Cruz et al., 2002; Barbosa et al., 2014). Khoja et al. (2015) found that sugarcane molasses are a better fermentation medium for the bacteria *Zymomona mobilis*, which performed better in this particular medium.

Conclusions

The yeast isolates showed distinct morphological characteristics among each other and were compared to the reference strains. Same observation was made with the molecular characterization, which showed intra-specific differences for all *S. cerevisiae* isolates. These isolates also differed from the reference strains, thus this necessitates constant monitoring from the producers, in order to verify the yeast cells that initiates fermentation which will be the ones to complete it. Despite the small sample sizes, a diversity of molecular morphophysiological traits has been revealed. This work show morphophysiological and molecular characterization of yeast to be useful to industry as it contributes to the selection of new suitable strains for alcoholic fermentation.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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