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ISOLATION OF WILD YEAST FOR POTENTIAL USE IN BEER PRODUCTION

Priscila Treviño-Aguilar[1], Myriam Elias-Santos[1], Claudia López-Alvarado[1], Benito Pereyra-Alférez[1], Jorge Hugo Garcia-Garcia[1]*

Address (es):
[1] Universidad Autónoma de Nuevo León, Facultad de Ciencias Biológicas, Instituto de Biotecnologia, Pedro de Alba s/n, 66451, San Nicolas de los Garza, Nuevo León, 8183294000 ext. 6438.

*Corresponding author: jorge.garciagr@uanl.edu.mx

ABSTRACT

There is a limited amount of yeast strains that are currently used in industrial beer brewing. Wild yeasts could provide an alternative to common domesticated brewer's yeasts by offering a new range of sensory characteristics and improved performance in harsh brewing conditions such as high gravity and high ethanol concentration in wort. High gravity brewing is practical and profitable as it increases production capacity, therefore reducing investment and energy costs. Exploiting the existing natural diversity could lead to finding superior industrial yeasts as well as a better understanding of biodiversity. The aim of the present study was to determine if wild yeast strains isolated from the ecosystem at a regional level (Northeast Mexico) showed favorable characteristics in the brewing process. Sixty-three yeast isolates were obtained from diverse samples including flowers and fruits. Five isolates were selected after three rounds of beer fermentation based on their sensory characteristics. Tests to assess growth over time, flocculation potential, ethanol yield, osmotolerance and ethanol tolerance were applied to two reference yeasts (commercial beer brewing strains) and to the selected isolates of interest which were identified as *Saccharomyces cerevisiae* by *MEX67* amplification and ITS sequencing. The results indicated that the selected wild isolates exhibit characteristics comparable to commercial reference strains in terms of growth and stress tolerance.

Keywords: wild yeasts, beer, *Saccharomyces cerevisiae*

INTRODUCTION

Beer is generally made from four main ingredients: water, barley malt, hops, and yeast. All of them are involved in beer's final flavor, including yeast, which is responsible for the production of diverse flavor compounds and ethanol (**Mosher, 2009**). Beer fermentation is done mainly by members of the *Saccharomyces* genus. There are few yeast strains that are used industrially in the brewing process, they can be grouped in two main classes: Ale (*Saccharomyces cerevisiae*) and Lager strains (*Saccharomyces pastorianus*). Yeasts represent a very diverse group of organisms and even strains that are classified as the same species often show a high level of genetic divergence (**Steensels et al., 2014**). Lager strains are a natural interspecific hybrid organism between *S. cerevisiae* and *S. eubayanus*. This hybrid obtained improved growth rates and biomass yields at low temperature from its *S. eubayanus* subgenome and the ability to consume maltotriose from *S. cerevisiae* (**Hebly et al., 2015**). Commercial strains of *S. cerevisiae* have shown specific selection for stress tolerance, sugar utilization and flavor production (**Parapouli et al., 2020**). However, concentrated beer production may have caused a reduction in the number of strains used for brewing, thus it is relevant to explore opportunities to isolate or generate yeast variants that fulfill the interest for increased ethanol productivity, extensive substrate utilization and production of non-conventional compounds (**Huuskonen et al., 2010; Cubillos et al., 2019**).

Some strategies that have been carried out to provide new yeast strains to the brewing industry include experimental evolution, mutagenesis, breeding, and yeast isolation from the wild. Isolating wild yeasts from a particular geographical region to make the most of its inherent biodiversity may provide novel flavors and can conform to the demand of non-genetically modified microorganisms and use of Generally Recognized as Safe (GRAS) substances. Recent efforts across the globe have contributed to the expansion of the brewing yeasts' reserve by sampling from forests and orchards. Depending on the isolation method utilized there has been varying success in finding new *Saccharomyces* strains or species from the wild (**Cubillos et al., 2019**). Although ubiquitous, yeast strains are commonly associated with sugar-rich environments. Wild yeasts are commonly isolated from sources like fruits, plants (roots, leaves, flowers, and exudates) and grains. Other strains are associated with soil, orchard ambient air and specific sources, such as fruit juice, honey, and flower nectar. This is convenient since it implies an inherent ability to grow in an environment of high sugar concentration (**Herrera et al., 2009; Wang et al., 2015**). Wild yeasts most frequently associated with brewing are natural strains of *Saccharomyces* and *Brettanomyces* and, to a much lesser extent, *Candida* and *Pichia*, as well as other aerobic yeasts. During the production of Lambic beers, for example, wild strains of *Saccharomyces* and *Brettanomyces* are the main determinants of the sensory profile and the level of attenuation of beer (**De Keersmaecker, 1996**). The use of

wild yeasts in brewing, however, can lead to unpredictable fermentation results. Since they are undomesticated, they may be characterized by low fermentation yields and more sensitivity to ethanol stress, but usually provide a distinctive flavor, which could make them an adequate choice as a subsidiary fermentation agent. In addition, some may offer an advantage over commercial strains as they exhibit osmotolerance to variable degrees (**Wang et al., 2015; Capece et al., 2018**). Some documented successful findings of wild yeasts capable of producing beer with sensory desirable characteristics include a strain of *B. anomalus* that was also an effective as starter culture in batches of multiple gallons of beer and a strain of *Saccharomyces cerevisiae* called YH166, which was isolated from an open fermentation in a vacant lot in the United States and gave beer a pleasant fruity aroma reminiscent of pineapple and guava (**Lentz et al., 2014; Osburn et al., 2017**). However, it has also been found that some strains of wild yeasts may exhibit strong phenolic characters (plastic, medicinal and smoked), producing beers with a complex, spicy, fruity character and aromas that could be considered undesirable (**Bokulich and Bamforth, 2013**).

Yeast metabolism plays a vital role in flavor compound production. Alcohols, esters, organic acids, carbonyl compounds and sulphur compounds are some of the metabolites of importance for beer's flavor profile (**Olaniran et al., 2017**). Volatile esters are responsible for beer's fruity character, they can have a synergistic effect with one another and, as trace compounds, a minimal change in their concentrations can dramatically affect the taste of beer (**Verstrepen et al., 2003**). The concentrations of these compounds can be affected by the yeast strain of choice (genetics), nutritional factors, environmental conditions during fermentation, among other factors (**Nedovic et al., 2014**).

Intensification of the industrial brewing process has also created a need for yeast strains that can excel in high and very high gravity brewing (Gibson, 2011). Most commonly, beer wort (water with dissolved sugars from malted barley) has a sugar concentration of 10 to 12 degrees Plato (°P), in some cases also high gravity wort is used (14 to 17 °P), producing beer with higher concentrations of ethanol which is subsequently diluted to a normal drinking strength. Therefore, the use of high gravity brewing reduces investment costs, energy consumption and labor costs. A very high gravity wort is also applicable (18 to 25°P) to the brewing process representing an even greater economical and yield advantage (**Huuskonen et al., 2010**).

The approach of this study was to focus on the possibility that the natural biodiversity found in a geographical region might offer an opportunity to isolate novel yeasts with favorable characteristics for industrial beer production.

MATERIALS AND METHODS

Sample collection and isolation of wild yeast strains

In order to perform a pre-selection of wild yeasts that were suitable for brewing (ability to grow on beer wort nutrients), a barley malt culture medium (BMM) with a content of 6 ± 2 Brix degrees (°Bx) was used. In all cases the wort was brought to room temperature (25 °C) before measuring Brix degrees with a PAL-1 pocket refractometer (ATAGO, Tokyo, Japan). The BMM medium was prepared by mashing 150 g of pale malt 2 Row (Malteurop, Milwaukee, WI, USA) for every liter of water for 60 minutes at 66 °C and then autoclaving at 121°C for 15 minutes at 15 psi. Barley malt agar medium (BMA) was also prepared by adding 20 g of bacteriological agar per liter before autoclaving.

Samples of flowers, tree bark, fruits, fruits skin, leaves and soil from different orchards of three north east Mexican states (Nuevo Leon, Durango and Coahuila) were aseptically placed in sterile falcon tubes containing 40 mL of sterile BMM. Air samples from the same locations were collected in triplicates by exposing BMA plates to air for 30 minutes.

All tubes were incubated at 25 °C for 5 days as a yeast enrichment step. Then the liquid cultures of all samples were serially decimally diluted using sterile 0.85% (w/v) NaCl solution and were spread onto BMA plates in triplicates and they were incubated at 25 ± 2 °C for 3 days. Cells of every colony type were isolated by repetitive streak plating, then morphological distinction of yeast cells was performed by observing them under a microscope at 1000X magnification. Pure cultures were maintained on 1% yeast extract, 2% peptone and 2% dextrose (YPD) agar and liquid medium and glycerol stocks of the isolates were prepared and stored at -40 °C.

Screening of yeast isolates
The wild yeast isolates of interest in this study were selected from all the isolates obtained based on their ability to ferment beer wort and produce beer with a pleasant sensory profile. To ensure that the isolates to be selected were able to maintain their vitality and continue to produce pleasant aroma and flavor compounds, 3 fermentation rounds were carried out in duplicates in 50 mL tubes, for 5 days each. After initially providing the respective pitching rate (0.75 million cells/mL/°P)[14], the same bottom yeast sediment was reused each time. In all cases the same beer recipe was prepared by mashing 3.5 kg of 2-row pale malt and boiling 60 minutes with 30 g of cascade hops (specific gravity of around 1.035).

For each fermentation round, a tasting was carried out by a panel composed of 8 judges to characterize the product obtained by means of the 9-point hedonic scale test (affective test method) and a flash profile analysis (rapid descriptive analysis for sensory characterization).

The 9-point hedonic scale test was implemented as described by **Lim (2017)**, panelists were asked to indicate overall, how much they liked or disliked each sample and to fill out a ballot containing the following scale:

- Like Extremely
- Like Very Much
- Like moderately
- Like Slightly
- Neither Like nor Dislike
- Dislike Slightly
- Dislike Moderately
- Dislike Very Much
- Dislike Extremely

For flash profiling (FP), the method described by **Dairou and Siefferman (2002)** with some modifications was implemented. An attribute generation session was held first by presenting all the samples to the panelists one at a time. The samples were evaluated, and the perceived attributes were recorded (flavor and aroma attributes). Differently to the referenced method, the attributes were discussed by the judges and a consensus was reached (usually avoided in FP in order to save time). Afterwards, a sample rating session was held where a ballot containing the descriptors recorded before was given to every panelist to evaluate the samples once more, this time ranking them in order of intensity for each attribute generated. The following five-point system to rate attribute intensities, as recommended by **George and Laurie Fix (1997)**, was used:

1) Not detectable
2) Slightly detectable
3) Detectable, but not strong
4) Strong
5) Overpowering

All data was entered to a database afterwards and factor analysis was implemented for FP taking into account the mean intensity score for each attribute. Analysis was conducted in R (**R Core Team, 2021**) and all figures in this paper were produced using the packages ggplot2 and fortify (**Wickham, 2016; Tang et al., 2016**).

The reference yeasts in this study were included for comparison in each tasting, which consisted of the same beer wort recipe fermented by a commercial Ale type yeast (SafAle ™ S-04 Fermentis, Milwauke, USA) fermenting at room temperature (25 ± 2 °C) and a Lager type yeast (Saflager ™ S-189 Fermentis, Milwauke, USA) fermenting at 15 °C $\pm$ 2, according to the manufacturer recommendation. It was relevant to select reference yeasts from an industrial brewing yeast global manufacturer to be able to compare their fermentation properties to the wild isolates. In addition, the flavor profile of both references favored contrast during the sensory evaluation process. In the ale strain's case providing typical characteristics present in a large range of ales (balanced fruity and floral notes as described by the manufacturer). And in the lager strain's case allowing for a neutral flavor brew (mild flavor, low ester production) as opposed to the widely used strain Fermentis Saflager W-34/70, which provides more of a pronounced floral and fruity aroma.

The first tasting round consisted of sampling beer produced by all the yeast isolates collected fermented at 25 °C $\pm$ 2 °C for 5 days. For the second tasting round, each isolate was used in 2 different batches in duplicates at 2 different temperatures, 25 °C $\pm$ 2 °C (Ale type fermentation) and 15 °C $\pm$ 2 °C (Lager type fermentation), to determine which of them generated a more desirable final product and set it as its optimal fermentation temperature. Finally, the third tasting round consisted of using the isolates to produce beer only at their respective optimal fermentation temperature (referred to this way from this point on), which means that they received a higher hedonic score at this type of fermentation.

Identification of selected yeast isolates
Selected isolates were identified by obtaining their genomic DNA from overnight YPD medium cultures with 1×10^6 cells/mL by the method reported by **Osorio et al. (2009)**. Genomic DNA was used for ITS-5.8S amplification using primers ITS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-CTTGGTCATTTAGAGGAAGTAA-3') for sequencing and restriction analysis using HaeIII (**Gardes and Bruns, 1993; McCullough et al., 1998**); species identification primers for *S. paradoxus*, *S. mikatae*, *S. kudriavzevii*, *S. bayanus*, *S. arboriculus* and *S. cerevisiae* were used as reported by **Muir et al. (2011)**. *S. cerevisiae* is identified by gene MEX67 using primers ScerF2 (5'-GCGCTTTACATTCAGATCCCGAG-3') and ScerR2 (5'-TAAGTTGGTTGTCAGCAAGATTG-3'). The PCR products, 5 μL, were loaded on an agarose gel and electrophoresed at 70 V/cm2, stained with ethidium bromide and visualized. We used a 100 BP DNA ladder (Promega, Wisconsin, USA) and Lambda HindIII as molecular size markers (Promega, Wisconsin, USA). DNA bands for ITS-5.8S were purified using the Wizard SV 96 PCR Clean-Up System (Promega, Wisconsin, USA) and sequenced with the AB 3500 (Applied Biosystems, California, USA), to ensure the detection of the studied genes.

Growth kinetics: growth curve and flocculation potential assay
Growth performance over time of selected isolates was determined through a growth curve while also employing the commercial Lager type reference yeast for comparison (Saflager ™ S-189 Fermentis, Milwauke, USA) incubated at 15°C $\pm$ 2°C. The selected isolates and the reference yeast were inoculated in duplicate in tubes containing 50 mL of BMM, and then diluted (1:10) using sterile BMM, which was also used as blank. The growth curve was obtained by performing Optical density readings at 600nm (OD600) every 24 hours for 6 days with a SmartSpec 3000 spectrophotometer (Biorad, California, United States). The selected isolates were incubated at their optimal fermentation temperature and before performing every reading the culture was homogenized using a vortex mixer. Yeast cell counts were also performed using a Neubauer haemocytometer (Marienfeld). In all cases the respective pitching rate was calculated and provided (0.75 million cells/mL/°P) (**Fix, 1999**).

After reaching the maximum growth point in all cases (no cell density increases in 24 hours), some fluctuation in the measured absorbance levels was observed. Thus, the residual absorbance of the yeast suspension throughout fermentation was investigated (residual absorbance before and after flocculation and settling). This method is described as an adequate parameter to characterize yeast flocculation potential, usually involving agitation, and resettling before measuring absorbance, and can also be combined with a direct observation of floc formation in the yeast culture (**Kihn et al., 1988; Vidgren and Londesborough, 2011**). However, the approach taken in this study was modified in accordance with the static fermentation method, which is considered to be an *in vivo* style test because it is carried out under conditions more closely akin to the static fermentation conditions encountered in a typical brewery (Stewart, 2018). The yeasts of interest were inoculated in 450 mL of BMM in 500 mL glass flasks and taking OD readings (600nm) every 24 hours for 10 days using the same method described before excepting without homogenizing the culture. This assay also served as a screening method

High gravity and ethanol tolerance assay

The selected isolates' tolerance to adverse fermentation conditions was determined by growing yeasts in culture medium simulating such conditions and then performing viable cell count by plate dilution method over time. The conditions tested were high glucose and high ethanol concentrations. The isolates of interest and both commercial reference yeasts (Lager type and Ale type) were inoculated in duplicates in tubes containing 10 mL of sterile YPD medium enriched with different glucose concentrations: 12°P, 17°P and 25°P, and ethanol concentrations: 4%, 8%, 12% and 16%. In all cases the tubes were inoculated with $9x10^6$ cells/mL and yeasts were incubated at their optimal fermentation temperatures (15 ± 2 °C or 25 ± 2 °C). A control was used in all cases (2°P, no added ethanol). Tubes with 0.9 mL of sterile 1M NaOH buffer were used to make dilutions (1:10 to 1:10,000,000) of the yeast cultures which were inoculated in petri dishes with YPD agar medium, and were left to grow for a period of 3-4 days at 25 °C $\pm$ 2 °C. The plates that showed growth in the range of 10 to 150 colonies were used to calculate the CFU/mL by multiplying the number of colonies by the dilution factor. The viable count by plate dilution method was repeated thrice. Once 24 hours after inoculating the tubes (T0). Once again at the yeasts' maximum growth point (T1, 4 days after T0) and then the last measurement was conducted at its stationary phase (T2, 9 days after T0).

To simply monitor sugar consumption, Brix degrees decline over time in the enriched growth medium of all cultures was also measured using a PAL-1 pocket refractometer (ATAGO, Tokyo, Japan). Readings of the medium's initial °Bx, as well as those of 5, 8, 15 and 25 days after the initial time were taken in triplicates.

Ethanol yield estimation assay

Selected isolates and both commercial reference yeasts (Ale type and Lager type) were used to carry out 0.5-liter fermentations (3.5 kg of 2-row pale malt, 30 g of cascade hops boiled 60 minutes with a specific gravity of around 1.035) in duplicates, they were incubated at their optimal fermentation temperatures (15 ± 2 °C or 25 ± 2 °C) for 17 days. The respective pitching rate was calculated and provided (0.75 million cells/mL/°P) in all cases. To calculate an estimation of the yeasts' ethanol production the original and final specific gravity of all beer wort was measured using a hydrometer (alla france, Chemillé, France) and to monitor sugar consumption, a measurement of the initial and final °Bx in beer wort was also taken. The decrease in specific gravity was correlated with the percentage of alcohol by volume present (ABV) by means of the following standard formula:

$((1.05$ x (Original gravity – Final gravity$)) /$ Final gravity$) / 0.79$ X $100 = \%$ ABV. Where 1.05 is grams of ethanol produced for every gram of CO_2 produced, and 0.79 is the density of ethanol. The apparent attenuation of the wort was also be calculated using the following formula:

$$AA = (OG - FG) / OG \text{ (Gravities are in specific gravity units)}$$

RESULTS

Wild yeast strain isolation and screening

The results of this study's sampling assay show that wild yeasts can be found in sources like flowers, fruits, and ambient air. As is the case of several of the studies referenced, it was assumed that the isolates are wild yeasts given the locations from where they were obtained. From all the collected samples, 63 wild yeasts isolates were obtained.

Simple parametric statistics were used to analyze the data from the hedonic scales. This is consistent with the fact that the scales are described as ordinal in nature (**Cliff *et al.,* 2016**). Normality tests to confirm non-normality of the data were performed (data not shown).

Attributes generated in flash profile analysis (Table 1) are consistent with those mentioned in literature. Some unpleasant sensory characteristics are the presence of acetaldehyde (poor wort fermentation), relatively high production of sulfur and phenolic compounds, as well as overproduction of esters (**Gibson, 2011**). The presence of these compounds in some of the samples may have earned them a low hedonic score. Whereas attributes considered as positive are pleasant

aroma and mouthfeel, as well as characteristic flavors like alcohol and fruitiness (esters) (**Verstrepen *et al.,* 2003**).

Table 1 Attributes generated by panelists in flash profile analysis for all isolates throughout the 3 tastings. Aroma and in-mouth flavor are presented as acronyms A and F

Attributes	Frequency of mention
Sweet	18
F_Estery	16
F_Cooked Vegetable	10
F_Phenolic	10
F_Solvent	10
Astringent	8
Alcohol	8
A_Floral	8
F_Acetaldehyde	5
F_Sour	5
F_Diacetyl	4
F_Butyric	2
F_Earthy	2
A_Butyric	1
A_Diacetyl	1
A_Almond	1
A_Peach	1
Bitter	1

The factor analysis plot (Figure 1) shows that out of the 10 wild isolates with the highest hedonic scores (a mean liking of 7 or higher), there were 5 of them highly correlated to each other and the references (to a less extent to the lager reference), forming a group of samples at the upper right side of the plot in proximity to the attributes ester, solvent, alcohol, astringent and floral, which means that those attributes described them. The rest of the wild isolates formed a group in contiguity to the attributes sweet and cooked vegetable, except for one that was better described by phenolic.
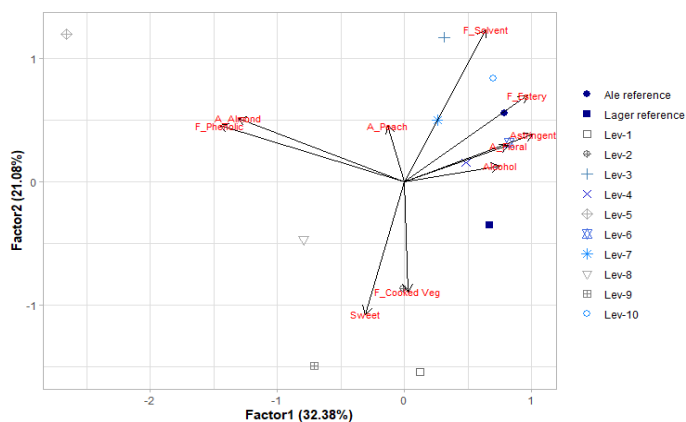

Figure 1 Factor analysis scores and loadings plot of flash profile sensory analysis of the 10 wild isolates with the highest hedonic scores and the reference yeasts

After the screening process, five yeasts of interest were selected (Table 2) based on their pleasant sensory characteristics (similarity in attributes to the references), their ability to maintain such characteristics without producing any detectable off flavors through all the fermentation cycles and their high hedonic rating.

Table 2 Initial and final °Bx and density values of wort from the ethanol yield estimation assay and estimated ABV and attenuation.

Isolate	Source	Fermentation temperature	Original Specific Gravity	Final Specific Gravity	ABV (%)	Attenuation (%)
Ale type reference	Commercial	25 °C	1.036	1.004	4.23	88.88
Lager type reference	Commercial	1 5°C	1.036	1.004	4.23	88.88
Lev-3	Wild berries surface	25 °C	1.034	1.010	3.16	70.59
Lev-4	Orange tree flowers	15 °C	1.034	1.010	3.16	70.59
Lev-6	Orange orchard ambient air	15 °C	1.034	1.009	3.23	72.06
Lev-7	Apple fermentation	15 °C	1.034	1.008	3.36	75.00
Lev-10	Apple fermentation	15 °C	1.034	1.006	3.70	82.35

Identification of yeast isolates of interest

The selected yeast strains (Lev-3, 4, 6, 7 and 10) were in all five cases identified as *Saccharomyces cerevisiae* according to the amplification of *MEX67* (Figure 2) as well as by restriction of the ITS with *HaeIII* (results not shown). None of the other primers specific for *S. paradoxus, S. mikatae, S. kudriavzevii, S. bayanus, S. arboriculus* showed PCR amplification. The sequences of the ITS-5.8S from the amplification of Lev-3 (Genbank MN525555), Lev-4 (Genbank MN525556), Lev-7 (Genbank MN525557 and Lev-10 (Genbank MN525558) all had more than 96% identity with *S. cerevisiae* (Genbank CP006466.1).
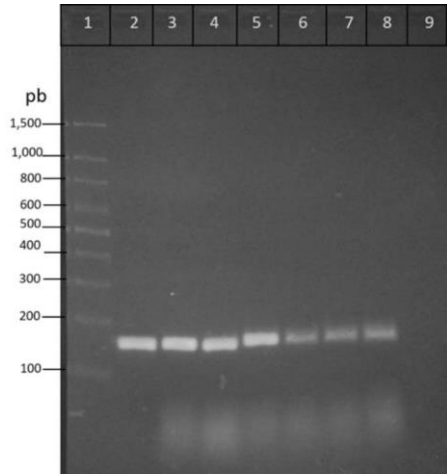

Figure 2 1.5% Agarose gel electrophoresis for MEX67 amplification (150 bp). In lane 1: 100 bp Ladder; Lane 2: *S. cerevisiae* S288c; Lane 3: SafAle S-04; Lane 4: Lev-3; Lane 5: Lev-4; Lane 6: Lev-6; Lane 7: Lev-7; Lane 8: Lev-10 and Lane 9: Negative control.

Growth curve and flocculation potential assay

When comparing the selected isolates to the Lager type reference it was observed that all wild yeasts showed comparable absorbance values at their maximum growth point (Figure 3A). In all cases, the selected isolates reached their maximum growth point before the reference yeast.

The isolated yeasts showed comparable values of absorbance to the reference (Figure 3B). It is worth noting that we used the lager (Saflager ™ S-189 Fermentis) yeast as a reference for growth and flocculation potential because all selected wild yeasts except for Lev-3, were incubated 15°C (Table 2).
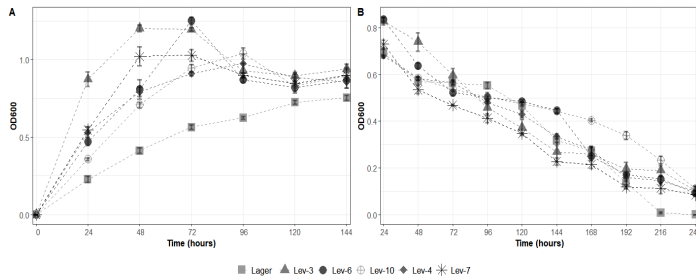

Figure 3 Optical density (A) and flocculation potential assay results (B) of selected isolates and Lager type reference strain

High gravity and ethanol tolerance

In this study's growth kinetics assay, the selected isolates reached an OD600 equivalent to 10^7 cells/mL (Figure 3A). This value was also observed in the control condition of this assay for all yeasts, including the references. At the 12°P of added glucose condition of this assay (concentration of sugars equivalent to a standard gravity industrial fermentation), all yeasts were able to maintain the expected number of viable cells (1×10^7 cells/mL) while Lev-3, Lev-4 and Lev-7 thrived in this condition reaching up to 1×10^8 viable cells/mL. At 17°P (high gravity) and 25°P (very high gravity), it was observed that the reference strains were inhibited to varying degrees, in all cases the wild isolates exhibited higher cell density, with Lev-4 performing the best in very high gravity wort (Figure 4A). Sugar consumption was monitored by measuring °Bx over time, at 9 days (T2) of the very high gravity wort fermentation there was a high amount of sugar left (Figure 4B), so we kept measuring for 25 days.

At up to the 8% of added ethanol condition of this assay, the selected isolates and reference yeasts' growth was not affected. While at 12% of added ethanol it was

observed that the reference strains were completely inhibited, Lev-3, Lev-6 and Lev-7 presented the greatest ethanol stress tolerance being able to reach and maintain the expected viable cell number (1×10^7 cells/mL) (Figure 5). At 16%, all yeasts except for Lev-7 were completely inhibited.
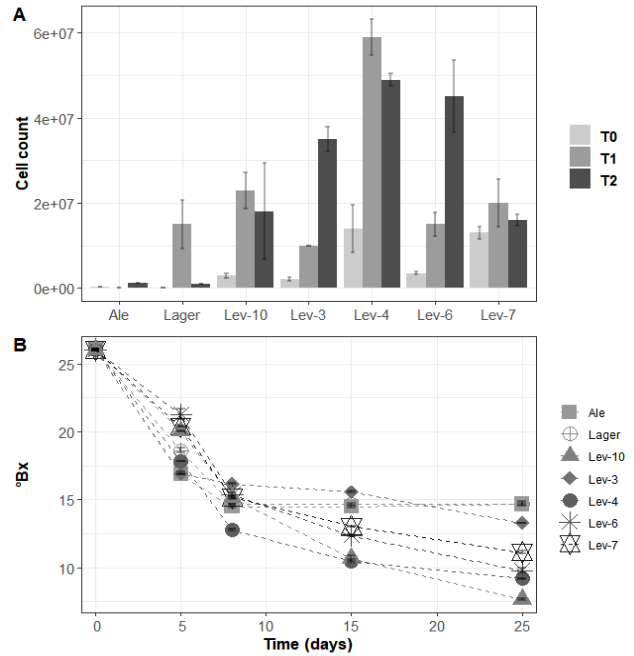

Figure 4 Cell number over time of selected isolates and the Lager and Ale type reference strains in the 25°P (added glucose) condition, T0: 1 day after inoculation; T1: 4 days after inoculation; T2: 9 days after inoculation (A). Brix degrees decline over time (B).
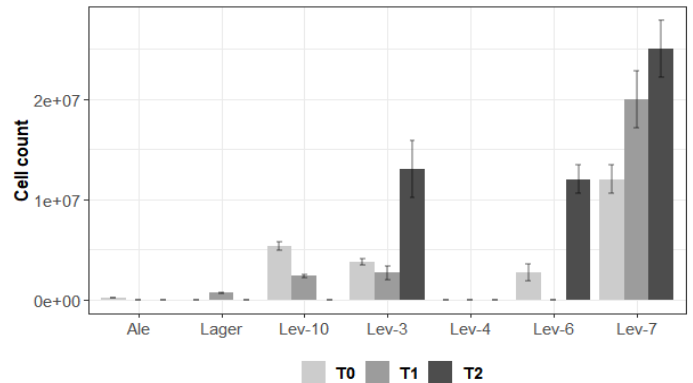

Figure 5 Cell number growth over time of selected isolates and the Lager and Ale type reference strains in the 12% added ethanol condition, T0: 1 day after inoculation; T1: 4 days after inoculation; T2: 9 days after inoculation.

Ethanol yield estimation

Reference yeasts (Lager type and Ale type) showed a very similar performance compared to each other regarding their ethanol production capacity and wort attenuation, which was higher than that of the selected wild yeasts in all cases. All selected yeasts showed similar attenuation values except for Lev-10 which exhibited a performance closer to that of the reference yeasts (Table 2).

DISCUSSION

We obtained 63 isolates from the samples gathered, of which 5 were selected based on their sensory characteristics (Table 2). The presence of wild yeasts found in the samples collected in this study corresponds to that reported in previous studies where diverse geographical locations were sampled. In a study performed in two widely separated areas: the Iberian Peninsula (Spain) and the Yucatan Peninsula (Mexico), the presence of wild yeasts in flower nectar was evaluated, finding high yeast cell population densities in the species sample (**Herrera *et al.,* 2009; Cliff *et al.,* 2016**). In another study, osmotolerant wild

yeasts were successfully isolated from a total of 90 randomly collected samples of orchard soil, apple, and apple leaves from 10 apple orchards located in the Shaanxi province in China (**Wang *et al.,* 2015**). Wild yeasts were also isolated from fruits such as grapes, molasses and cashew apples in a study conducted in India (**Tikka *et al.,* 2013**). The screening process of the wild isolates in this study was based on the yeasts' ability to produce desirable sensory compounds, as well as to maintain those sensory characteristics throughout all the fermentation cycles. The latter aspect was also relevant because, normally, industrial scale fermentations are carried out with recycled yeast from previous fermentations, and this recycled yeast differs physiologically from the freshly propagated yeast from the pure culture (**Huuskonen *at al.,* 2010**).

The nature and magnitude of yeast populations can vary widely and depend on the inherent composition of their habitat. There are surprising metabolic differences (rate of fermentation and flavor production) even among yeast species that are explained by inter-individual genetic variability (**Breunig *et al.,* 2014; Michel *et al.,* 2016; Cubillos *et al.,* 2019**). Therefore, the varying sensory characteristics of the wild isolates of interest in this study, despite them being the same species, may be explained mainly by two reasons: the specific location and sample from which the yeast was isolated and the strain's metabolic characteristics (which depend to some extent on the former). *Saccharomyces cerevisiae* is predominantly found in association with human activities, but it can be frequently isolated from sugary foods and other sources unrelated to alcoholic beverage production (**Fay and Benavides, 2005**), non-*Saccharomyces* wild strains in the total of isolates in this study may have been excluded because of the screening process.

When comparing growth of the selected isolates to the reference, all wild yeasts showed comparable absorbance values at their maximum growth point and in all cases, the selected isolates reached their maximum growth point before the reference yeast. The drop in OD600 at 96 hours in the growth curve (Figure 3A) may be explained by floc formation and thus, not all yeasts cells being resuspended, even after homogenization (**Stewart, 2018**). The flocculation potential assay served as an indicator of the number of cells in suspension in the cultures (Figure 3B), which is an important characteristic in industrial yeast strains. Since the selected wild yeasts presented a similar behavior to a commercial lager strain, they may be appropriate for industrial fermentations (**Stewart, 2018**).

As shown in the results, the selected isolates and reference yeast reached a similar number of cells while growing under normal conditions (Figure 3A). However, the high gravity assay showed that Lev-4 performed the best in very high gravity wort, while Lev-3, Lev-4 and Lev-7 thrived under normal conditions (Figure 4A). After 25 days of fermentation, the beer fermented by Lev-10 had the least number of sugars left (Figure 4B), and although 25 days is not a normal fermentation time for beers, with further studies this yeast could be used for other fermented beverages such as fruit wines.

These results agree with the inherent characteristics in *S. cerevisiae* strains, as there is large evidence of its efficient glucose utilization and high stress tolerance in general (**Stanley *et al.* 2010; Kayikci and Nielsen, 2015**). Osmotolerant wild yeasts have also been successfully isolated in other similar studies obtaining diverse results in osmotic tolerance and species variability, **Wang *et al.* (2015)** used a glucose-enriched malt culture medium with different concentrations: 0% (control), 2%, 20%, 40% and 50% (p/v) to screen wild yeasts isolated from apple orchards for osmotic stress tolerance. Thirteen isolates were obtained and identified by the amplification of the ITS1-5.8S-ITS2 regions, the most common species were *Kluyveromyces marxianus*, *Hanseniaspora uvarum*, *Saccharomyces cerevisiae*, *Zygosaccharomyces rouxii*, *Candida tropicalis* and *Pichia kudriavzevii*. In another study conducted by **Suranska *et al.* (2016)** a YPD medium with 40% (w/v) and 50% (w/v) glucose was employed to screen wild isolates from vineyard grapes and spontaneously fermented musts for eonological properties. Four of the isolates, all of them identified as *Saccharomyces cerevisiae* by amplification of 5.8S rDNA gene regions, exhibited higher growth rate than a commercial *S. cerevisiae* reference strain (BS6) in these high sugar concentration conditions.

Sugar tolerance is an aspect with substantial variance among wild yeasts (**Ok and Hashinaga, 1997**), besides the influence of the natural habitat and fermentation conditions on yeasts' osmotic stress tolerance, this trait is linked to the genetic factor, there is substantial evidence that the MAPK HOG (high osmolarity glycerol) pathway participates in the response to high sugar concentrations allowing *S. cerevisiae* cells to perceive and respond rapidly to altered osmolarity (**Gomar-Alba *et al.,* 2015**).

The ethanol tolerance threshold observed in our assay is consistent with other studies where ethanol tolerant strains were obtained from similar samples. In a study conducted by **Arekar and Lele (2015)**, an ethanol tolerance of up to 12% with a radical biomass decrease observed thereafter was also observed. YPD broth containing varying concentrations of added ethanol from 6 to 15% (v/v) was used to screen wild yeasts isolated from tropical fruits. Two of the isolates

identified as Saccharomyces cerevisiae showed similar ethanol tolerance to a *S. cerevisiae* reference strain (NCIM 3215). In another study carried out by **Tikka *et al.* (2013)** YPD medium with different concentrations of added ethanol: 6%, 7%, 8%, 9%, 10%, 11%, 12%, 12.5% 13%, 13.5%, 14% and 14.5% was used to screen wild yeasts isolated from fruits for ethanol stress tolerance. It was found through RAPD analysis that 7 out of 15 wild isolates obtained were *S.cerevisiae*. The most tested strains exhibited tolerance in a range of 7 to 12% of added ethanol with a very low tolerance rate afterwards. Some important effects of ethanol toxicity on yeasts are growth inhibition, reduced cell viability and enzyme inactivation. Cell membranes appear to be the main sites of ethanol damage; numerous studies conducted on *S.cerevisiae* have identified the composition of the plasma membrane essential for ethanol tolerance. The composition of the brewer's yeast membrane is influenced by the wort's composition and the fermentation temperature, which in turn are influenced by gene expression to allow adaptation to the environment (**Gibson, 2011**).

A promising approach has been suggested by **Gibson *et al.* (2007)** to possibly improve brewing yeast performance in high gravity wort by supplementing with specific metal ions, lipids, and lipid components such as fatty acids and sterols, among others. This strategy may counter the toxic effects of these harsh brewing conditions on enzyme activity and the yeast cell membrane.

The attenuation and ethanol production of the isolated yeasts was like the reference strains (Table 2). Although usually domesticated strains show a more vigorous maltose fermentation than non-industrial yeast strains (thus, higher attenuation), there are cases where wild isolates may outperform commercial strains in maltose utilization as a carbon source (**Bell *et al.,* 2001; Lee *et al.,* 2011**). Lower attenuation in some beer fermentations may be explained by the yeasts' glucose repression pathway (gene expression changes triggered by the presence of glucose), which has an impact in sugar uptake and produces sluggish fermentations and decreased yeast vitality (**Piddocke *et al.,* 2009; Kayikci and Nielsen, 2013; Lei *et al.,* 2016**). In some cases, supplementing beer wort with maltose and malt triose has been shown to increase wort fermentability, even in high gravity brewing (**Herrera *et al.,* 2009; Piddocke *et al.,* 2009**). It could be fruitful to perform additional high gravity assays supplementing with different sugars that could increase this study's isolates' attenuation percentage.

CONCLUSION

The data discussed in the present study demonstrates the wide possibilities of finding wild *Saccharomyces cerevisiae* strains in rich biodiverse environments that could provide new less conventional flavors to the beer industry and better fermentation performance; taking advantage of the rich biodiversity in microbial organisms that can be found in different parts of the world. The wild yeast isolates evaluated in this study were able to produce beer with desirable sensory attributes and exhibited characteristics that might be favorable for the brewing process such as vigorous growth and varying tolerance to high glucose and ethanol concentrations in comparison to the commercial reference strains. More specifically, Lev-4 and Lev-6, performed well in high gravity wort; Lev-7 had a high tolerance for ethanol and Lev-10 yielded similar ethanol concentrations to the commercial brewing yeasts and produced the beer with less residual sugars from a high gravity worth at 25 days of fermentation.

Most of the tests cited in this study, as well as those performed here, were carried out on a laboratory scale; hence with further studies on a larger scale these new yeasts could be adapted to use in commercial production of beer or other fermented beverages.

Declaration of interest
The authors declare that there are no conflicts of interest.

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