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ORIGINAL ARTICLE

New sources and methods to isolate vinasse-tolerant wild yeasts efficient in ethanol production

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Abstract In most Colombian distilleries, fuel alcohol is produced by a continuous operation mode, recycling vinasse to dilute B molasses, which is the main byproduct of the sugar refining process and, more recently, the main feedstock for ethanol production. Commercial yeasts are used as catalysts during the fermentation stage; however, their yield is significantly affected by hyperosmolarity-induced stress and high organic acid concentration, both of which gradually reduce the performance of these microorganisms after a 3-month period of vinasse recycling. In this study, a methodology for isolating wild yeasts obtained from molasses storage tanks and sugarcane buds and leaves was standardized. These yeasts exhibited natural tolerance to vinasse and 85 % of the isolates were characterized as *Saccharomyces cerevisiae* by biochemical testing. Based on intraspecific characterization by interdelta markers 12–21 and subsequent confirmation by the AFLP method, isolates were classified into five groups of strains with genetic distances ranging between 15 and 30 %. A culture medium containing 50 % vinasse and around 16 % (w/v) reducing sugars was used to determine their fermentation capacity; ethanol yield (Yp/s) ranged from 5.69 to 6.82 % (w/v), volumetric productivity (Qp) from 2.37 to 2.84 g L^{-1} h^{-1}, and efficiency was 71.22–85.09 % after 24 h.

Keywords *Saccharomyces cerevisiae* · Wild yeasts · Ethanol efficiency · Vinasse · Interdelta fingerprinting

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Introduction

Batch fermentation for production of ethanol from cane juice or molasses generates approximately 13 L vinasse L^{-1} ethanol (Goldemberg et al. 2008). Researchers from Brazil report a production of 191.1 million m^3 vinasse $year^{-1}$. Although this residue is used in fertirrigation, the debate continues about its potential impact on soil acidification, accumulation of potassium or phenols (Martinelli et al. 2013; Rajagopal et al. 2014), and entrainment of cadmium and lead to the groundwater (Pereira and Pereira 2008).

In the Colombian Cauca river valley, five sugar mills have coupled distilleries to their process, achieving an overall production of 1,250,000 L alcohol daily, using B molasses, the byproduct of sugarcane crystallization, as raw material (Quintero et al. 2008; Fernández-López et al. 2012). At all the distilleries, this feedstock is converted into ethanol using three 100,000-L tandem fermenters that operate continuously as well as commercial yeasts of the species *Saccharomyces cerevisiae*, provided by Praj-Delta T Industries Limited. This process has been linked with the recycling of 40–55 % vinasse (distilled ethanol byproduct) to reduce water usage and pollution in watersheds. In addition to the recycling scheme, the vinasse is concentrated in Flubex evaporators to reduce its volume to an average of 2–3 L L^{-1} ethanol (Castillo et al. 2010).

Under normal fermentation conditions, the population dynamics of commercial strains undergoes changes attributable not only to the high concentrations of volatile and non-volatile organic acids, osmotic pressure, potassium salts and ethanol concentrations, but also to the entry into the system of several species of non-*Saccharomyces* wild yeasts transported by feedstock feeding pipes (Da Silva-Filho et al. 2005a; Ingledew et al. 2009). These phenomena have already been described in the case of Colombian distilleries, where increases in organic acid concentrations in the vinasse, particularly lactic, acetic, and butyric acids, have been associated with periods of reduced ethanol production efficiency (Socarras Díaz et al. 2009). Wild yeasts of the species *S. cerevisiae* have also been isolated from fermentation tanks during the process itself to determine their potential use in fermentation (Da Silva-Filho et al. 2005a). In fact, researchers are now paying more attention to this practice of Brazilian distilleries because of the potential uses of these strains adapted to the substrate and efficient in ethanol production (Basso et al. 2008; Da Silva-Filho et al. 2005a, b).

Although there is no consensus on which technique should be used to characterize yeast isolates, it is increasingly important that the microbiology labs of sugarcane distilleries agree on one specific molecular method to quickly identify strains with potential use in distilleries. Different studies conducted by Lopes et al. (2007), Basso et al. (2008) and Lopandic et al. (2008), indicate that intraspecific molecular characterization is key to studying the population dynamics of commercial strains in industrial fermentations, identifying the most efficient strains and solving cases of contamination with wild yeasts by identifying and isolating the dominant strain. Several molecular methods have also been proposed to characterize different strains. However, the routine application of most of these methods has proven to be either time-consuming, expensive, or with little discriminatory power (Lopez et al. 2003; Ayoub et al. 2006). Of all the PCR methods, fingerprinting (interdelta typing) could be appropriate for routine characterization of *S. cerevisiae* strains. The delta elements form part of the flanking region of the *S. cerevisiae* TY1 and TY2 retrotransposons. Some elements are separated from these structures and scattered throughout the genome thus allowing greater polymorphism between different strains and facilitating their identification (Legras and Karst 2003; Schuller et al. 2004).

This paper describes a method for the specific isolation and selection of vinasse-tolerant wild yeasts belonging to the genus *S. cerevisiae* with potential to be used in the ethanol production industry. It also compares the yield and efficiency in ethanol production of a wild type group isolated with two culture media: chloramphenicol glucose agar (CGA) and molasses-vinasse-yeast selection (MVYS). The interdelta fingerprinting methodology was evaluated for intraspecific characterization and selection of strains isolated from both molasses storage tanks and sugarcane leaves and buds. The physiological characterization by lab-scale fermentation studies, using B molasses at 15 % (w/v) TRS and 50 % (v/v) vinasse as substrate, enabled the selection of strains with the greatest efficiency and potential to be used in distilleries.

Materials and methods

Strain isolation

Isolates were obtained from a dual-purpose sugar mill-ethanol distillery, initially from the T-103 molasses storage tank, from which three 20-L samples of B molasses were taken in three different months (May, June, and July 2012) and on which serial dilutions 10^{-1} to 10^{-3} were performed. The amount of 100 μL of each dilution was placed in Petri dishes containing MVYS medium (containing B molasses adjusted to 7 % w/v total reducing sugars (TRS), 300 ppm ammonium biphosphate, 70 % (v/v) vinasse, 100 μg mL^{-1} chloramphenicol, 3.5 % (w/v) bacteriological agar, and pH 4.5. Samples were then incubated at 30 °C for 72 h. In addition, 75 mL macerated sugarcane leaves and buds were diluted with 175 mL sterile vinasse, adjusting the dilution to 70 % (v/v), then incubated at 30 °C, and constantly shaken at 150 rpm for 16 h. The samples were serially diluted 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}, and 100 μL of each dilution were placed in Petri dishes containing MVYS medium and incubated again at 30 °C for 72 h.

Yeast colonies with a diameter greater than 2.5 mm were collected from the Petri dish containing MVYS medium diluted 10^{-4}, and subcultured in 7 mL yeast extract-peptone-dextrose (YPD) medium (1 % yeast extract, 2 % peptone, 2 % glucose) in 15-mL Falcon tubes. Colonies were then incubated at 30 °C and 150 rpm for 15 h until a cell population of 2×10^{8} cells mL^{-1} (OD=1.3 at 660 nm) was obtained. These cultures were cryopreserved in 30 % glycerol at −80 °C.

Other strains belonging to the yeast bank from Cenicaña's Factory Processes Program, which had been isolated previously from the T-124 feeder tank, the H-124 heat interchanger, and the R-311 fermentation tank, were also included in this study (Buzón 2008; Mora 2008). These yeast strains were isolated using CGA (dextrose 20 g L^{-1}, yeast extract 5 g L^{-1}, chloramphenicol 0.1 g L^{-1} and agar 15 g L^{-1} at final pH 6.6).

Strains and culture conditions

The strains were denominated with the acronym LCC, which stands for Levadura (Yeast) Cenicaña Colombia. The molecular characterization included yeasts previously isolated from the T-103 molasses storage tank and the T-124 feeder tank. *Saccharomyces cerevisiae* isolates LCC1-1, LCC1-2, LCC1-

3, LCC1-5, LCC1-6, LCC1-7, LCC1-9, LCC1-13, LCC1-16, LCC1-17, and LCC1-18 were obtained from the Providencia Sugar Mill using CGA (Buzón 2008). Strain LCC2-13 was isolated using CGA from the outflow of the H-124 heat interchanger from the Mayagüez Sugar Mill (Mora 2008); strains LCC2-14 to LCC2-31 were isolated from the T-103 tank at the Mayagüez Sugar Mill using MVYS; and strains LCC0-1 to LCC0-20 were isolated from sugarcane cultures obtained from Lot 1 at Cenicaña, also using MVYS. Commercial strains GRX, GR, and XP were also included. All yeasts were cultured on YPD agar at 30 °C for 24 h and then stored at −80 °C in 30 % glycerol in the yeast strain bank of Cenicaña's Factory Processes Program.

Inter- and intra-specific biochemical and molecular characterization

Biochemical characterization was based on the carbohydrate assimilation pattern using the Analytical Profile Index (API) 20C AUX kit (BioMérieux S.A. France). Isolation of genomic DNA and molecular characterization by interdelta sequence typing were performed using the primers delta 12 and delta 21 following the protocol described by Legras and Karst (2003). Amplification reactions were performed using an EBSCO thermocycler with the following thermal profile: one cycle for 4 min at 95 °C followed by 35 cycles for 30 s at 95 °C, 30 s at 46 °C, and 90 s at 72 °C, and a final cycle for 10 min at 72 °C.

For analyses based on the amplified fragment length polymorphism (AFLP) technique, 2.5 ng DNA μL^{-1} were digested with 0.2 U μL^{-1} of each *Eco*RI/*Mse*I enzyme. The digestion product was ligated with the adapters of the two respective enzymes and 0.04 U of T4 DNA ligase. The ligation product was diluted at 1:10, and 5 μL of this dilution taken for PCR amplification with 0.1 U Taq polymerase μL^{-1}, using 40 nmol L^{-1} of each nonspecific primer EcoRI-0 and MseI-0, 100 mmol L^{-1} of each dNTP and 3 mmol L^{-1} MgCl2, and 0.2U Taq DNA polymerase (Fermentas Inc.). PCR conditions were 2 min at 94 °C, followed by 20 cycles for 30 s at 94 °C, 1 min at 58 °C, and 1 min at 72 °C. The PCR product was visualized with SYBR Safe after running agarose gel electrophoresis (2 %) at 80 V for 45 min. A second amplification was performed, using 5 μL of the PCR product from the first amplification diluted 1:25, 10× PCR buffer, 5 μM dNTPs, 0.09 U Taq DNA polymerase μL^{-1}, and primers MseI-C, EcoRI-AG, MseI-C, and EcoRI-AC, similar to those proposed by De Barros-Lopes et al. (1999) with minor modifications (Table 1 of supplementary information). Thermal conditions of the second PCR were 2 min at 94 °C, followed by 23 cycles for 20 s at 94 °C, 30 s at 60 °C, and 90 s at 72 °C, and a final 30-min extension at 72 °C. The amplification product was run on 5 % polyacrylamide gel and visualized with silver nitrate (De Barros-Lopes et al. 1999).

Analyzing genetic relationships between wild type yeast strains

The Tools for Population Genetic Analyses (TFPGA), program Version 1.3 (Miller 1997), was used to determine the genetic relationships between commercial and wild yeast strains. The genetic analysis included a mathematical model by the unweighted pair group method with arithmetic mean (UPGMA), which was based on the following equation:

$$\frac{1}{|A|.|B|}\sum_{x\in A}\sum_{y\in B} d(x,\mathrm{y}.)$$

At each stage, the closest clusters were combined in a top-level group. The distance between any given cluster A and B was taken as the average of all pairwise distances between objects "x" in A and "y" in B, representing the average of the distances of elements in each cluster. The dendrogram was computed based on Ward's clustering method using the minimum variance algorithm. A simple hierarchical clustering was performed, based on overall similarity. The number of characters available, depicted in a dendrogram of genetic distances, was taken into account (Swofford and Olsen 1990).

Lab-scale fermentation using a high-vinasse culture medium

The yeast for inoculation was grown in Erlenmeyer flasks filled with medium containing 50 g glucose L^{-1}, 20 g peptone L^{-1}, and 10 g yeast extract L^{-1}. After incubation at 30 °C and 150 rpm for 16 h (OD600 of 7–7.5), the cell suspension was aseptically collected by centrifugation (10 min at 7500 × *g*, 4 °C) and resuspended in distilled water to a concentration of 200 mg fresh yeast mL^{-1}. The yeast cells were then poured into the fermentation medium to adjust the population inoculum to $50–55\times10^6$.

Kinetic studies were performed in 500 mL Erlenmeyer flasks containing 250 mL culture medium based on B molasses (w/v), 15 % TRS, 60 % vinasse (v/v), and 300 ppm diammonium phosphate (DAP). Initial pH was adjusted to 4.5 with H_2SO_4. Four replicates were performed. Physical conditions were maintained at 150 rpm agitation and 30 °C temperature. Cell count was performed in a Neubauer chamber at the start of fermentation and again after 24 h.

Analytical methods

Fermentable sugars and ethanol were measured by high-performance liquid chromatography (HPLC) using a Sugar-Pak 1 separation column and calcium edetate as the regenerative mobile phase. Ethanol content was contrasted with the Alcotest technique, measuring the distillate with a Mettler Toledo DE40 densitometer. Volatile acidity of the culture

medium was performed using standards methods (Praj 2004). Kinetic parameters and yield coefficients were determined according to the following equations:

Product/substrate yield:

$$Yp/s = \frac{\Delta P}{\Delta S} = (P_f - P_o)/(S_f - S_{o,})$$

where P_o is the initial and P_f the final concentration of ethanol and S_o the initial and S_f the final concentration of fermentable sugars.

Rate of product formation or volumetric productivity (g L^{-1} h^{-1}):

$$Qp = \Delta P/\Delta t = (P_f - P_o)/(t_f - t_{o,})$$

where Qp is the volumetric productivity, (ΔP) the cumulative ethanol concentration (g L^{-1}), and (Δt) the time interval during which fermentation occurs.

Ethanol production efficiency:

$$E = (Actual\,E/Theoretica\,l\,E)100 = ((\Delta P)/(S \times 0.51))100,$$

where E is ethanol production efficiency, actual E is the concentration of ethanol produced in the must or (ΔP), and theoretical E is the ethanol concentration that should be produced (S×0.51), taking into account the initial concentration of fermentable sugars (S) and the value of theoretical ethanol (0.51) that should be produced from 1 mol of glucose.

Results and discussion

The productivity and efficiency of ethanol production depend largely on the implantation capacity of the yeast strain during the process. In environments such as those of industrial fermentation, these microorganisms struggle in the presence of increased concentrations of sugars, salts, and organic acids.

Strain isolation

Substrates with high sugar concentrations provide a suitable environment for the growth of xero and osmotolerant yeasts (Lievens et al. 2015). The T-103 tank provides the environment to isolate wild yeast, useful for fermentation at an industrial scale, given that those tanks store B molasses, which has a high sugar content (sucrose 57–59 % w/v) and low water activity (a_w<90) that increase hyperosmolar stress allowing only survival of strains having the potential to tolerate industrial conditions, as previously described (Ruyters et al. 2015).

Yeast strains are usually isolated from the environment using common yeast culture media containing chloramphenicol, which only inhibits the accompanying bacterial flora and do not discriminate strains suitable for use at the industrial level. Besides osmotolerance, ethanol tolerance, and productivity, tolerance to the toxic components of vinasse is one of the main characteristics of strains suitable for use in industrial scale ethanol production in Colombia. Lactic, acetic, and butyric acids are the main toxic components of vinasse. It has been shown that acetic and lactic acids can cross the cell membrane and then dissociate into cytoplasm due to higher internal pH, the excess of hydrogen ions causes an increase in ATP requirements to pump protons out of the cell, decreasing yeast biomass production by the negative effect on the metabolism and energy consumption (Ingledew et al. 2009).

The methodology accordingly included a solid medium in which was adjusted the chemical conditions that strains endure in fermentation tanks containing an aggressive substrate such as vinasse. The MVYS culture medium used in this study for isolation of yeast strains should provide stress conditions similar to those to which the organisms are exposed during the industrial process. In this case, 38 isolates were obtained with this medium. Based on the observed colony size (>4 mm), 18 isolates were obtained from the T-103 molasses storage tank (Fig. 1), being designated as LCC2-14 to LCC2-31. Most of the isolates (80 %) were characterized biochemically as *S. cerevisiae* using the API 20C AUX kit, showing specificity for the isolation of *S. cerevisiae* strains when MVYS culture medium was used as compared with previous studies in which CGA was used. Strains of the genus *Candida* with high vinasse tolerance but low ethanol production were isolated using CGA media (Mora 2008; Buzón 2008). Most of the strains isolated from the T-103 tank (61.54 %) presented molecular markers identical to those of the commercial strains used in distilleries. Four strains (30.8 %) had an interdelta molecular profile different from that of commercial strains and similar to LCC2-26 (Fig. 2). Given the chemical characteristics of sugar cane leaves and buds, such as high concentrations of volatile and nonvolatile organic acids this prompted the search for new isolates from this agroindustrial waste. Previous studies had indicated that, depending on the method of isolation, sugarcane can contain 12 different genera of basidiomycetes yeasts, seven genera of ascomycetous yeasts (De Azeredo et al. 1998), and up to 11 different kinds of molds (Ahmed et al. 2010). The development of a selective culture medium (MVYS) made it possible to isolate 20 vinasse-tolerant yeast colonies capable of growing in sugar concentrations as high as 150 g L^{-1}. Of these 20 isolates, 85 % were characterized by biochemical methods as *S. cerevisiae*, and only three strains showed a biochemical pattern corresponding to that of *Candida* sp.

Interspecific characterization by interdelta molecular fingerprinting and AFLP

Although some distilleries in Brazil start the fermentation process with pressed baker's yeast, during fermentation these

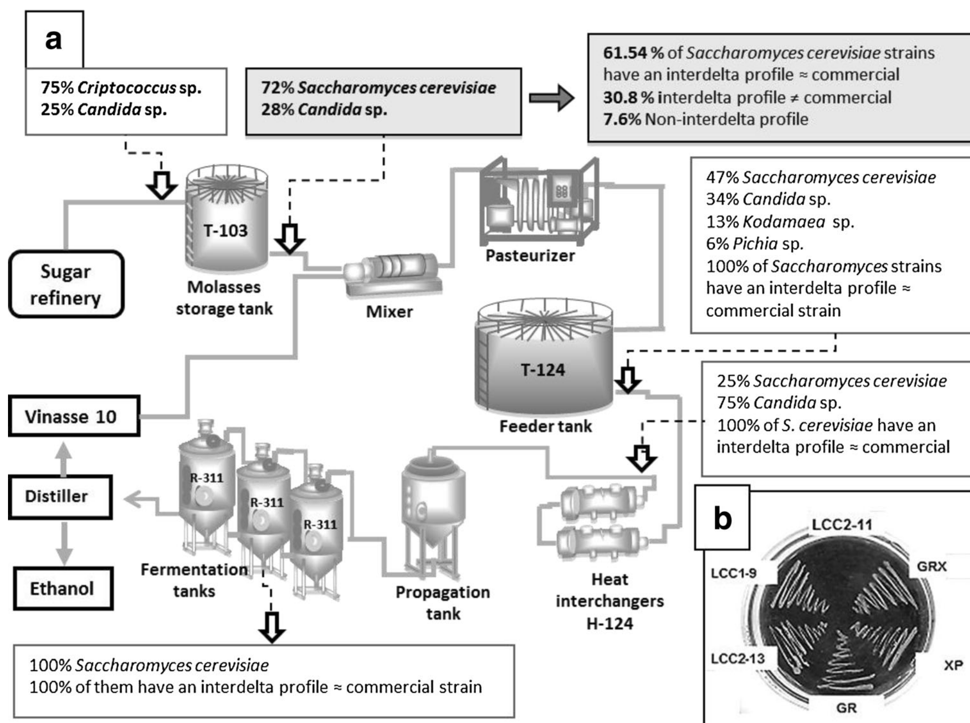

Fig. 1 **a** Sampling points of native strains. In this study, all strains were isolated from the T-103 molasses storage tank. Some microorganisms belonging to the yeast strain bank of Cenicaña's Factory Processes Program were also included as part of the characterization. These microorganisms had been isolated previously from the T-124 feeder tank, the H-124 heat interchanger, and the R-311 fermentation tank (Buzón 2008; Mora 2008). **b** wild type *Saccharomyces cerevisiae* strains LCC1-9 (T-124) and LCC2-13 (H-124) and commercial strains GRX, XP, GR, after 72 h growing in selective MVYS medium and vinasse-susceptible strain *Candida pelliculosa* LCC2-11 as a control

strains are replaced with wild yeasts that contaminate the process. In most distilleries, this contamination triggers a microbial succession of wild *S. cerevisiae* strains that are ultimately responsible for fermentation at an industrial scale (Basso et al. 2008). In Colombia, ethanol fermentation starts with a single commercial strain of *S. cerevisiae* GRX or XP in the propagation tank (Fig. 1a). However, after approximately 3 months of fermentation, both ethanol productivity and yield decrease. Although numerous studies have compared different molecular techniques, there is no consensus about how to differentiate, at the intraspecific level, those commercial *S. cerevisiae* strains that serve as starters from other wild strains contaminating the process through feedstock feeding lines or about how to identify those wild strains, which may be used in ethanol production.

The microbiology labs of wineries are currently using interdelta fingerprinting as a fast, reliable, and low-cost technique for routine intraspecific characterization of yeasts (Ness et al. 1993; Legras and Karst 2003). But even though this method has been used in several studies, there is no consensus about its widespread use among microbiology laboratories in distilleries. Although the reproducibility of the interdelta PCR characterization technique, using primers delta 1 and delta 2, was initially an issue of concern (Ness et al. 1993; Fernández-

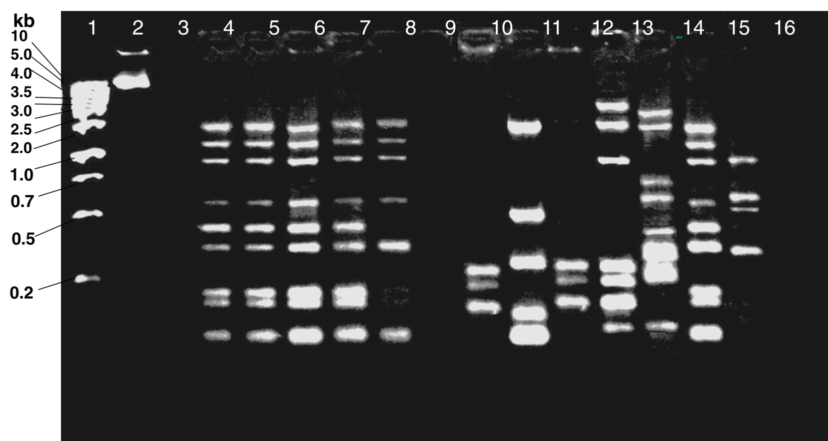

Fig. 2 PCR genetic fingerprinting of interdelta molecular profile 12–21 obtained from the genomic DNA of native yeasts isolated from tanks T-103 and T-124, where: lane 1 is the molecular weight marker; lane 2, phage λ; lane 3, LCC1-1; lane 4, LCC1-5; lane 5, LCC1-6; lane 6, LCC1-9; lane 7, LCC1-13; lane 8, LCC1-17; lane 9, LCC2-3 (*Candida colliculosa*); lane 10, LCC2-18; lane 11, LCC2-26; lane 12, LCC2-29; lane 13, GRX; lane 14, GR; lane 15, XP; lane 16, ethanol red; and lane 17, primers control

Espinar et al. 2001), with the standardization of the DNA concentration and the use of primers delta 12-delta 21 or delta 2-delta 12, this technique has become a reliable method such as electrophoretic karyotyping (pulsed-field electrophoresis) or mtDNA RFLPs (Legras and Karst 2003; Franco-Duarte et al. 2011). In this study, the amplification of the interdelta region indicated that the molecular pattern of 40.5 % of the isolates obtained from the T-103 tank coincided with the interdelta fingerprint of the commercial strain *S. cerevisiae* GRX while 21 % of the isolates matched the genetic fingerprint of the commercial strain *S. cerevisiae* XP (ten bands between 100 and 2000 bp). Four strains, approximately 30.8 %, showed a molecular pattern similar to that of LCC2-26 (Fig. 2), whereas only one strain characterized as *S. cerevisiae* (7 %) presented no interdelta pattern. Intraspecific characterization by genetic fingerprinting showed a clear polymorphism between several isolates (Fig. 2), which allowed them to be classified as seven different strains. Of the strains isolated from the juice of sugarcane leaves and buds, 17 were characterized as *S. cerevisiae* and showed an interdelta molecular pattern. Strains LCC0-3, LCC0-4, and LCC0-15 were characterized as *Candida* sp. and showed no interdelta molecular profile.

AFLP markers have been used for the intraspecific molecular characterization of *S. cerevisiae* strains for some time. De Barros-Lopes et al. (1999) used primer pairs EcoRI-C/MseI-AC to characterize 26*S. cerevisiae* strains of the yeast culture collection of the CBS-Knaw Fungal Biodiversity Centre in the Netherlands, while Gallego et al. (2005) determined that AFLP markers could detect the greatest number of polymorphic bands in a single assay. The purpose of using AFLPs in this study was just to corroborate the results of interdelta molecular markers. Hence, the combinations of primers used (EcoRI-AG/MseI-C and EcoRI-AC/Msel-C) showed high polymorphism, up to 53 bands per primer pair (106 in total) were visualized. Molecular weights ranged from 50 to 550 bp, and 85 bands (81.13 %) were polymorphic and showed an average heterozygosity of 0.2562 (Fig. 1 of supplementary information).

A simple hierarchical clustering, based on the overall similarity of available characters, was performed using the TFPGA program version 1.3 (Miller 1997). This clustering was then depicted in a dendrogram of genetic distances between commercial and wild ethanol-producing yeast strains isolated from both the molasses tank and sugarcane leaves and buds (Swofford and Olsen 1990) (Fig. 3). Based on cluster analysis results, six groups were identified. Group 1 comprised strains related to the commercial strain XP (LCC1-2, LCC1-13, and LCC1-17) with genetic distances ranging between 0.029 and 0.15. Group 2 was composed of commercial strain GRX and LCC2-13, with a genetic distance of 0.052. Group 3 was comprised of strain GR, presenting a genetic distance of 0.1825 regarding the GRX group. Group 4 was formed by strains LCC2-22 and LCC2-27, isolated from the T-103 molasses tank, with a genetic distance of 0.2754 regarding the group of GRX, XP, and GR. Strains LCC0-2 and LCC0-17, closely related to each other (genetic distance of 0.12) and isolated from sugarcane leaves and buds, formed Group 5 with the largest genetic distance (0.3295) from the former groups (Fig. 3).

According to Nei (1972), the genetic distance between a strain and its parental ranges between 0.02 and 0.15 so it is feasible to affirm that strains LCC1-2, LCC1-13, and LCC1-17 are related to strain XP and that strain LCC2-13 shares the same molecular markers with GRX. In addition, testing using AFLPs indicated that the genetic distance of strains LCC0-2 and LCC0-17 isolated from cane leaves and buds from all commercial strains is greater than 30.4 %, which agrees with the interdelta pattern observed (Fig. 3). Although AFLP analysis has a higher polymorphism, it was used in this study to confirm interdelta analysis results. As previously mentioned, the routine application of AFLP at the industrial level has so far faced certain limitations because it is expensive, inefficient, and time consuming (Fernández-Espinar et al. 2001).

Lab-scale fermentation using a culture medium containing B molasses and high vinasse concentration

Ethanol has been produced in Colombia since 2005, using B molasses as feedstock. However, when molasses is used as raw material for fermentation, the high soluble solids content and the osmotic pressure exerted on cells are major growth inhibitors (Ingledew et al. 2009). The stress caused by high osmotic pressure activates gene expression mechanisms for the production of glycerol, trehalose, and metabolites, which helps restore the inner balance of cells, so that its activities do not deteriorate. However, the energy demand of this defense mechanism of the cell competes for carbon sources, thus decreasing ethanol yield and productivity (Fernández-López et al. 2012).

Recent studies have focused on the factors that affect cell viability in the ethanol fermentation, analyzing the response of yeast strains to each stressor independently (Thammasittirong et al. 2013; Fakruddin et al. 2013); however, it has been demonstrated that inhibitors such as high acids, salts, and ethanol concentration act synergistically in industrial fermentations (Narendranath et al. 2001; Ingledew et al. 2009), particularly in Colombia, those factors act over yeast from the beginning of fermentation, given that vinasse is recycled from distillation to the fermentation tank continuously (Socarras Díaz et al. 2009), furthermore, it has been hypothesized that stress factors such as a high concentration of KCl, causes cell toxicity by cations excess rather than hyperosmotic stress (Mukherjee et al. 2014).

The above justified the use of a culture medium in which those stressors act synergistically to select wild yeast suitable for molasses fermentation under high acid, ethanol, and osmotic pressures. In this study the MVYS made it possible to

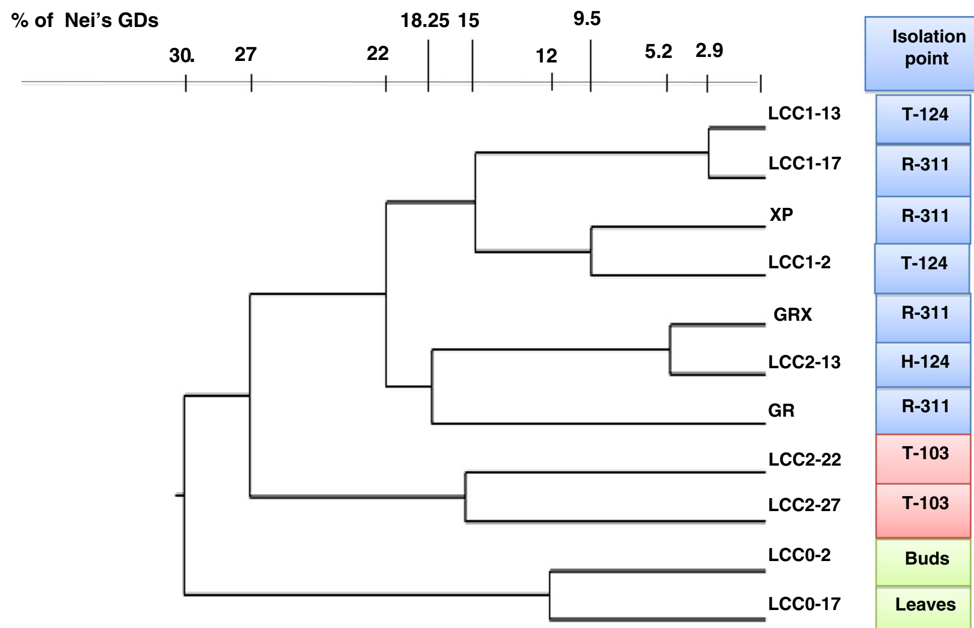

Fig. 3 Cluster analysis using UPGMA to determine intraspecific genetic similarities of wild type and commercial yeasts based on AFLP. Isolation point of each strain is showed in the front column, *Saccharomyces cerevisiae* LCC1-17, GRX, and XP were isolated from the fermentation tank (R-311), LCC1-2 and LCC1-13 were isolated from the feeder tank (T-124), LCC2-13 outside of the heat interchanger (H-124), LCC2-22 and LCC2-27 from the molasses storage tank (T103), and LCC0-2 and LCC0-17 from sugarcane buds and leaves, respectively

obtain a number of strains that tolerate the high organic acid concentration of vinasse as well as high osmotic pressures, reducing the time required to evaluate large numbers of yeast strains. In this study, the fermentation with wild yeasts started with 50–55 × 10^6 cells and, after 24 h, ethanol production ranged between 5.7 and 6.5 % (w/v), values very similar to those of the commercial strain (6.8 %, w/v) at lab scale (Table 1). The strain *S. cerevisiae* LCC2-27 presented an interdelta pattern different from that of the commercial yeast showing the highest ethanol production when industrial molasses-vinasse culture media was used (6.1 w/v). This yeast was isolated from the molasses storage tank T103 and presented an interdelta molecular pattern that coincided with that of strain LCC2-26 (Table 1).

Regarding reducing sugars consumption, a statistically significant difference ($P<0.05$) was observed among tested strains, ranging between 90 and 82 % total consumption. Sugar consumption was directly related to ethanol production in most yeast strains, except LCC1-17 (isolated from the fermentation tank), which presented a fermentable sugars consumption of 13.51 % (*w/v*). This strain also presented the lowest population and its ethanol production remained below 60 g L^{-1}, yielding 0.44 g ethanol g^{-1} glucose (Table 1).

Several authors have reported the isolation of wild yeasts from distilleries, which present a greater adaptation to musts that contain B molasses from sugarcane. Characteristics such as tolerance to high osmotic pressure and ethanol allow these strains to present yields close to 93 % and achieve ethanol productivities of 2.5 g L^{-1} h^{-1} (Pereira et al. 2011). In the present study, yeast strains LCC2-22 and LCC2-27 isolated from the T-103 molasses storage tank showed higher yields (Yp/s), productivities (Qp), and efficiencies (E) than strains

Table 1 Fermentable sugars consumed, ethanol production, yield, efficiency, and productivity of wild *Saccharomyces cerevisiae* strains isolated from a distillery environment and sugarcane leaves and buds using B molasses (15 % TRS) and vinasse (50 %) as substrate after 24 h of fermentation

Strain	Isolating point	Culture media for isolating	Interdelta pattern	Sugar consumed	Ethanol % *w/v*	Yield Yp/s	Productivity Qp	Efficiency E %
GRX	Commercial	–	GRX	13.15	6.82	0.51	2.84	85
LCC1-2	T-124	CGA	GRX	12.69	5.85	0.51	2.43	73
LCC1-13	T-124	CGA	GRX	12.76	5.70	0.45	2.37	71
LCC1-17	R-311	CGA	XP	13.51	5.9	0.44	2.47	74
LCC2-13	H-124	CGA	GRX	12.82	6.6	0.51	2.72	83
LCC2-22	T-103	MVYS	LCC2-22	12.66	5.9	0.51	2.46	73
LCC2-27	T-103	MVYS	LCC2-22	12.86	6.0	0.47	2.51	75
LCC0-2	Buds	MVYS	LCC0-2	12.77	5.9	0.46	2.46	73
LCC0-17	Leaves	MVYS	LCC0-17	12.36	5.9	0.48	2.45	73

LCC1-2 and LCC1-13 isolated from the T-124 feeder tank, and strain LCC1-17 isolated from the R-311 fermenter using CGA as selective media. The former two presented Yp/s values of 0.51 and 0.47 g g^{-1}; Qp values of 2.46 and 2.51 g L^{-1} h^{-1}; and efficiencies of 73 and 75 %, respectively. The latter three presented Yp/s values ranging from 0.44 to 0.46 g g^{-1}; Qp values from 2.37 to 2.47 g L^{-1} h^{-1}; and efficiencies from 71 to 74 %. Strains LCC0-2 and LCC0-17, isolated from sugarcane leaves and buds, presented the following parameters: Yp/s, 0.46 and 0.48 g g^{-1}; Qp, 2.46 and 2.45 g L^{-1} h^{-1}; and efficiencies, 73.4 and 73.2 % (Table 1). Based on the foregoing, the molasses storage tank is a potential source of vinasse-tolerant wild yeasts that could be used on an industrial scale in distilleries.

In general, strains isolated using MVYS media from the T-103 molasses tank and sugarcane leaves and buds presented higher ethanol productivity and efficiency than wild strains isolated from the R-311 fermentation tanks. The volumetric ethanol productivity of strain LCC2-27 was similar to that previously reported for Brazilian strain PE-2 (2.50 g L^{-1} h^{-1}), also isolated from distillery environments. The efficiency reported for this strain (86 %) is considerably higher than that reported in the present study (78.5 %) (Pereira et al. 2010). Two aspects, however, should be emphasized: first, fermentations with strain PE-2 were performed under very different conditions of sugar concentration and inoculum size and, second, these strains have been submitted to adaptations in the same fermentation tank for over 15 years (Basso et al. 2008).

Although the fermentations with strains PE-2, CA1185, CAT-1, and JP-1 were performed without the addition of vinasse due to diametral differences in the processes of ethanol production in Brazil and Colombia (Pereira et al. 2010, 2011), it should be highlighted that microbial research carried out in Brazil in the field of population dynamics of alcohol-producing yeasts has allowed the process to achieve a higher level of technification, which enables the selection of strains with yields higher than 90 %, and the capacity to introduce and adapt those strains to different environmental conditions (Della-Bianca et al. 2013). However, the high volume of vinasse (13 L) produced per volume of ethanol evidences the need not only to recycle this byproduct, but to isolate vinasse-tolerant microorganisms to make this process much more environment-friendly. This study may serve as a reference for those distilleries that are planning to include the recycling of vinasse in the fermentation stage as it offers a quick method for isolating yeast strains suitable for industrial processes.

Conclusions

The design of isolation and characterization schemes of wild yeasts adapted to fermentation conditions such as those presented in this study offers a fast and specific way to identify yeast strains that are better adapted to industrial processes and can optimize fermentation. The culture medium used is as important as the whole method of isolation and should provide stress conditions as high as those to which the organism is submitted during industrial processes. In this study, 38 isolates were obtained with the MVYS culture medium. Between these, strains LCC2-22, LCC2-27, LCC0-2, and LCC0-17 were preselected for their high alcohol potential, with productivities ranging between 2.4 and 2.5 g L^{-1} h^{-1} and yields above 0.45 g g^{-1}, which are considered high enough for fermentations in culture media with high organic acid content such as vinasse. Of all possible sources of microorganisms in the distillery environment, the molasses storage tank was the ideal place to isolate suitable wild yeast strains due to an aggressive environment that enables yeast to adapt for higher tolerance to osmotic and acidic stress, which explains higher ethanol productivity using molasses and vinasse as substrates.

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Conflict of interest The authors declare that they have no conflict of interest.

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