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# Isolation and characterization of *Saccharomyces* species for bioethanol production from sugarcane molasses: Studies of scale up in bioreactor

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ABSTRACT

Decreases in oil reserves and gas fields around all over the world justify the deepening of studies to render viable the larger-scale use of new energy sources. Therefore, the use of microorganisms to convert sugars into ethanol is a feasible process to be performed in a short period of time and at low costs. In this context, this study aimed to select ethanol-producing yeasts, after isolating samples in molasses obtained from companies in the Province of Tucumán (Argentina) and grapes obtained from farms located in Cafayate (Salta, Argentina). Among the twenty-nine samples studied A2, A10 and A11 isolates showed higher ethanol productions of 12.87; 13.64 and 13.46% respectively. A2 showed a homogeneous growth meanwhile the growth of strains A10 and A11 was flocculent. Molecular taxonomic characterization of these isolates showed a percentage of similarity of 100% with the strain *Saccharomyces cerevisiae*. The behavior of the non-flocculent A2 strain at laboratory scale was faster using a sugarcane molasses based medium, reaching 11.36% ethanol without adding nutrients and other growth factors, probably because its disperse form facilitates the transfer of nutrients and products. These values were improved to 12.02% when the process was scaled up to a 10L bioreactor. All these studies allowed concluding that *S. cerevisiae* A2 strain is a promising microorganism for the production of bioethanol with potential environmental, energy and economic benefits to be projected into industrial scale.



## 1. Introduction

Biofuels are fuels of biological origin, derived from renewable organic biomass, where biomass represents a potential source of carbohydrates for microbial fermentation [1]. Interest in its use has increased to the extent that governments seek to reduce and even eliminate dependence on fossil fuels, to ensure future, greater energy security, while benefiting the environment. Considering that oil accounts for 97% of the energy used for transport and industry [2], governments around the world have actively promoted the identification, development and commercialization of technologies for the production of alternative fuels in the last 20 years [3], including the production of ethanol [4].

Brazil was the first country to promote a program in order to replace some of the gasoline with ethanol. This program, called National Alcohol Program (PROALCOOL) [5], allowed encouraging ethanol production and consumption [6]. The momentum for bioethanol in Argentina, from sugarcane was shaped legally with the sanction of Law 26.334 "*Arrangements for the Promotion of Bioethanol Production*" in 2008. The objective of ensuring sustainability in the production of ethanol is enough stimulus in the search for alternative technologies that would satisfy or refine the existing ones.

Molasses are one of the main products obtained from the manufacture of raw sugars in the sugar industry. Molasses are widely used as raw material for alcoholic fermentation due to its abundance and low price compared to other available raw materials. Molasses are a complex and heterogeneous mixture and its composition may vary considerably depending, among others, on the sugarcane variety, soil, climate, culture period, production

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process and efficiency of operation in the factory [7].

*Saccharomyces cerevisiae* is the main species of yeast employed for ethanol production at industrial level since this microorganism is easy to handle, shows no high nutritional needs, can produce ethanol concentrations above 15% [8], tolerates high concentrations of sugars, is not expensive, produces low levels of by-products, is osmotolerant and presents high viability for recycling [9].

Programs for isolation and selection of yeast strains have had positive results, due to higher yields of fermentation. The decrease in glycerol production and foaming resulted in higher levels of ethanol production and the consequent reduction in production costs. The rich biodiversity of yeasts found in environments of ethanol plants production could be an important source of new strains. This is due to, among other factors, the selective pressure on the cells which occurs during the recycling of yeast cells generates strains with increased tolerance to stress conditions in industrial fermentation: high concentrations of ethanol, sugar and $CO_2$ pressure and low $O_2$ pressure and low pH [10].

This paper deals with the isolation of yeasts from molasses and grapes, and further evaluation of bioethanol production in commercial media. Selected strains were taxonomically characterized and assayed for optimizing some operational variables and the use of sugarcane molasses as economic fermentative medium. Finally, the no flocculent A2 strain was employed to scale up the process aiming to achieve an industrial application.

## 2. Material and methods

### 2.1. Isolation of yeasts from sugarcane molasses and grapes

Two culture media were employed in the present study: YPS proliferation medium (yeast extract 10 g/L, peptone 10 g/L and sucrose 50 g/L) for reactivation and propagation of yeasts; and YPS fermentation medium (yeast extract 10 g/L, peptone 10 g/L and sucrose 250 g/L) for fermentation [11]. Additionally, sugarcane molasses was evaluated after collecting sugarcane molasses from local sugarcane mills (Tucumán, Argentina) and diluted to achieve 25% of total reducing sugars (TRS). All these media were sterilized in autoclave at 121 °C during 15 min. Agarized YPS proliferation medium, used to isolate yeasts, was prepared with YPS proliferation medium and 15 g/L agar.

Twenty-nine strains isolated from sugarcane molasses and grapes were assayed for their ability to produce ethanol. Yeast samples were aseptically collected from local sugarcane mills (Tucumán, Argentina) and vineyards located in Salta (Argentina), plated individually on YPS agar supplemented with antibiotics (ampicillin 20 g/L, tetracycline 10 g/L, chloramphenicol 20 g/L, and eritromicin 20 g/L) to suppress bacterial contaminants [10], serially diluted, plated and growth at 30 °C for 24 h. Isolated colonies were dispensed into fresh medium containing 40% (v/v) glycerol as cryoprotectant, and maintained at −20 °C for further assays.

### 2.2. Preparation of inocula and fermentation experiments

Single colonies of isolates were grown overnight in 50 mL YPS proliferation media placed in 200 mL bottles. Cultures were performed in thermostatic baths (model G-76, New Brunswick Scientific Co., Edison, NJ, USA) at 30 °C and 200 rpm.

The ability to produce ethanol was assayed by triplicate after inoculation of 50 mL YPS fermentation medium with 0.50 g/L biomass in 200 mL bottles. Fermentations were assayed during 144 h in oven at 30 °C.

Fermentation trials to study the influence of temperature on three selected strains (A2, A10 and A11) were carried out by triplicate after inoculation of 50 mL YPS fermentation medium with 0.50 g/L biomass. Fermentations were performed during 144 h in 200 mL bottles placed in oven at 25, 28, 30 and 35 °C.

Studies for the selection of initial sucrose concentration were performed on the selected strains during 120 h by triplicate in 200 mL bottles containing 50 mL YPS fermentation medium, changing the initial amount of sucrose to 250, 300, or 350 g/L. Flasks were inoculated with 0.5 g/L of biomass and incubated in oven at 30 °C.

Sugarcane molasses based medium was evaluated as economic culture medium for the development of A2 and A10 strains without supplementation. Fermentations were conducted by triplicate in 200 mL bottles, inoculated with 2% dry matter and incubated in oven at 30 °C.

In all cases, samples were taken at selected times. For each sample the whole volume of one flask was centrifuged and the supernatant used to analyze total reducing sugars (TRS), direct reducing sugars (DRS), and ethanol concentration. The yeast growth was evaluated by dry weight [12]. The supernatant was separated and stored for determination of sugars using the volumetric method of Fehling Causse-Bonnans (FCB) [13,14] and ethanol by Rezex Organic Acid HPLC with precolumn, mobile phase 10 mM $H_2SO_4$, flow rate 0.6 mL/min, 55 °C, Gilson 305 pump, detector LKB Model 2142, differential refractometer, and recorder/integrator Shimadzu CR3A.

### 2.3. Use of the yeast S. cerevisiae A2 to scale up the process in a 10L bioreactor

The proliferation of the yeast *S. cerevisiae* A2, isolated from sugarcane molasses was carried out in a 10 L Bioreactor Model N°: MF-214 (New Brunswick Scientific CO. INC., Edison: New Jersey, USA). The bioreactor was inoculated with 250 mL of *S. cerevisiae* A2 suspension ($1.14 \times 10^4$ cel/mL), obtained after 12 h incubation in a thermostatic bath at 30 °C, 150 rpm, and 2.5 vvm oxygen. In order to maximize the proliferation of biomass and minimize the formation of ethanol, the process was conducted using a fed-batch process, where the sucrose present in the YPS proliferation medium was added intermittently, starting with 20 g/L inside the reactor and adding three consecutive pulses of 10 g/L after 4, 8 and 12 h.

Fermentation was conducted using 8L culture medium in the same 10 L Bioreactor without mechanical stirring and aeration, at 30 °C and pH 5.0. The fermentation medium was prepared using sugarcane molasses and 10% of sterilized cane juice, to reach 25% (w/v) TRS. The inoculum was originated from 1L of concentrated *S. cerevisiae* A2 suspension ($2.79 \times 10^{10}$ cel/mL) previously obtained from the proliferation process.

Determination of cell growth was performed by optical density at 640 nm using a UV–Visible spectrophotometer: Zeltec, model ZL-5000 plus and cell counting in a Neubauer chamber [15]. To determine the concentration of ethanol, 50 mL of the fermented medium was distilled and then quantified by HPLC as previously described. TRS were determined using the volumetric method of FCB [14].

### 2.4. Characterization and molecular taxonomy

The selected isolates were subcultured on YPS fermentation medium for 24 h at 30 °C. 1500 μL were transferred into micro centrifuge tubes and the cells were recovered by centrifugation at 10,000 × g for 2 min. The supernatant was discarded and the DNA extraction was realized following the methodology proposed by Yamada et al. [16]. The D1/D2 domain of 26S rDNA region was amplified using the primers NL1 (5′-GCATATCAA-TAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-

**Table 1**
Origin of isolates, aspect of the colonies, and ethanol concentration produced after 144 h for isolates grown in YPS fermentation medium. Results are the media of three fermentations and standard deviations are provided.

| Isolate | Origin | Appearance of the colony | Ethanol (%) |
|---|---|---|---|
| C1 | Grapes-Cafayate | Small, circular, brown | 3.20 ± 0.21 |
| C2 | Grapes-Cafayate | Small, circular, brown | 5.66 ± 0.32 |
| C3 | Grapes-Cafayate | Small, circular, brown | 4.42 ± 0.22 |
| C4 | Grapes-Cafayate | Small, circular, brown | 3.21 ± 0.14 |
| C5 | Grapes-Cafayate | Small, circular, brown | 3.11 ± 0.12 |
| Q1 | Grapes-Cafayate | Big, round, buttery, white | 5.86 ± 0.28 |
| A1 | Molasses | Medium, round, buttery yellow | 11.2 ± 0.74 |
| A2 | Molasses | Medium, round, buttery yellow | 12.87 ± 0.83 |
| A4 | Molasses | Medium, round, buttery yellow | 8.45 ± 0.41 |
| A5 | Molasses | Medium, round, buttery yellow | 5.71 ± 0.28 |
| A9 | Molasses | Medium, round, buttery yellow | 11.87 ± 0.68 |
| A10 | Molasses | Medium, round, buttery yellow | 13.20 ± 0.81 |
| A11 | Molasses | Medium, round, buttery yellow | 13.20 ± 0.88 |
| J1 | Molasses | Big, round, creamy | 5.00 ± 0.29 |
| J3 | Molasses | Small, yellowish | 2.10 ± 0.14 |
| J6 | Molasses | Small, yellowish | 4.70 ± 0.18 |
| J7 | Molasses | Big, round, creamy | 4.26 ± 0.17 |
| J8 | Molasses | Small, yellowish | 2.10 ± 0.02 |
| J9 | Molasses | Big, round, creamy | 1.50 ± 0.2 |
| J10 | Molasses | Medium, round, buttery yellow | 0.70 ± 0.02 |
| J11 | Molasses | Medium, round, buttery yellow | 3.51 ± 0.14 |
| J13 | Molasses | Big, round, creamy | 1.50 ± 0.10 |
| J14 | Molasses | Big, round, creamy | 1.86 ± 0.13 |
| YN1 | Grapes-North Yacochuya | Big, round, creamy | 5.87 ± 0.21 |
| YN2 | Grapes-North Yacochuya | Big, round, creamy | 6.40 ± 0.22 |
| YS1 | Grapes-South Yacochuya | Big, round, creamy | 7.66 ± 0.24 |
| YS2 | Grapes-South Yacochuya | Big, round, creamy | 4.33 ± 0.15 |
| T | Grapes-Tolombón | Medium, round, white | 1.60 ± 0.13 |
| AN | Grapes-Animaná | Big, yellow | 2.40 ± 0.16 |

3′) [17,18]. The amplification was carried out by PCR under the following conditions: initial denaturation at 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min; final extension at 72 °C for 10 min. Amplified products were separated in agarose gel (1.0% wt/vol) stained with ethidium bromide and visualized under UV illumination.

The PCR fragments (550 bp) were sequenced by Macrogen (Korea) in an ABI prism 373A (PE Applied BioSystems) automated sequencer. The sequences obtained were aligned with 26S rDNA sequences of databases present in the NCBI (National Center for Biotechnology Information) using the software MEGA version 4.0 [19]. Phylogenetic trees were constructed with MEGA 4.0 using a Neighbor-Joining algorithm [20] and bootstrap analyses for 1000 replicates were performed.

### 2.5. Scanning electron microscopy

Scanning electron microscopy was carried out following the method described by Karnovsky [21] under high vacuum using a microscope Zeiss Supra 55VP (Carl Zeiss, Oberkochen, Germany). Samples were processed and observed in the Centro Integral de Microscopía Electrónica (CIME), CCT-CONICET-UNT, San Miguel de Tucumán (Argentina).

### 2.6. Statistical analysis

Determinations of total reducing sugars, ethanol and biomass concentrations were carried out in triplicate, and the results expressed as mean values. The mean values were submitted to analysis of variance (ANOVA) by the Statistica Software 12. They were compared using the Tukey's test at significance level ($P$) < 0.05, and different letters were used to label values with statistically significant differences among them.

## 3. Results and discussion

### 3.1. Isolation and selection of yeasts

The ability to grow and produced ethanol was evaluated in 29 yeast strains isolated from both sugarcane molasses (17 strains) and grapes (12 strains) using YPS fermentation media, starting with an initial inoculum concentration of 0.5 g/L. Table 1 shows the origin of isolates, appearance of colonies, and the final ethanol concentration. Results showed that 13 strains produced reasonable amounts of ethanol oscillating between 5 and 13%. The most outstanding results were achieved with strains A2 (12.87%), A10 (13.20%) and A11 (13.20%); all of them isolated from sugarcane molasses. Fig. 1

**Table 2**
Kinetic and stoichiometric parameters after 144 h fermentation, during the growth of *Saccharomyces cerevisiae* strains A2, A10 and A11 in YPS fermentation medium at 30 °C. Results are the media of three fermentations and standard deviations are provided. Different letters in the same column mean statistically significant difference according to the test of Tukey ($P < 0.05$).

| Strain | $TRS_0$ (g/L) | $TRS_f$ (g/L) | $Q_S$ (g/L·h) | $P_0$ (g/L) | $P_{max}$ (g/L) | $Q_P$ (g/L·h) | $Y_{P/S}$ (g/g) | $X_0$ (g/L) | $X$ (g/L) | $Q_X$ (g/L·h) | $Y_{X/S}$ (g/g) | $q_S$ (g/g·h) | $q_P$ (g/g·h) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A2 | 250 | 16.5 ± 0.13$^a$ | 1.622 | 0 | 128.7 ± 0.80$^b$ | 0.894 | 0.55 | 1.5 | 4.4 ± 0.09$^a$ | 0.0199 | 0.012 | 0.565 | 0.311 |
| A10 | 250 | 25.5 ± 0.11$^b$ | 1.559 | 0 | 132 ± 0.78$^a$ | 0.917 | 0.59 | 1.5 | 4.7 ± 0.11$^b$ | 0.0223 | 0.014 | 0.486 | 0.286 |
| A11 | 250 | 31.5 ± 0.18$^c$ | 1.517 | 0 | 132 ± 0.83$^a$ | 0.917 | 0.60 | 1.5 | 4.3 ± 0.12$^a$ | 0.0194 | 0.013 | 0.542 | 0.327 |

$TRS_0$ = initial concentration of TRS; $TRS_f$ = TRS concentration at the end of fermentation; $Q_S$ = volumetric rate of TRS consumption; $P_0$ = initial ethanol concentration; $P_{max}$ = ethanol concentration at the end of fermentation; $Q_P$ = global volumetric productivity; $Y_{P/S}$ = product yield; $X_0$ = initial biomass concentration; $X$ = biomass concentration at the end of fermentation; $Q_X$ = biomass volumetric productivity; $Y_{X/S}$ = biomass yield; $q_S$ = specific volumetric rate of TRS consumption; $q_P$ = specific ethanol productivity.

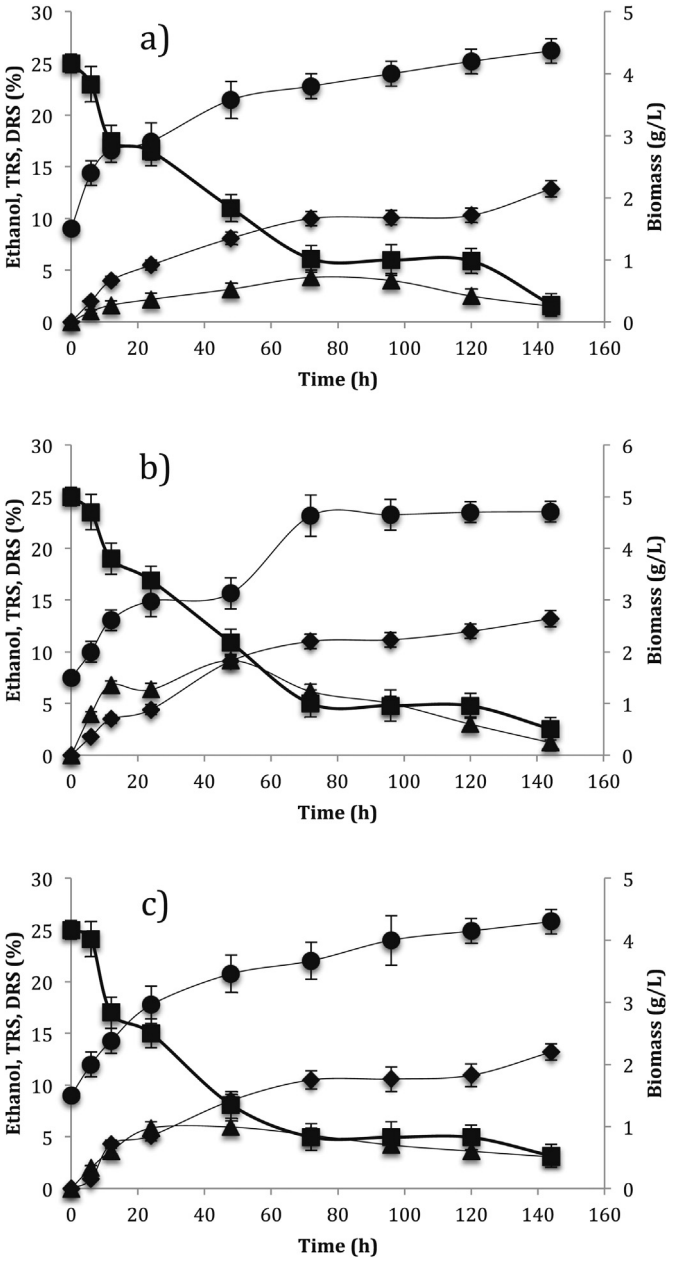


**Fig. 1.** Course with time during the growth of *Saccharomyces cerevisiae* strains a) A2, b) A10 and c) A11 in YPS fermentation medium at 30 °C. TRS (■); Ethanol (◆); DRS (▲); Biomass (●). Results are the media of three fermentations and bars represent mean ± standard deviation.

shows the course with time for total reducing sugars (TRS), direct reducing sugars (DRS), ethanol concentration and biomass. The tendency was similar in the three strains, although the final ethanol concentration was slightly higher in strains A10 and A11. Table 2 collects the kinetic and stoichiometric parameters of fermentation. A2 strain presented higher values of volumetric rate of TRS consumption (1.622 g/L·h) and specific volumetric rate of TRS consumption (0.565 g/g·h), meanwhile A10 and A11 strains showed higher global volumetric productivities (0.917 g/L·h). Conversely, the three strains showed a narrow interval of biomass yield (0.012–0.014 g/L h). These three strains were isolated from sugarcane with high levels of sugars. Chandel et al. [22] used *S. cerevisiae* 174 producing up to 8.13 g/l of ethanol after 72 h fermentation, meanwhile Martin et al. [23] observed a maximum ethanol concentration of 7.4 g/L using *S. cerevisiae* ATCC 96581, and Chandel et al. [24] achieved an ethanol production of 19.45 ± 0.55 g/L from natural *S. cerevisiae* VS3. Previously, Díaz Montaño et al. [25] studied the fermentative capability of five yeast strains isolated from *Agave tequilana* Weber juice, founding that three strains identified as *S. cerevisiae* were able to produce 5.18 ± 0.15% ethanol after 24 h, showing higher tolerance to ethanol than the other two strains, which were classified as *Kloeckera africana* and *K. apiculata*, which showed a poor growth and produced less than 2.90 ± 0.20% ethanol.

The results of Fig. 1 also pointed out that the hydrolysis of sucrose was successfully performed during the whole trial, and that both glucose and fructose were not found in limiting concentrations. Additionally, the hydrolysis of sucrose was not inhibited by the presence of ethanol. Furthermore, the concentration of TRS was similar to the amount of DRS after 144 h, for the three strains, due to most of the sucrose had been hydrolyzed. Finally, it can also be observed the amount of biomass, which was similar for the three strains thought the fermentation; being noticeable that the three strains continued to develop even when the ethanol concentration had exceeded 10%.

### 3.2. Morphologic and growth characteristics of the selected microorganisms

The selected yeasts after growing on agarized YPS proliferation medium presented white-creamy colonies. When strains were developed in liquid medium the strain A2 showed a homogeneous and dispersed growth, whereas the growth of the strains A10 and A11 was flocculent. The biomass was associated forming clusters or 1 mm diameter flocs, being deposited at the bottom of the culture flasks at the end of fermentation. The three strains were observed by optical microscopy, showing the 3 isolates an ovoid shape, which is characteristic in yeasts. It was also observed cell division by gemmation and the absence of pseudomycelia (Fig. 2).

Furthermore, no significant differences were observed in the

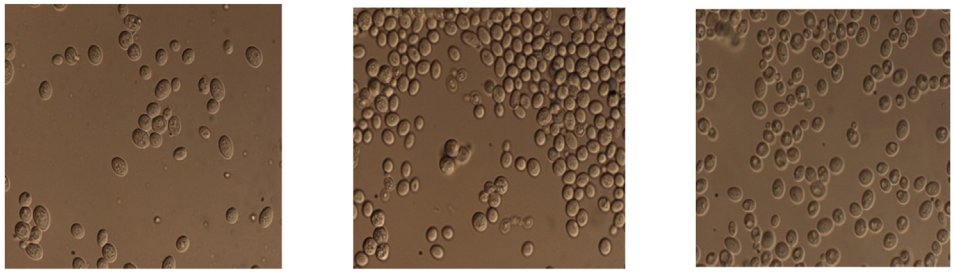


**Fig. 2.** Optical microscopy (1000 ×magnification) of isolated yeasts: a) A2, b) A10 and c) A11.

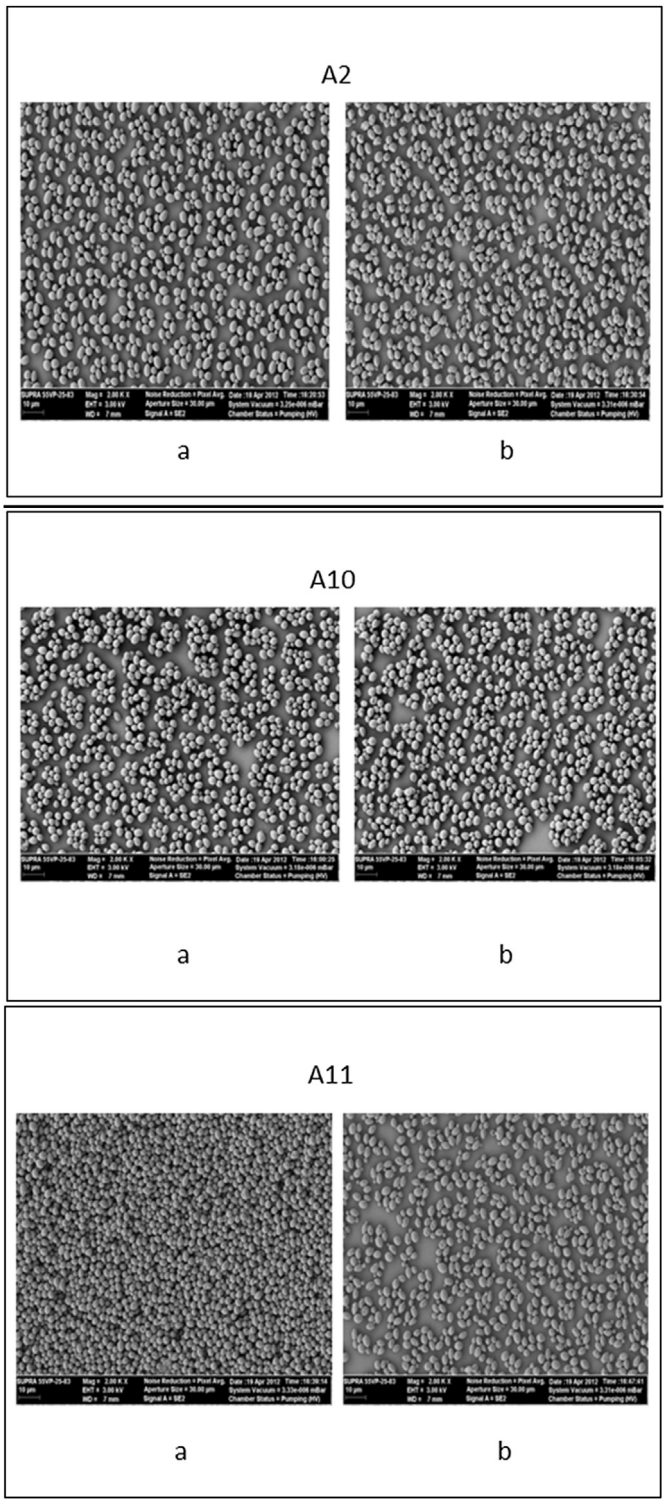


**Fig. 3.** Comparison of yeast strains A2, A10 and A11 before (a) and after (b) 24 h fermentation at 30 °C using a Scanning Electron Microscope (2000× magnification).

surface characteristics of A2, A10, and A11 strains before and after fermentation in the images obtained by scanning electron microscopy (Fig. 3) with 2000× magnification, although yeast cells exhibit a slight increase in surface roughness after fermentation, possibly due to the transport of substances across the plasma membrane. Ma et al. [26] studied the effect of different concentrations of ethanol on a *S. cerevisiae* strain, concluding that concentrations from 7% alcohol produced a marked reduction of cell size and a deformation of the plasma membrane. These results would suggest that the selected yeasts were not subjected to oxidative stress, maintaining its shape and size, with the possibility of using them in cell recycles maintaining reliability of viable strains for the production of bioethanol.

### 3.3. Identification of selected microorganisms: molecular characterization and taxonomy

Selected microorganisms A2, A10 and A11 were identified by sequencing the 26S rDNA D1/D2 domain and compared with sequence of type strains from the database of the NCBI (accession number indicated in Fig. 4). Alignment results of the rDNA sequences of these isolates show that the sequences of strain A2 was found to have 100% similarity with *S. cerevisiae* strain 810 GDB (EF554822). Strain A10 was shown to have 100% similarity *S. cerevisiae* strain CBS 1907 (AJ508581). Finally, A11 strain showed 99% similarity with *S. cerevisiae* strain CBS 1907 (AJ508581) and 99% of identity with *S. cerevisiae* strain 810 GDB (EF554822). To confirm the position of each strain in phylogeny, a number of sequences were selected from the NCBI database for the construction of a phylogenetic tree using the MEGA4 program. As shown in Fig. 4 the isolates A2, A10 and A11 share the same clade cluster of the phylogenetic tree of D1/D2 26S rDNA sequences corroborating all they belong to the genus *Saccharomyces*.

### 3.4. Influence of temperature

The behavior of the strains A2, A10, and A11 at different temperatures (see Fig. 5) showed that although high concentrations of ethanol were reached at 25 and 28 °C, the highest concentration was achieved when incubation was carried out at 30 °C, meanwhile at 35 °C the strains produced lower ethanol concentrations. These results showed that strains were highly sensitive to changes in temperature being 30 °C the optimal temperature for the production of ethanol for the three strains. These results are in agreement with the studies reported by Zabed et al. [27] who postulated that it is generally believed that the ideal fermentation temperature range is between 20 and 35 °C, meanwhile certain problems derived from the use of higher temperatures in almost all fermentation processes. Using other strains, for instance in the case of *Zymomonas mobilis*, the best ethanol concentration (55.57 g/L) was found at 30 °C, while the lowest (4.6 g/L) was found at 40 °C. Similarly, harmful effect on ethanol concentration using this microorganism was also observed at above 37 °C by several investigators. For instance, Lee et al. [28]

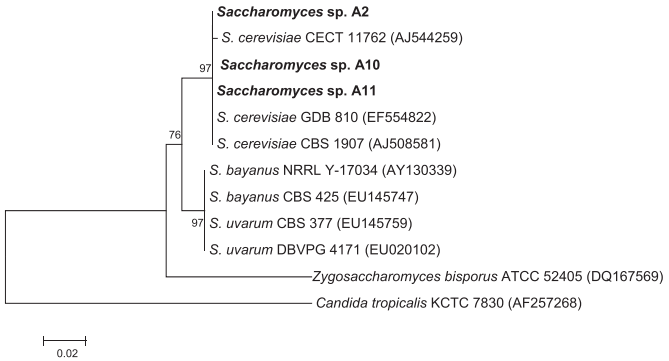


**Fig. 4.** Phylogenetic tree obtained by the Neighbor-Joining method, based on the analysis of 26S rDNA of the isolates A2, 10 and 11. The strains were identified within the genus *Saccharomyces*. Access numbers to the type strains are in parentheses.

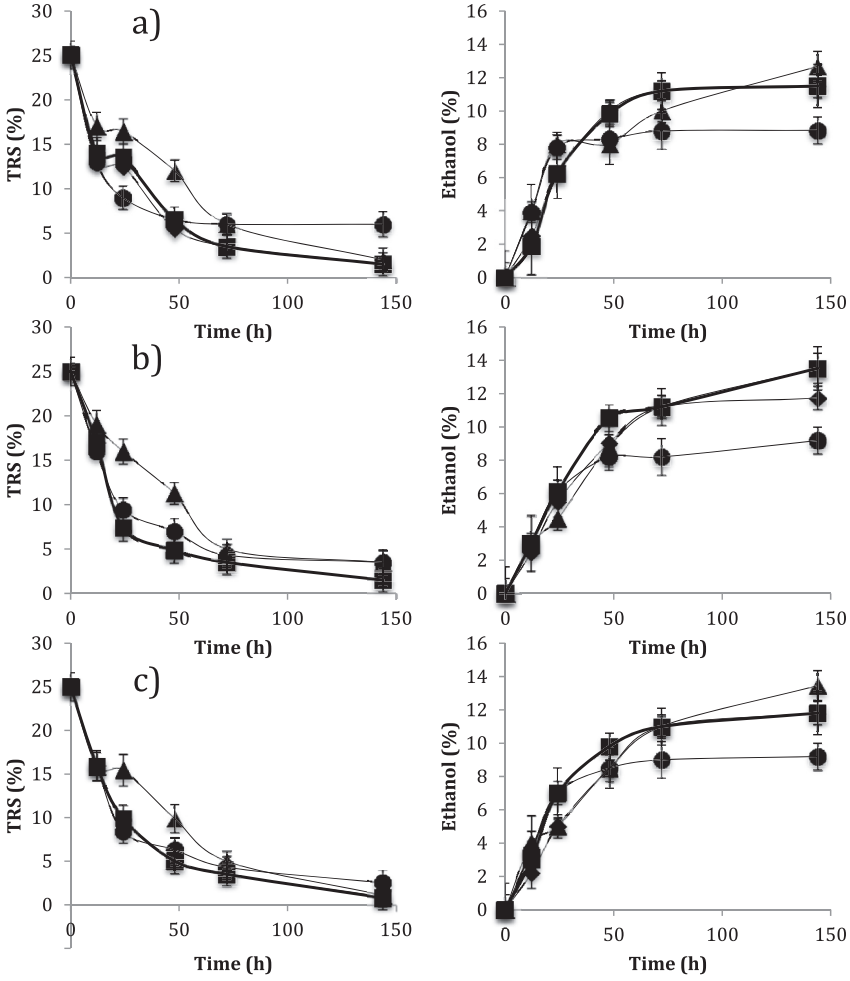


**Fig. 5.** Influence of temperature using isolates strains A2 (a), A10 (b) and A11 (c) grown in YPS fermentation medium starting 250 g/L sucrose and 0.5 g/L d.w. biomass. 25 °C (◆); 28 °C (■); 30 °C (▲); 35 °C (●). Results are the media of three fermentations and bars represent mean ± standard deviation.

studying the strain *S. cerevisiae* BY4742 at 35 and 40 °C concluded that a difference of 5 °C can affect the production of ethanol by yeasts, since ethanol production decreased from 28.98 to 5.52 g/L with increasing temperature from 35 to 40 °C.

### 3.5. Influence of the initial sucrose concentration

Due to the low levels of residual TRS at the end of fermentation during the previous study, higher initial sugar levels were tested to avoid the possibility of limiting the generation of higher concentrations of alcohol. Thus, strains A2, A10 and A11 were evaluated by triplicate during 120 h in YPS fermentation medium containing 250, 300 and 350 g/L sucrose, using 0.5 g/L inoculum and 30 °C. The profiles of ethanol production (see Fig. 6) using 250 and 300 g/L of initial sucrose were very similar using the strain A2, with a maximum final ethanol concentration of 11.61% at 120 h. This concentration was higher compared to that produced using 350 g/L of initial sucrose, where a decrease of 20% was evident in the final ethanol concentration, reaching a maximum amount of 9.70%. Identically, with the strains A10 and A11, using 250 and 300 g/L of sugar, the similar final ethanol concentration of 12.49% and 12.25% were quantified respectively, meanwhile raising sucrose concentration to 350 g/L, the final ethanol production reached 11,7% (with A10 strain) and 11.27% (with A11 strain) evidencing a decrease of around 10%. This decrease in the production of ethanol could be probably caused by the high concentration of initial sugar (substrate inhibition) and possibly by osmotic pressure.

Notably, the higher decrease was observed with the non-flocculent strain A2. Considering that the flocculation of yeasts is an asexual process reversible and calcium dependent, wherein the cells are adhered together to form flocs [29], and that *S. cerevisiae* is mediated by specific proteins of the cell surface; it can be said that flocculation can act as a protective mechanism in yeast stress conditions, such as acidification, high concentration of sugars, etc. [30]. For these reasons, the results do assume that the flocculent characteristics of strains A10 and A11 would protect a high osmotic pressure generated by high sugar concentration and decreased production of ethanol is lower compared with non-flocculent strain A2.

Similar results were observed by Lee et al. (2013) who testing the strain *S. cerevisiae* NK28 at different initial concentrations of sucrose, obtained a maximum ethanol concentration of 85.56 g/L with 200 g/L sucrose, but when the sugar concentration increased to 300 and 400 g/L, ethanol concentration decreased considerably to 46.58 and 1.11 g/L respectively. In our case, although *S. cerevisiae* strains A2, A10, and A11 tolerated up to 300 g/L without decreasing its production of ethanol, it can be deduced from the above results that the concentration that ensures high concentrations of alcohol

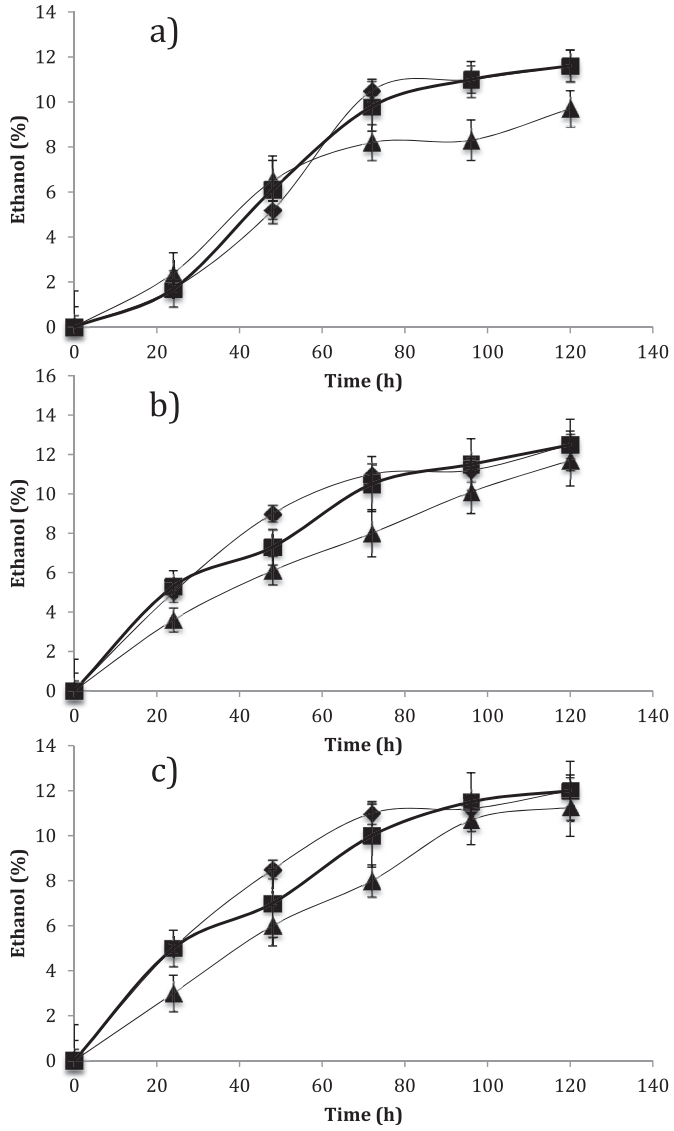


**Fig. 6.** Influence of initial sucrose concentration using isolates strains A2 (a), A10 (b) and A11 (c) grown in YPS fermentation medium at 30 °C starting with 0.5 g/L d.w. biomass. 250 g/L (◆); 300 g/L (■); 350 g/L (▲). Results are the media of three fermentations and bars represent mean ± standard deviation.

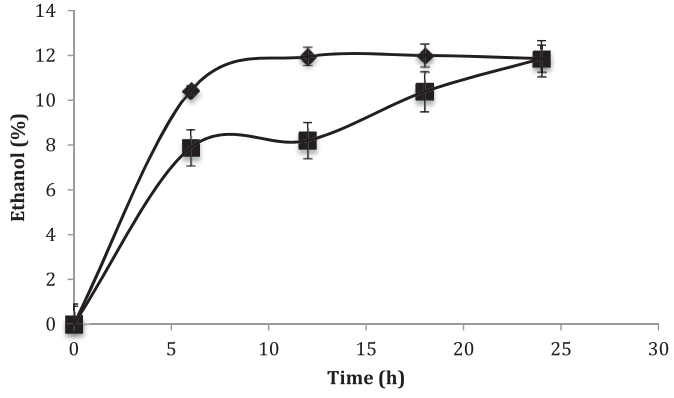


**Fig. 7.** Ethanol produced by strains A2 (non-flocculent) and A11 (flocculent) grown at 30 °C in molasses media (containing 250 g/L TRS), starting with 2% d.w. biomass. A2 (◆); A11 (■). Results are the media of three fermentations and bars represent mean ± standard deviation.

is 250 g/L of sugar.

### 3.6. Influence of high inocula concentrations using molasses media

To evaluate the performance of the selected strains and compared with those currently used in distilleries at industrial scale, it was necessary to reproduce the concentrations of inoculum used on a larger scale. Ethanol production generally recycles yeasts to minimize the time of fermentation. This procedure is based on centrifuging the fermented broth, recycling the yeasts after a pretreatment with sulfuric acid to pH 1 within 1 h. Generally, it is recovered 10% of the volume of the broth and the cream has about 20% dry matter. Thus, the recycled inoculum is about 2% dry matter, leading to fermentation times ranging between 12 and 16 h to reach the concentration of ethanol considered profitable for distillation (10% approximately).

Isolates A2 (non-flocculent) and A11 (flocculent) were grown in molasses medium containing 250 g/L of TRS, incubated at 30 °C starting with an inoculum of 2% dry matter. The A2 strain produced 10.43% ethanol at 6 h (see Fig. 7), reaching a maximum value of 11.95% after 12 h. Considering that the concentration values obtained in the ethanol industry after 10 h of incubation are about 10%; the values obtained with the A2 strain contribute to argue that its potential application to industrial level. The fermentative characteristics of A2 strain allow producing high ethanol concentrations in a shorter time, thus decreasing the costs of the production process. The behavior of the non-flocculent A2 strain was faster, probably because its dispersed form facilitated the transfer of nutrients and products.

The A11 strain with an inoculum 2% dry matter, produced 7.88% ethanol at 6 h of fermentation; reaching the maximum concentration (10.38%) at 18 h. No differences were observed in peak concentrations of ethanol achieved (11.86%) for A2 and A11 after 24 h fermentation.

The reduced production of ethanol achieved by A11 strain may be due to the flocculating property, which could hamper the contact of the yeast with the substrate, thereby increasing the time of fermentation [10]. Plessas et al. [31] studding the production of ethanol, obtained similar conclusions, since they need 14 h fermentation to produce 7.40% ethanol using the flocculent strain *S. cerevisiae*.

### 3.7. Use of the yeast S. cerevisiae A2 to scale up the process in a 10L bioreactor

Finally, the process was scaled up to a 10L bioreactor. Fig. 8 shows a short lag phase in the first 4 h followed by an

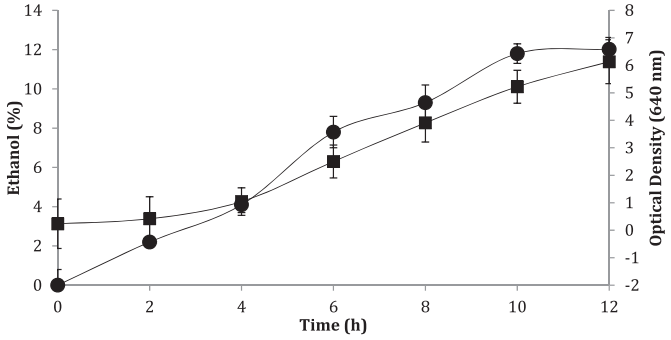


**Fig. 8.** Ethanol produced (●) and optical density growth (■) during the scale up process in a 10L Bioreactor using the yeast *Saccharomyces cereviceae* A2. Results are the media of three fermentations and bars represent mean ± standard deviation.

exponential phase typical of the microbial kinetics, and the production profile corresponding to a rapid fermentation caused by adding a high concentration of initial inoculum in order to minimize the production time, making the process more effective from an economical point of view. The size of the inoculum, corresponding to 2% dry matter, is the average number of yeasts recycled by the process of Melle-Boinot applied industrially in the region. It was observed 12% of alcohol production after 10 h of fermentation, slightly higher to the value currently obtained in the local industry at the same time (10%). Consequently, the scale up from lab scale reactors to 10L bioreactors was successful both in biomass and ethanol production using the yeast *S. cerevisiae* A2. Therefore, the biomass production of yeast strains isolated naturally in sugary environments is a key point in this sustainable circuit oriented to achieve technological and industrial improvements.

## 4. Conclusions

Argentine molasses and grapes were used to isolate 29 ethanol-producing yeasts, finding three isolates with higher ability to produce ethanol. One of these isolates showed a homogeneous growth (A2) meanwhile the other two presented a flocculent growth (A10 and A11) when they were grown in commercial media. It was also observed that the three isolates presented a percentage of similarity of 100% with the strain *S. cerevisiae*. The three strains were highly sensitive to changes in temperature, finding an optimal temperature for the production of ethanol at 30 °C, and although they could tolerate up to 300 g/L without decreasing the production of ethanol, the best results were achieved using 250 g/L of initial sugar. Higher sugars concentrations (350 g/L) represented a decrease in ethanol concentration, particularly using the non-flocculent strain A2, thus concluding that flocculation can act as a protective mechanism in yeast stress conditions such as with high concentration of sugars, and consequently the flocculent characteristics of strains A10 and A11 protected a high osmotic pressure generated by high sugar concentration. These strains were also able to grow in molasses medium although the behavior of the non-flocculent A2 strain was faster, probably because of its dispersed form facilitates the transfer of nutrients and products. The process can be successfully scaled up from laboratory scale to 10L bioreactor, thus demonstrating the feasibility of the *S. cerevisiae* A2 strain to be used as a novel strain to produce ethanol.

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