RIVERS OF TUCUMAN CONTAMINATION BY VINASSE SPILLS.ALTERNATIVE TO REDUCE THE LEVELS OF THIS POLLUTANT AND PROMOTE ITS USES

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Sugar and alcohol industrial sector, produces ETHANOL, which is used for fuel, food, beverage, pharmaceutical and chemical industries; SUGAR, sold for food, BAGASSE, solid waste grinding to obtain paper and electricity and VINASSE, which is the main liquid waste generated during the production of ethyl alcohol. Discharge of this type of untreated effluents produced water eutrophication of rivers characterized by nutrient enrichment in an aquatic ecosystem. The aim of this work is to deal the problem of pollution of rivers of Tucuman proposing an alternative to decrease the amount of vinasse produced in the process and promote its many uses.

INTRODUCTION

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.
Biofuels are fuels of biological origin, derived from renewable organic biomass, where biomass represents a potential source of carbohydrates for microbial fermentation (Gutiérrez Rivera et al., 2005). 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

(Putsche & Sandor. 1996), 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 (Sheehan, 1994), including the production of ethanol (Mielenz, 2001).
Biofuels have many advantages:
• They are renewable: biofuels are a convenient alternative to fossil fuels. They come from agricultural or livestock raw materials, which can be grown or raised.
• They are cleaner: one of its great advantages is that they are more biodegradable than fossil fuels, so they are potentially less harmful in case of spills and emit less pollutants into the atmosphere when when they are burned.
• Generate jobs: they are an alternative to encourage investment and employment in agriculture and the countryside.
• Take advantage of materials traditionally considered as waste: garbage, fats and animal excrement are raw materials for producing biofuels.
The momentum of bioethanol in Argentina, from sugar cane was shaped legally with Law 26,093, "Regime Regulation and Promotion for Sustainable Production and Biofuels Uses", in 2010 and established that must supply with at least 5% ethanol to all gasoline, a percentage which was gradually increased to 12% in 2016. This meant an opportunity for the province of Tucuman, in the north of Argentina, to expand its industrial production counting with nearly 300,000 hectares planted with sugar cane, 15 sugar mills and 11 distilleries (Figure 1, Ingenios azucareros, 2015).

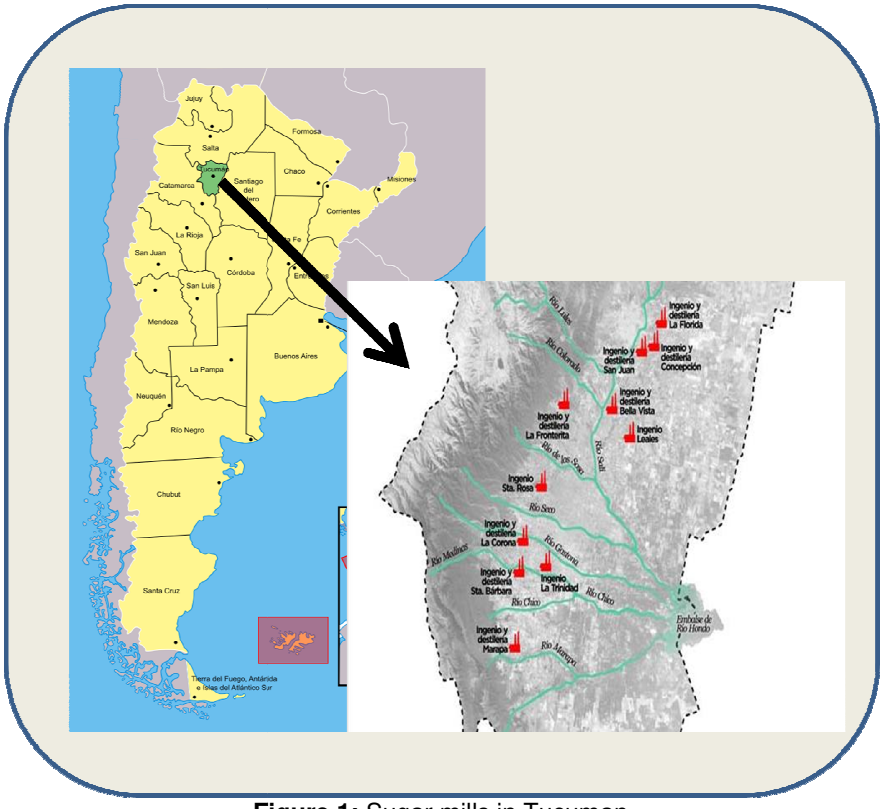


Figure 1: Sugar mills in Tucuman

Sugar and alcohol industrial sector, produces ETHANOL, which is used for fuel, food, beverage, pharmaceutical and chemical industries; SUGAR, sold for food,

BAGASSE, solid waste grinding to obtain paper and electricity and VINASSE, which is the main liquid waste generated during the production of ethyl alcohol.
Discharge of this type of untreated effluents produced water eutrophication of rivers characterized by nutrient enrichment in an aquatic ecosystem (Figure 2, El Liberal, 2011).

Figure 2: Eutrophication of rivers.

The explosion of algae that accompanies the first phase of eutrophication causes a clouding that prevents light penetrates to the bottom of the ecosystem. As a result at the bottom becomes impossible photosynthesis, and the environment becomes anoxic. Therefore this waste is highly polluting for water bodies, where it can cause the collapse of aquatic life due to the large amount of organic matter, so it is necessary to properly handle it. All approaches seek to eliminate or mitigate the effects of pollution on rivers, where traditionally these derivatives have been discharged without treatment or insufficient treatment. However, vinasses are a rich material with many potential uses.

Nature and composition of vinasse

The rational use of vinasse is based on the knowledge of its basic composition, the study of the physical and chemical conditions of the soil that should receive them, the crop and the variety to be fertilized, the source of vinasse used, climatic characteristics (Mainly precipitation), infrastructure of the area of supply, forms and means of application and economic considerations, among many others.
The chemical composition of the vinasse is quite variable, depending mainly on the raw material used in the distillation, which can come from 3 sources: molasses (concentrated), directly from the juice of the mills and mixed (mixture of juice and molasses). Table 1 shows the general characteristics of the vinasse (Perera, 2009:p.6).

Table 1: General characteristics of the vinasse

pH	**5,2**
Conductivity	**28,7 mS/cm**
QOD	**99100 ppm**
BOD	**40800 ppm**
Total Solids	**11,10%**
Ca	**0,26%**
Mg	**641 ppm**
K	**1,42%**
Na	**760 ppm**
Cu	**4,5 ppm**
Zn	**2,9 ppm**

It is produced from 13-15 liters of vinasse per liter of alcohol produced from sugar cane molasses (Perera, 2009).
The organic matter is the main component, also highlighting the contents of solids, potassium and calcium.
The high value of BOD and high acidity (pH) of vinasse and its high content of biodegradable organic matter, modifies the physical, chemical and especially microbiological conditions of the waters, affecting and limiting aquatic life and occupation as a source of human supply (Gloria & Filho, 1983; Castillo Valle, 1984).
The great power of contamination of the vinasse is favored for several reasons: (Silva & Filho, 1981; Castillo Valle, 1984).
1. Existence of unpleasant odors.
2. High acidity, affecting biochemical conditions of soils and wáter.
3. High degree of concentration of volatile and fixed solids, which favors sedimentation processes where they are evacuated.
4. High biochemical oxygen demand (BOD), which is defined as the amount of oxygen that the microorganisms responsible for the stabilization (oxidation) of organic matter require, its value represents a measure of the concentration of biodegradable matter in water.

Possible uses of vinasse

There are several ways of using vinasse (Filho, 1983).
a. Production of unicellular protein, through aerobic fermentation.
b. Methane gas production, through anaerobic fermentation.
c. Concentration (around 60º Brix), with the following possibilities of use
- Components of animal rations.
- Using yeast as fertilizer.
- Incinerated to produce fertilizer.
d. Agricultural use of the "in nature" residue, totally or partially substituting mineral fertilizations.

Ethanol production at industrial level since this microorganism is easy to handle, shows no high nutritional needs, can produce ethanol concentrations above 15% (Laopaiboon et. al, 2009), tolerates high concentrations of sugars, is not expensive, produces low levels of by-products, is osmotolerant and presents high viability for recycling (Carballo, 2000).

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 etanol 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 (Basso et. al., 2008).

The aim of this work is to address the problem of pollution of rivers of Tucuman proposing an alternative to decrease the amount of vinasse produced in the process. The advantages of the possibility of increasing the values of fermentative power of yeast for industrial use 10 to 12% ethanol in the wort, reduce the amounts of vinasse obtained. Traditionally yeasts are the main microorganisms used in the production of alcohol and finding strains hyper-producer yeast it is increasing the production capacity of bioethanol, with lower costs distillation and guarantee the supply of a renewable biofuel and reduce pollution of rivers.

MATERIAL AND METHODS

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 (Nishida et. al., 2004). Additionally, sugarcane molasses was evaluated after collecting sugarcane molasses from local sugarcane mills (Tucuman, 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 (Tucuman, 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 (Basso et. al., 2008), 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.

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.

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 (Hernández Muñoz, 1997). The supernatant was separated and stored for determination of sugars using the volumetric method of Fehling Causse-Bonnans (FCB) (Association of Official Analytical Chemists, 1985; Abate et. al., 1985) 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.

Characterization and molecular taxonomy

The selected isolates were subcultured on YPS fermentation medium for 24 h at 30°C. 1500 mL were transferred into micro centrifuge tubes and the cells were recovered by centrifugation at 10,000 x g for 2 min. The supernatant was discarded and the DNA extraction was realized following the methodology proposed by Yamada et al. (2002). The D1/D2 domain of 26S rDNA region was amplified using the primers NL1 (50-GCATATCAATAAGCGGAGGAAAAG-30) and NL4 (50-GGTCCGTGTTTCAAGACGGM- 30) (Lott, Kuykendall, & Reiss, 1993; Kurtzman & Robnett, 1997). 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 forBiotechnology Information) using the software MEGA version 4.0 (Tamura et. al., 2007). Phylogenetic trees were constructed with MEGA 4.0 using a Neighbor-Joining algorithm (Fitch & Margoliash, 1967) and bootstrap analyses for 1000 replicates were performed.

Scanning electron microscopy

Scanning electron microscopy was carried out following the method described by Karnovsky (1965) under high vacuum using a microscope Zeiss Supra 55VP (Carl Zeiss, Oberkochen, Germany). Samples were processed and observed in the Centro Integral de Microscopía Electronica (CIME), CCT-CONICET-UNT, San Miguel de Tucuman (Argentina).

RESULTS AND DISCUSSION

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 2 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. Figure 2 shows the course with time for total reducing sugars (TRS), direct reducing sugars (DRS), ethanol concentration and biomass. The tendencywas similar in the three strains, although the final ethanolconcentration was slightly higher in strains A10 and A11. The results of Figure 2 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%.

Table 2: 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

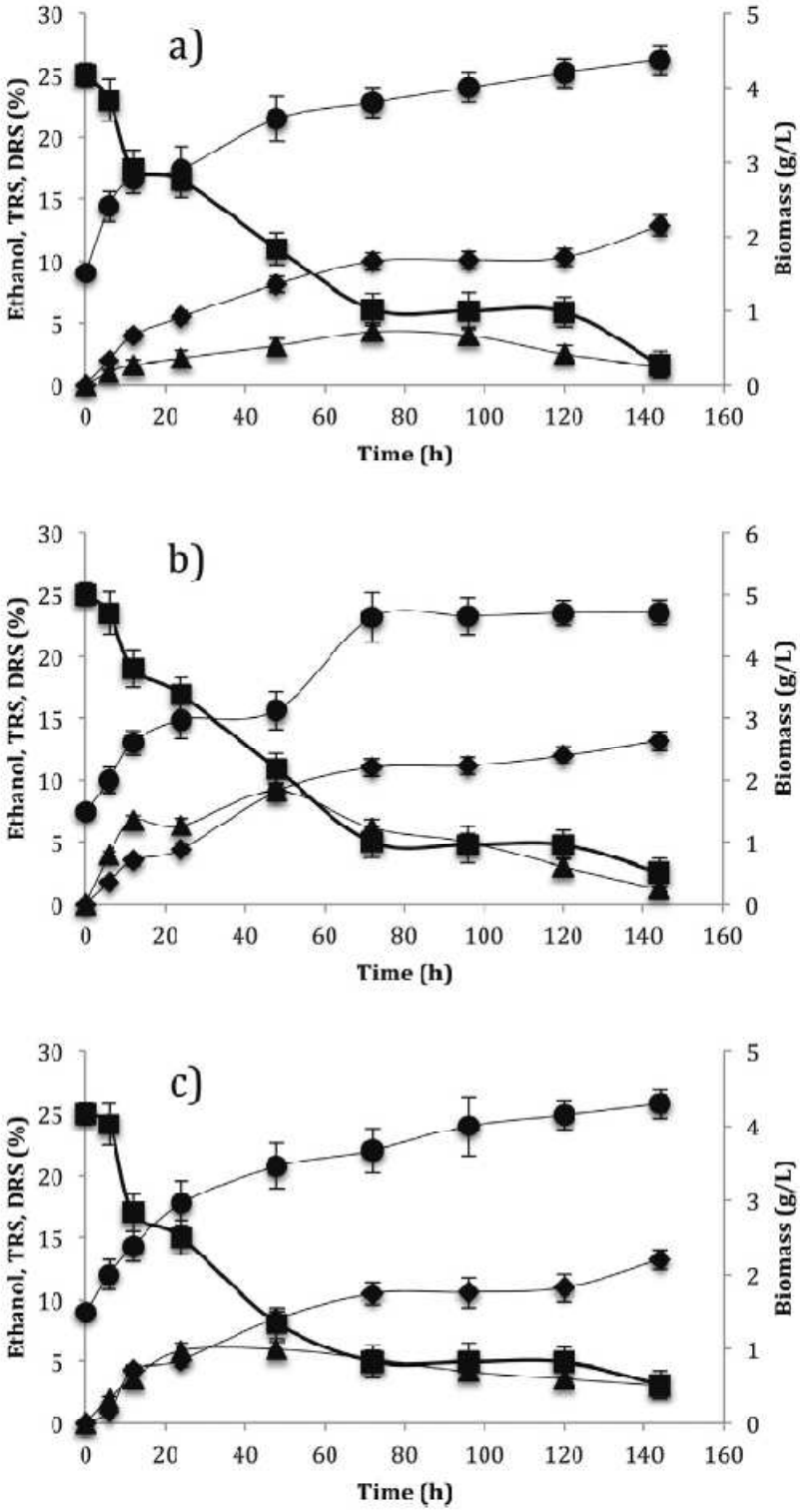


Figure 2: Course with time during the growth of Saccharomyces cerevisiae strains a) A2, b) A10 and c) A11 in YPS fermentation medium at 30 °C. TRS (■); Ethanol (◆); DRS (▲); Biomass (●). Results are the media of three fermentations and bars represent mean ± standard deviation.

Morphologic and growth characteristics of the selected microorganisms

The selected yeasts after growing on agarized YPS proliferation medium presented white-creamy colonies (Figure 3).

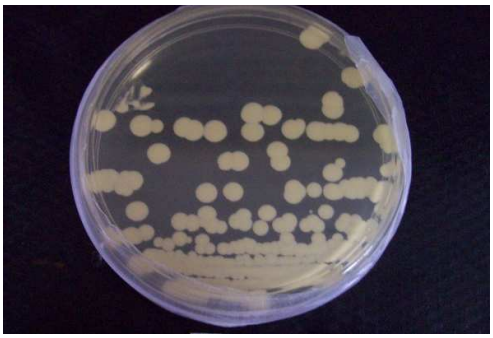


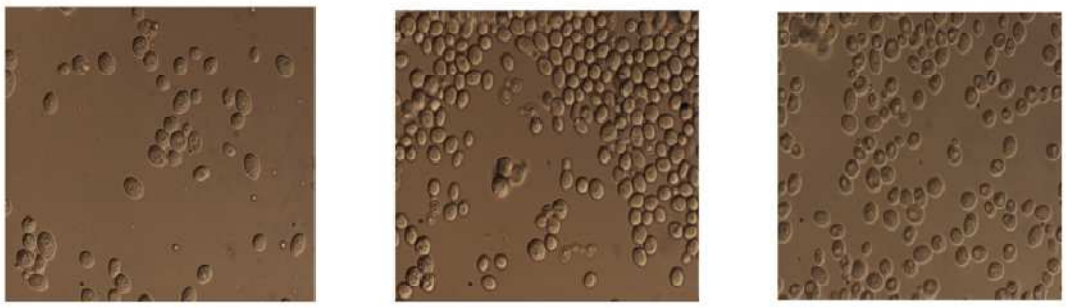
Figure 3: White-creamy colonies

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 (Figure 4).

Figure 4: Homogeneous and dispersed growth

The biomass was associated forming clusters or 1 mm diameter flocs, being deposited at the bottom of the cultura 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 (Figure 5). Furthermore, no significant differences were observed in the 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.

Furthermore, no significant differences were observed in the surface characteristics of A2, A10, and A11 strains before and after fermentation in the images obtained by scanning electron microscopy (Figure 6) with 2000 x magnification, although yeast cells exhibit a slight increase in surface roughness after fermentation, possibly due to the transport of substances across the plasma membrane.



a) A2 b) A10 c) A11

Figure 5: Optical microscopy (1000 x magnification) of isolated yeasts: a) A2, b) A10 and c) A11.

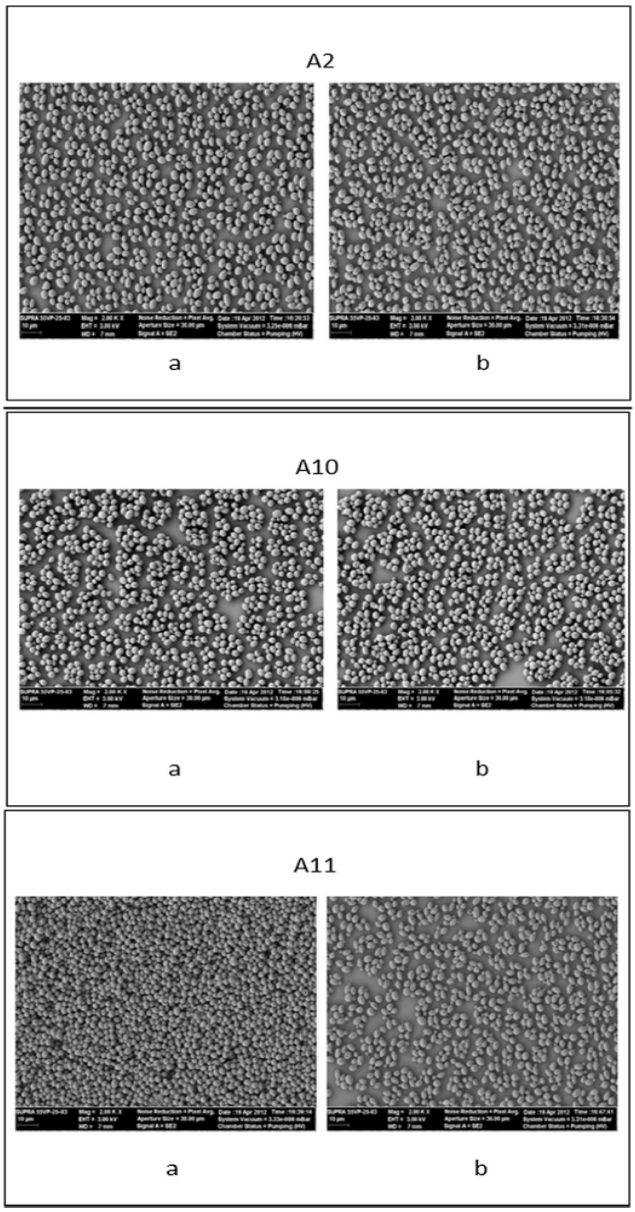


Figure 6: Comparison of yeast strains A2, A10 and A11 before (a) and after (b) 24 h fermentation at 30°C using a Scanning Electron Microscope (2000 x magnification)

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 Figure 7). 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.

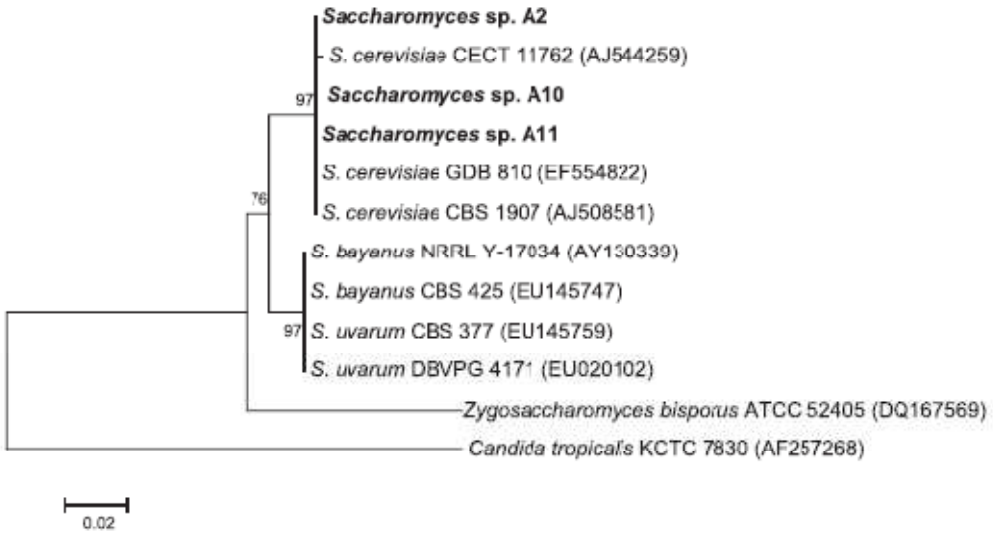


Figure 7: Phylogenetic tree obtained by the Neighbor-Joining method, based on the analysis of 26S rDNA of the isolates A2, 10 and 11. The strains were identified within the genus Saccharomyces. Access numbers to the type strains are in parentheses.

Use of strain *S. cerevisiae* A2 for the production of ethanol and to reduce the amount of vinasse

A model was generated where the variables that interested for this work were introduced (Table 3).

Table 3: Use of yeast A2 and its environmental and industrial considerations.

Alcohol percentage	10%	11%	12%
Total cane consumption (%)	47,22	46,50	45,77
Steam consumption at distillery (Kg. steam/liter of alcohol)	4,50	4,25	4,00
Liters of vinasse per liter of alcohol ($L_{vinasse}/L_{alcohol}$)	13	11	9

Table 3 shows that total steam consumption decreases by 3.07% when the final concentration of alcohol is improved to 12%, and this decrease is mainly due to a saving of 11.1% in the consumption of steam at the distillery. It can also be seen that the production of vinasse is reduced by 30%.
Currently a distillery that produces 100 m^3 of alcohol 96° per day, discharges 1300 m^3 of vinasse, that is, 13L vinasse is generated per liter of alcohol produced (Perera, 2009).
All the Tucuman mills begin their fermentation processes using commercial baking yeast. Table 4 compares the commercial strain of baking and the strain isolated, studied and selected in this work (*Saccharomyces cerevisiae* A2).

Table 4: Comparison of Commercial yeast bakery and *Saccharomyces cerevisiae* A2

Microorganism	Origin	Time fermentation	Concentration of Ethanol	Total cane consumption	Steam consumption at distillery	Liters vinasse per liter of alcohol
Comercial yeast bakery	CALSA-ARGENTINA Company	10 h	10%	47,22%	4,5 Kg. steam/liter of alcohol	13 $L_{vinasse}/L_{alcohol}$
Saccharomyces cerevisiae A2	Isolated from molasses	10 h	12%	45,77%	4 Kg. ateam/liter of alcohol	9 $L_{vinasse}/L_{alcohol}$

When comparing both strains, it was observed that *Saccharomyces cerevisiae* A2 produces a higher percentage of ethanol (12%) than the commercial strain of baking (10%) at the same time of fermentation (10h) and would reduce the levels of vinasse by 30% from 13 Lvinasse / Lalcohol to 9 Lvinasse / Lalcohol which would confer a significant environmental benefit.

CONCLUSIONS

It was possible to isolate 29 different yeasts from samples of molasses, cane juice from different sugar mills of Tucuman, canefield soil and grapes from the region of Salta (north of Tucuman).
Of the total yeasts isolated from samples of molasses, juices and grapes, 16 produced ethanol percentages lower than 5% while the remaining 13 produced ethanol concentrations higher than 5%.
Isolates A2, A10 and A11 were selected as good ethanol producers with ethanol concentration values recorded of 12.87, 13.20 and 13.20% respectively.
It was observed that the A2 strain showed a homogeneous growth in liquid medium, this characteristic is compatible with the technology currently used in the industry. These results showed to strain A2 as a candidate to be used in the industrial production of ethanol without needing to make technological changes.
Strains A10 and A11 showed a flocculent nature in liquid medium.
Isolates A2, A10 and A12 were taxonomically identified and the analysis of the sequences allowed to assign a 100% identity with Saccharomyces cerevisiae.
The scaling of this yeast strain with high production of ethanol, naturally isolated from the environment is a key point in the sustainable circuit to improve technological and industrial level.
From the environmental point of view when using the strain *Saccharomyces cerevisiae* A2 would be achieved to reduce in 30% the levels of vinasse generated passing from an average of 13 Liters of vinasse / Liter of alcohol to 9 Liters of vinasse / Liter of alcohol which would confer a significant environmental benefit.
Therefore increasing the fermentative power by at least two points means:
-Increase Production Capacity.
-Lower energy consumption for distillation.
-Decrease the liters of vinasse produced per liter of alcohol.
These characteristics make the strain *Saccharomyces cerevisiae* A2, a producer of bioethanol with potential environmental, energy and economic benefits, to project it on an Industrial scale.

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