Vol.56, n.2: pp. 161-169, March-April 2013
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

Alcoholic Fermentation by the Wild Yeasts under Thermal, Osmotic and Ethanol Stress

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ABSTRACT

This study aimed to explore the variability in the metabolism of nine wild yeasts isolated from the sugarcane juice from a distillery in the Brazilian State of Mato Grosso. Cell viability under the stress conditions was evaluated. The yeasts were inoculated in the test tubes containing sugarcane juice adjusted from 12 to 21º Brix, ethanol from 6 to 12% in volume and temperature at 30, 35 and 40ºC. The viability was established by the growth in Petri dishes and visually by the CO_2 production in the test tubes. None of the evaluated yeasts showed simultaneous resistance to the three stress conditions. The potential of yeast BB.09 could be emphasized due to its ability to ferment up to12% ethanol at 30°C.

Key words: Bio prospection, Alcohol distillery, Cellular viability, Fermentation

INTRODUCTION

Saccharomyces cerevisiae is used in the fermentation of sugarcane juice and molasses for ethanol production. The sugar, acidity and ethanol concentration varies significantly during the fermentation process and the yeast cells are subjected to this stress. The damages caused by the type and degree of stress is defined by many factors, including the yeast´s cycle and cellular division in the moment when the stress occurs (Folch-Mallol et al. 2004). One of the changes that the yeasts face during the fermentation process is the progressive decrease in the essential nutrients for the growth, which demands permanent adjustment of genetics and the cellular metabolic system. The alcoholic fermentation also includes other stress conditions, such as osmotic pressure and the increasing of ethanol concentration (Ivorra et al. 1999). The ethanol content may modify the degree of polarity of the cellular membrane. At high concentrations, ethanol content also reduces the multiplication and the viability of the yeast (Lind et al. 1991). The pathway of inhibition is complex and includes many mechanisms, which include denaturation and inhibition of enzymes and damage to the plasmatic membrane, through modifications to its permeability. The phospholipids present in the plasmatic membrane play an important role in the mechanism of tolerance to ethanol (D'Amore and Stewart 1987a). According to Alexandre and Charpentier (1998), the toxic effects of ethanol to *S. cerevisiae* involve the modifications in the lipid composition of the membrane, reduction of the metabolic activity, inhibition of the glucose's transportation into the cell, inhibition of the growth and viability of the cells and inhibition in the production of

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ethanol. The evaluation of the surviving capacity of the cells that are exposed to ethanol is a useful tool to compare the tolerance of different species and strains of the yeasts (Chi and Arneborg 2000a; Pina et al. 2004a). A frequently used method to determine the tolerance of ethanol involves the suppression of the cellular growth in the presence of alcohol (D'Amore and Stewart 1987b). Because of its simplicity, this method is very useful in the classification of a high number of strains by its ability to tolerate ethanol (Pina et.al 2004b).

The studies on the ethanol toxicity to the yeasts have been applied for *S. cerevisiae* species as a model (Chi and Arneborg 2000b). Despite the fact that this species has been hardly studied, the intraspecific variations could be significant on the fermentative capacity, because the *S. cerevisiae* strains have different sensibility to the alcoholic content. However, most of the strains are able to ferment sugar until alcohol content of 15 ºGL. The growth of yeasts generally occurs in lower alcoholic concentrations than the ones, which inhibit the fermentation (Guerra and Barnabé 2005).

Guimarães (2005) used methylene blue to study the viability of the yeasts and found that 87% of yeasts remained viable when incubated at 37 ºC, but the viability decreased to 83% when the yeast were subjected to 8% (v/v) of ethanol at 30ºC. Casimiro et al. (2000) evaluated five yeasts (L2, L3, L6, L7 and L8) for the fermentation of the clarified cashew juice adjusted to 16º Brix and to alcoholic stress by the addition of commercial ethanol at 0, 2, 4, 6, 8 and 10 ºGL. The results showed a variation of yeasts intolerance as a function of the different strains and the alcohol concentration. The highest performance was presented by the yeast L2, which produced a wine with an alcohol content of 10.05 ºGL. D'Amore and Stewart (1987c) reported that many factors could interfere with the strain's resistance to the ethanol stress, which included the adding of unsaturated fatty acids, accumulation of intracellular ethanol, temperature and osmotic pressure. The yeast's sensitivity to the ethanol also increases with increases in the temperature (Lima et al. 2001). At higher temperatures, the toxicity of the ethanol may prematurely stop the fermentation before the sugars are completely consumed (Chi andArneborg 2000c).

A high osmotic potential is caused by the sugar pressure in the must that may also increase the toxicity caused by ethanol (Guerra and Barnabé, 2005). Sugar concentrations over 25% can delay the beginning of the fermentation (lag phase) and establish adverse osmotic conditions for the yeast.

Bertoloni et al (1991) isolated the strains of yeasts able to ferment the sugarcane juice concentrated by saccharose addition. The strains converted the sugars to ethanol with yields from 89 to 92% at 30ºC. Among all the selected strains, the authors studied in detail the strains OSMO-6 and OSMO-8 and observed that the cell viability was invariably low, and that the higher values were obtained from the alcoholic fermentation of grape must with 30% sucrose. Higher concentrations of sucrose did not produce higher concentrations of ethanol in fermentation times from 24 to 28h.

The loss of cytoplasmatic water can occur in the yeasts exposed to osmotic shock and several mechanisms need to be then activated in order to protect the cell and its structures from dehydration (Estruch 2000). Walker (1998) reported that glycerol was the most effective osmosis regulator present in *S. cerevisae* cells and the trehalose was the most efficient carbohydrate for the stabilization of the plasmatic membrane when the yeast was submitted to osmotic stress. The survival of the yeast to the stresses during the alcoholic fermentation depends on its ability to quickly adapt to environmental changes. Because of the potential offered by the variation of *S. cerevisiae* metabolism, the selection of wild yeasts as fermentation agents could determine the efficiency of alcohol production.

The work evaluated the fermentative potential of nine wild yeasts isolated from the sugarcane juice from a distillery from the Barra do Bugres, Mato Grosso State, Brazil under thermal, osmotic and ethanol stresses.

MATERIALS AND METHODS

Yeast strains

Nine yeasts morphotypes were isolated from the sugarcane juice from the distillery Barralcool, Barra do Bugres, MT, Brazil. All the strains showed the ability to ferment the sugarcane juice. The yeasts (BB.01 to BB.09) were grown and preserved in a PDA medium.

Sugarcane juice

Sugarcane juice was collected from the Barralcool distillery in a sterilized flask.

Stress factors
Temperature, ethanol content and osmotic pressure were investigated as the stress factors. All the tests were carried out on sugarcane juice previously adjusted to each kind of test. A volume of 10.0 ml was put in test tubes with an inverted Durham tube and sterilized at 120ºC for 20 minutes. The pure cultures were reactivated by inoculation in 3.0 ml of 0.85% saline solution to provide the cells concentrations near 5.0 Mac Farland scale. The test tubes containing 10ml of sugarcane juice were inoculated with 0.02 ml of the suspension culture in triplicate for all the nine yeasts.

Temperature
The inoculated test tubes were incubated in a water bath at 30, 35 and 40ºC for 72 h. The fermentative activity was evaluated by the CO_2 formation in the Durham tubes.

Ethanol
The sugarcane juice was adjusted to 12º Brix with distilled water, keeping its natural pH of 5.0±0.1. The values of 6, 8, 10 and 12% alcohol were adjusted by addling commercial ethanol (Nobre 92.8ºINPM). Triplicate test tubes were incubated at 30, 35 and 40ºC for 72 h.

Osmotic pressure
The sugarcane juice was adjusted to 12, 15, 18 and 21º Brix by dilution with the distilled water, keeping its natural pH of 5.0±0.1. Triplicate test tubes were incubated in a water bath at 30, 35 and 40ºC for 72 h.
The viability of the yeast cells was determined by counting the colonies in Petri dishes (Speck, 1976). To inoculate the plates, aliquots (0.1 ml) were taken from the test tubes showing CO_2 formation (by serially diluted them in saline solution; 0.85%) and spreading with the Drigaslky's handle in Petri dishes containing LWYN medium. Ampicillin (500mg/l) was added to inhibit the growth of bacteria as described by Silva and Cereda (2009). The Petri dishes were incubated at 30°C for 72 h. The Petri dishes of the same dilution that presented between 30 to 300 colonies were chosen for counting. The average count from two Petri dishes was multiplied by the dilution factor and by 10^4 to express the counting for 1000 ml.

Analysis of results and statistics
The media were compared by Variance analysis using Tukey's test to the level of 5% of probability using the Statistica 7.0 program from Statsoft.

Results and Discussion
The measurement of cell viability as a function of stress associated with the ethanol/temperature and Brix/temperature allowed comparing the resistance of the morphotypes of nine wild yeasts. The higher count was found at the conditions without stress (30ºC, 12º Brix, 0% ethanol). None of the wild yeasts resisted well to all the stress factors.

Ethanol stress
At each temperature (30, 35 and 40 ºC), the viable cells numbers are shown in Figures 1 to 4 for the nine yeasts, as a function of the associated alcohol/temperature.
Some yeasts showed fermentative activity at 6 and 8% ethanol content at 30°C, but at 40°C, none was detected at the same alcohol content. The results of the stress conditions classified the yeast in three groups depending on the viability of the cells. In the first group, the yeasts BB.01, 02, 03 and 05 showed 10 to 50 UFC.10^{10}/l as viable yeasts number at 30 ºC. In this Group, the BB.01 yeast showed counts near ten times higher than the others (100 UFC.10^{10}/l. The second group contained the yeasts BB.04, 06, 07 and 08. These yeasts even under the best conditions for the growth (without stress) always showed low cell viability, with 2 UFC.10^{10}/l cells numbers. In the third group, the yeast B.09 showed low viability, although showing fermentative activity just at 40ºC.
The morphotypes BB.01 and BB.03 showed significantly higher viable cells counts with 6 and 8% of ethanol (v/v) at 30ºC. Similar results were found by Fernandes (2008) who evaluated the effect of the ethanol concentration/temperature on the cell viability of the yeasts. At first, the yeasts were submitted to 11% of ethanol at 30º C and then to 20% ethanol at 16 ºC. The cell viability was measured by the colony-forming units (CFU) in the Petri dishes with YM agar. For all the yeasts selected, the viability was significantly lower at the binomial 20% ethanol at 16 ºC, compared to all other cultivation conditions. Concerning the effect of the temperature of the fermentation on the yeasts viability, Torija et. al. (2002) found that the

temperature affected not only the fermentation kinetics, but also the yeasts metabolism. They found a high number of unviable yeasts at 35ºC.

In the present study, the ethanol concentration appeared as the major factor of stress. For the yeasts BB.04, 05 and 06, even at a favorable temperature of 30ºC, the fermentative activity occurred only at 6% of ethanol, which might be considered low if compared with the industrial conditions. With the increased, temperature and ethanol concentrations (6 to 12%), a significantly reduction in the cellular viability for all the analyzed morphotypes was observed and the fermentation was stopped. Nevertheless, both the yeasts (BB.07 and BB.08) showed fermentative activity at 6 and 8% of ethanol at 30 and 35ºC, without significant difference in the cell viability counts. At 10% ethanol, no fermentation was detected.

The yeast BB.09 was more resistant to the alcohol stress with fermentative activity in all the alcoholic concentrations tested. This strain also showed resistance up to 8% ethanol, even when the temperature was increased to 35°C, since the number of viable cells did not differ from that obtained under the conditions of lower stress (6% ethanol at 30°C). Although this yeast presented the cell viability at all the tested concentrations of ethanol, the fermentation was only at 6% and 30° C. The observed resistance of the yeasts to the ethanol content could be considered low when compared to Guerra and Barnabé, (2005), who found that the limit for alcoholic fermentation was 15 % of alcohol. The tested ethanol concentrations inhibited the viability and fermentation in a different way for each strain. A possible explanation would be the action of ethanol on the cell membrane (Lind et al. 1991).

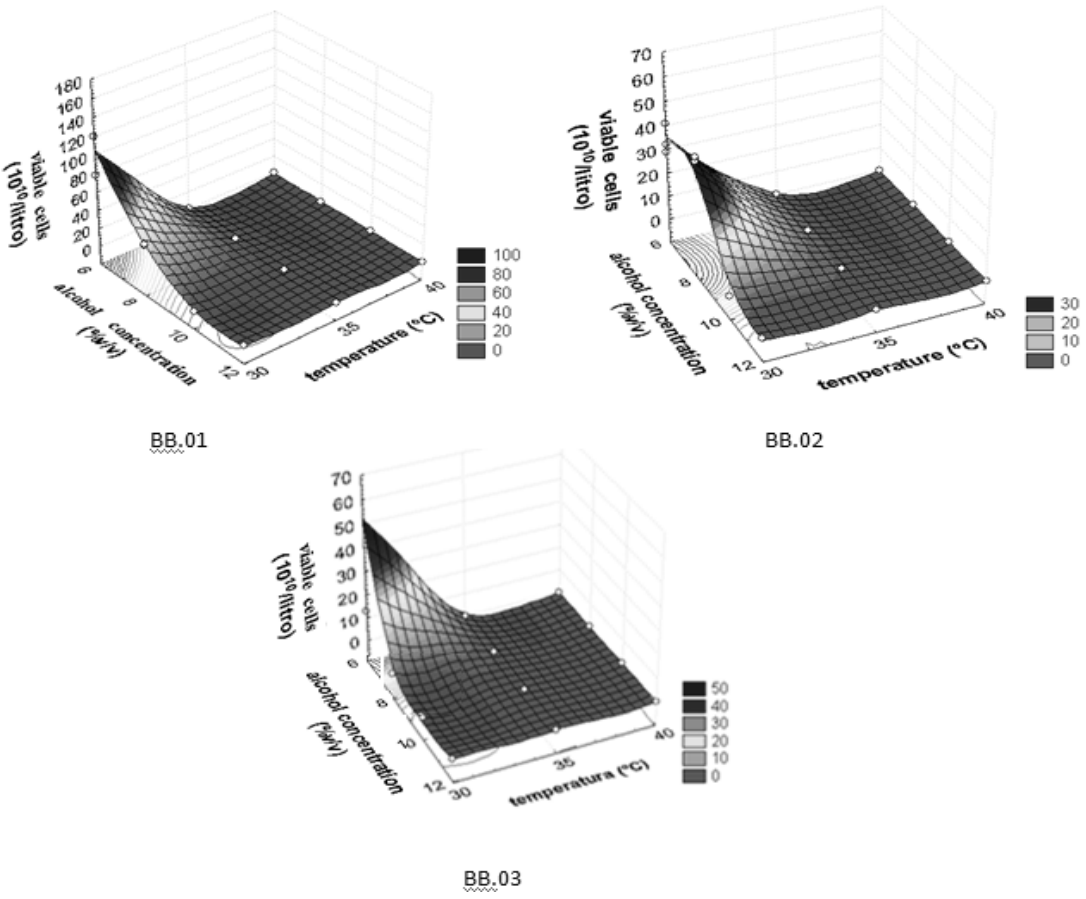

Figure1 - Variation of the wild yeasts viable cell numbers in ethanol and temperature stress conditions for the morphotypes BB. 01, 02 e 03.

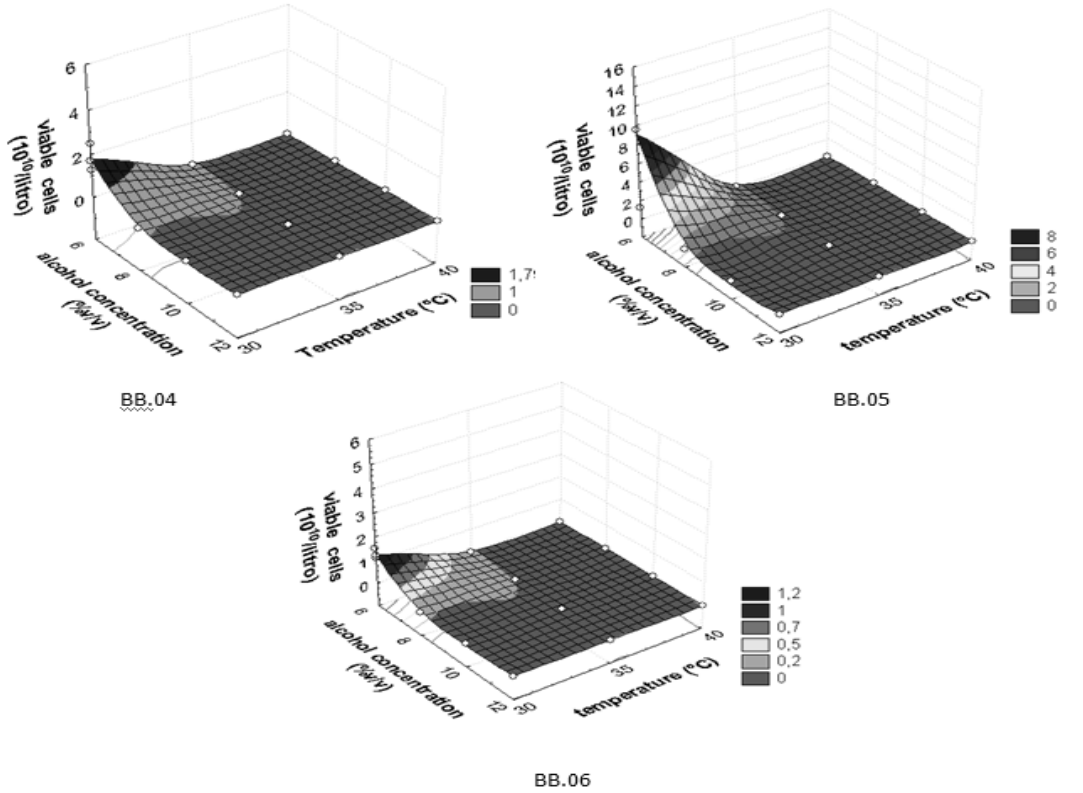

Figure 2 - Variation on the wild yeasts viable cells number ethanol and temperature stress conditions for the morphotypes BB. 04, 05 and 06.

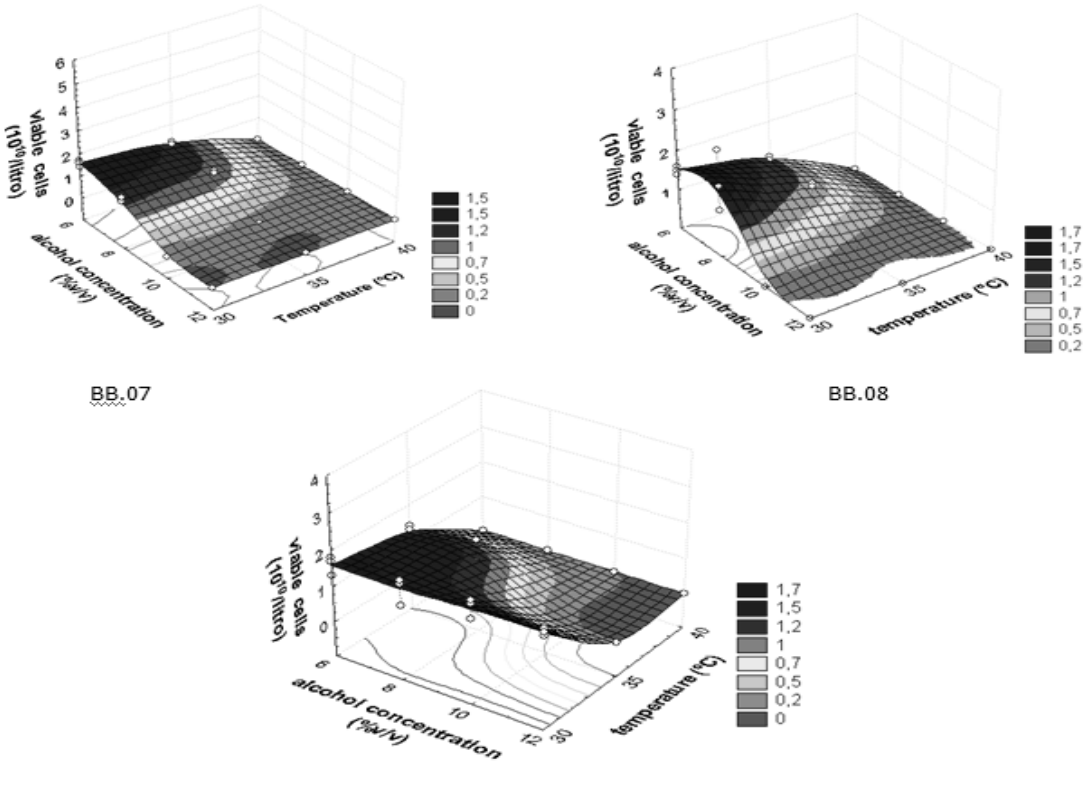

Figure 3 - Variation on the wild yeasts' viable cells number in ethanol and temperature stress conditions for the morphotypes BB. 07, 08 and 09.

In this work the limit for fermentation was 6% ethanol. However, this could not be extended to the industrial conditions. Nagodawithana and Steinkraus (1976) reported that the ethanol produced on the alcoholic fermentation process could have a greater toxic effect on the cells than the effect caused by the ethanol added in the laboratory conditions. That would be a question needing to be further studied, but there might be accumulation of other products of secondary metabolism that could also be toxic for the yeast (Lafon-Lafoucade and Ribéreau-Gayon 1984; Sá-Correia 1986). The results highlighted the yeast BB.9 as the most promising for alcoholic stress.

Osmotic stress

The effects of the osmotic stress by Brix increasing (12, 15, 18 and 21) on the cellular viability of the nine yeasts were studied at 30, 35 and 40 ºC. The 30 ºC and Brix from 12 to 15 were not really stress conditions (considering the environmental conditions in the Brazilian distilleries). The conditions of 30ºC with 25 Brix could be considered as normal to the environmental conditions at the distilleries in the Center-West region of the country, but for the fermentation of the sugar cane juice or molasses, the Brix^{o} would be 12 to 14. The viable cell counts from the nine yeasts evaluated in the stress due to the addition of saccharose and temperature are presented in Figures 4 to 6.

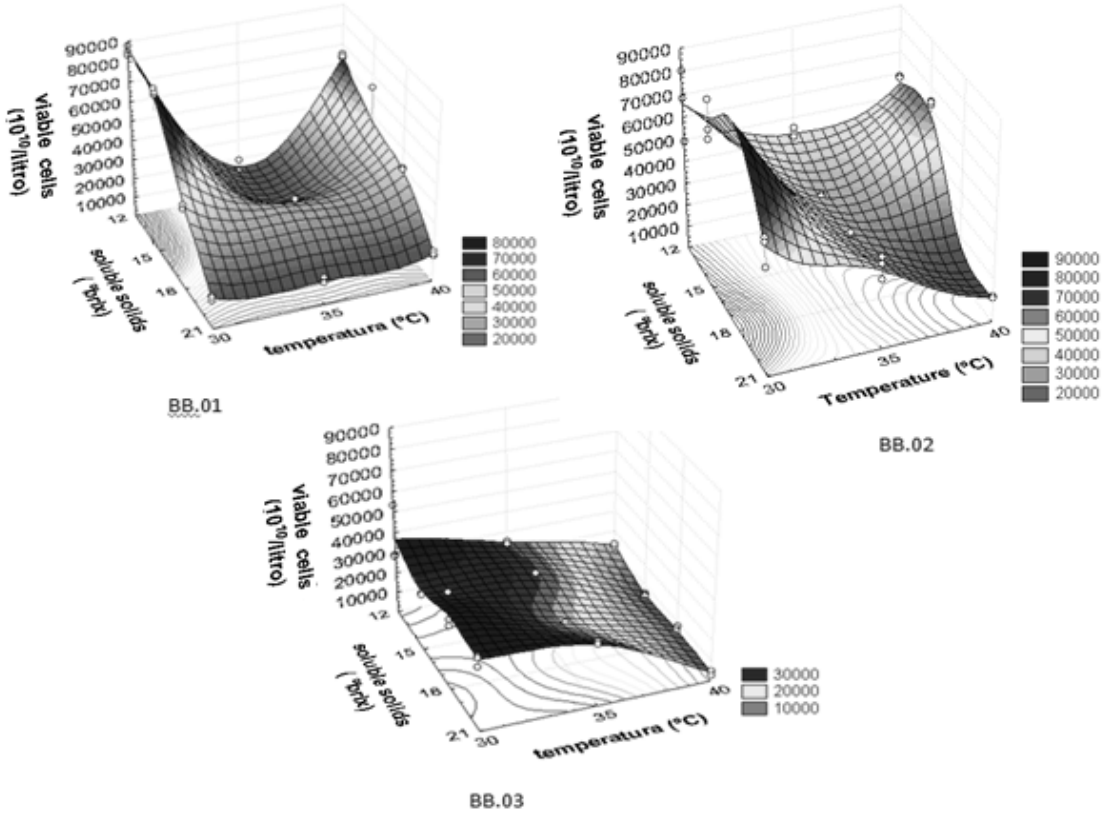

Figure 4 - Variation on the number of the wild yeasts' viable cells, fermenting in osmotic and temperature stress conditions for the morphotypes BB. 01, 02 and 03.

Apparently, there was no influence on the activity of the yeasts due to high Brix values or ethanol. It was possible to include most of the yeast in only one group that presented viable cells in a range from 12 to 90 000 CFU.1010/l. Another group (BB.06, 07 and 08) showed the lowest cell viabilities, between 180 and 450 UFC.10^{10}/l. Even so, the yeast BB.06 showed more resistance at 35ºC because their viable cells count did not differ from those at 30 at 35ºC. The yeast BB.07 was the most sensitive and presented a drop in the viability of cells with the increase of Brix and temperature.

The strain BB.09, however, was an exception, which presented low but stable cell viability (500 UFC.10^{10}/l) even at the higher Brix values. The highest count of viable cells was from 15 to 18º Brix, but fell when the Brix was adjusted to 21.

The yeast BB.03 (Fig. 4) and the BB.04 (Fig. 5) didn't differ significantly in the number of viable cells, showing more resistance to the osmotic pressure but not to the temperature. It was expected that the yeasts presented the highest counts of viable cells at 12 Brix, as occurred with the yeast BB.01, but for the BB.02, the highest cellular viability occurred at 18º Brix, both at 30ºC. At 35^0C, more variations on the influence of Brix concentration on the cells viability were observed, with a marked drop in viability. As a consequence, no fermentation occurred at 40ºC for the yeasts BB.08 and 09 that showed viability in 18 and 21º Brix at 35 and 40ºC (Fig. 4). Despite this resistance, yeasts might be under intense stress. The microscope examination showed the formation of the flocks, which was un-desirable for the industrial conditions.

The yeast BB.09 showed the best cellular viability between 15º and 18º Brix at 35ºC, with drop only on extreme values. This yeast had already been highlighted for its performance under ethanol stress.

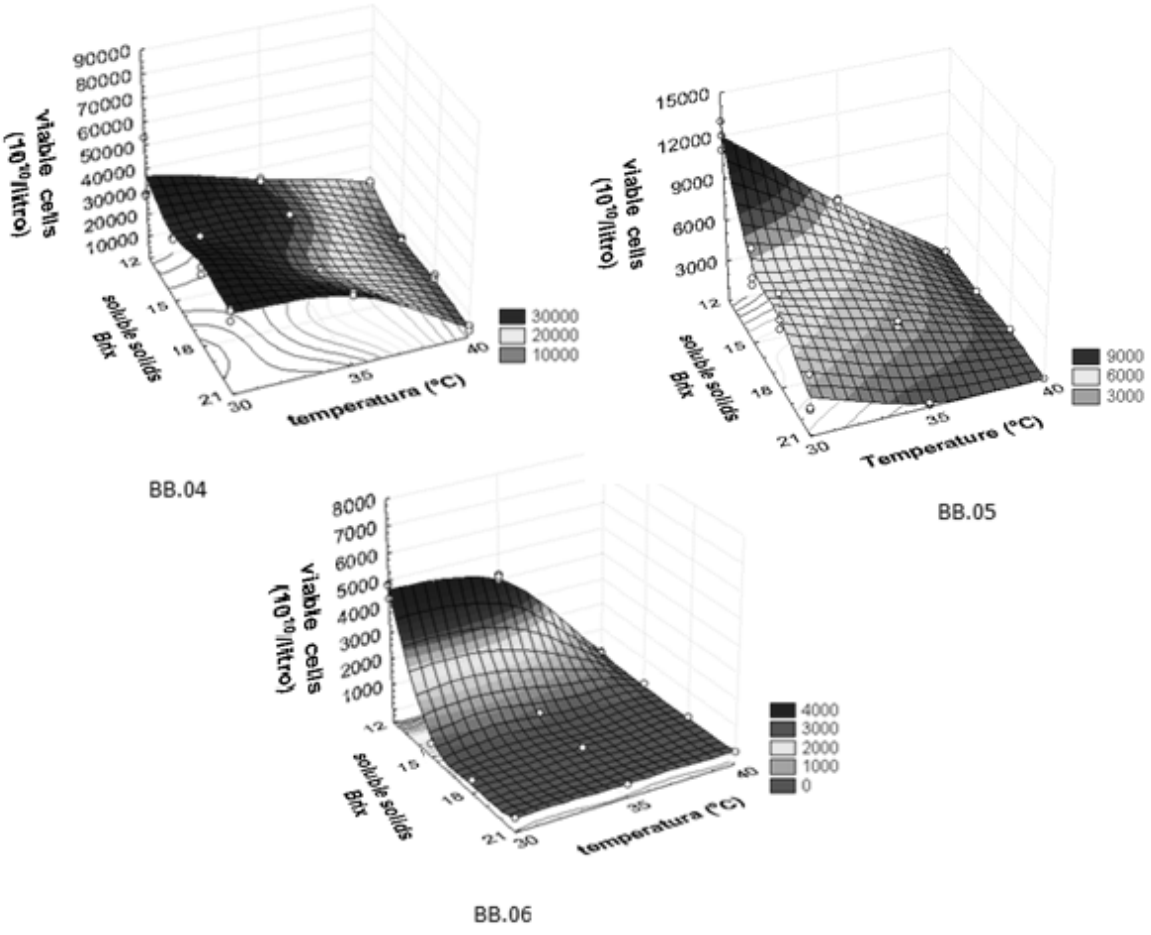

Figure 5 - Variations on the wild yeast viable cells number in osmotic and temperature stress conditions for the morphotypes BB. 04, 05 and 06.

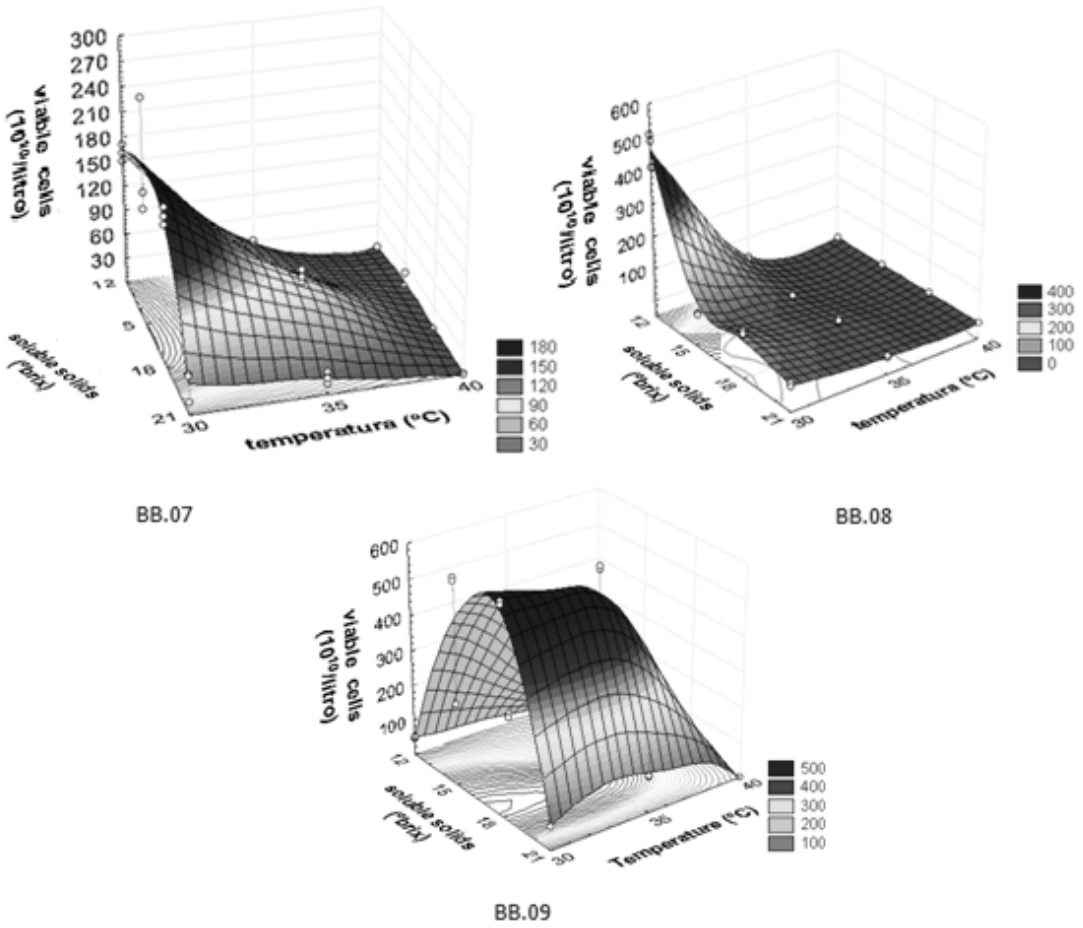

Figure 6 - Variations on the wild yeasts viable cells number in osmotic and temperature stress conditions for the morphotypes BB. BB. 07, 08 and 09.

CONCLUSION

The results showed high variability in resistance to stress conditions of wild yeasts isolated from the sugarcane juice, indicating the potential of bio prospection to select them. Generally, it was not possible to find the yeasts that could resist to all the stress condition at the same time. The temperature was the limiting condition most affecting the viability, followed by the ethanol concentration. The yeast BB.09 showed good potential because its ability to ferment at 10 and 12% of ethanol, but only at 30ºC. The BB.03 and BB.04 showed viability at all the Brix values at 30 ºC.

REFERENCES

Alexandre H, Charpentier C. Biochemical aspects of stuck and sluggish fermentation in grape must. *J Ind Microbiol Biotechnol*. 1998; 20: 20-27.

Ceccato-Antonini SR. Microbiologia da fermentação alcoólica. A importância do monitoramento microbiológico em destilarias. 1ª ed. São Carlos: EduFSCar, 2010.

Bertolini MC, Ernandes JR, Laluce C. New Yeast Strains For Alcoholic Fermentation At Higher Sugar Concentrations. *Biotechnol Lett*. 1991; 13: 197-202.

Casimiro ARS, Feitosa T, Borges M de F, Garruti D dos S, Campos JO de, Bringel MHF. Avaliação de leveduras industriais na fermentação de suco de caju. *Embrapa Agroindústria Tropical*. 2000; (circular técnica 4) 14p.

Chi Z, Arneborg N. *Saccharomyces cerevisae* strains with different degrees of ethanol tolerance exhibit diferent adaptative responses to produced ethanol. *J Ind Microbiol Biotechnol*. 2000; 24: 75-78.

D' Amore T, Stewart G. Ethanol tolerance of yeast. *Enzime Microbiol Technol. 1987;* 9: 322-330.

Dorneles D, Machado IMP, Chociai MB, Bonfim TM B. Influence of the use of selected and non-selected yeasts in red wine production. *Braz Arch Biol Technol*. 2005; 48: 747-751.

Estruch F. Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. Rev. *FEMS Microbiol*. 2000; 24: 469-486.

Fernandes APFV. Leveduras isoladas de produtos frutícolas: Capacidade Fermentativa e estudos sobre a H^+ATPase da membrana plasmática. (Tese de Doutorado). Lisboa, Portugal: Universidade Nova de Lisboa; 2008.

Folch-Mallol JL, Arroyo AG, Lledias F, Robles AAC. La respuesta a estrés en la levadura *Saccharomyces cerevisiae*. *Rev Lat Am Microbiol*. 2004; 46: 24-46.

Guerra CC, Barnabé D in Venturine Filho W G. Tecnologia de Bebidas. 1ª ed. São Paulo: Editora Edgard Blücher, 2005.

Guimarães TM. Isolamento, Identificação e Seleção de Cepas de Leveduras *Saccharomyces cerevisae* para Elaboração de Vinho. (dissertação de mestrado). Curitiba, Paraná: Universidade Federal do Paraná; 2005.

Ivorra C, Perrez-ortin JE, Olmo M. An inverse correlation between stress resistence and stuck fermentations in wine yeasts. A molecular study. *Biotechnol Bioeng*, 1999; 64: 698-708.

Lee SS, Robinson FM, Wang HY. Rapid determination of yeast viability. Biotechnol Bioeng Symposium. 1981; 11: 641-649.

Lima UA, Aquarone E, Borzani W, Schmidell W. Biotecnologia Industrial: Processos Fermentativos e Enzimáticos. 1ªed. Vol. 3 São Paulo: Ed. Edgard Blücher, 2001.

Lynd LR, Ahn HJ, Hill AGP, Klapatch T. Thermophilic ethanol production - investigation of ethanol yeld and tolerance in continuous culture. *Rev Biotechnol Appl Biochem. 1991;* 28: 549 - 569.

Longo E, Velázquez JB, Sieiro C, Cansado J, Calo P, Villa TG. Production of higher alcohols, ethyl acetate, acetaldehyde and other compounds by 14 *Saccharomyces cerevisiae* wine strains isolated from the same region. *World J. Microbiol Biotechnol*, 1992; 8: 539-541.

Longo VD, Liou LL, Valentine JS, Gralla EB. Mitochondrial superoxide decreases yeast survival in stationary phase. *Arch. Biochem Biophys*. 1999; 365: 131 - 142.

Melo DLFM, Santos FC; Barbosa Junior AM, Santos PO; Carnelossi MAG; Trindade RC. Identification of yeasts isolated from the pulp in nature and the production of homemade "Umbu" wine. *Braz. Arch Biol Techno. 2007;* 50: 887-892.

Nagodawithana TW, Steinkraus KH. Influence of the rate of ethanol production and accumulation on the viability of *Saccharomyces cerevisiae* in "rapid fermentation". *Appl Environ Microbiol*. 1976; 31: 158-161.

Panek AC, Vânia JJ, Paschoalin MF, Panek D. Regulation of trehalose metabolism in *Saccharomyces cerevisae* mutants during temperature shifts. *Biochem. 1990;* 72: 77-79.

Pina C, Antonio J, Hogg T. Inferring ethanol tolerance of *Saccharomyces* and non-*Saccharomyces* Yeasts by progressive inactivation. *Biotechnol Lett. 2004;* 26: 1521-1527.

Sá-Correia I. Synergistic effects of ethanol, octanoic and decanoic acids on the kinetics and the activation parameters of thermal death in *Saccharomyces bayanus*. *Biotechnol. Bioeng. 1986;* 28: 761-763.

Schmidell W, Lima U A, Aquarone E, Borzani W. Biotecnología Industrial: Engenharia Bioquímica. 1ª ed. Vol. 2. São Paulo: Ed. Edgard Blucher, 2001.

Speck ML. Compendium of Methods for the Microbiological examintion of foods. American Public Health Association: 1976.

Torija MJ, Roze N, Poblet M, Guillamo JMN, Mas A. Effects of fermentation temperature on de strain population of *Saccharomyces cerevisiae*. *Int J Food Microbiol*. 2002; 80: 47-53.

Walker GM. Yeast. Physiology and Biotechnology. London, UK: John Wiley and Sons Publishers, 1998.

Received: September 27, 2011;
Revised: January 18, 2012;
Accepted: October 17, 2012.

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