STRESS TOLERANCE OF *Saccharomyces cerevisiae* AND NON-*Saccharomyces* YEASTS REGARDING pH, TEMPERATURE, GLUCOSE AND ETHANOL

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ABSTRACT

Although *Saccharomyces cerevisiae* is the most important yeast species utilized in the bioethanol production from sugarcane musts, the unconventional (non-*Saccharomyces*) yeasts have demonstrated great genetic diversity, tolerance to various stresses and good fermentative performance, which indicated them to a variety of biotechnological processes. The aim here was to evaluate the tolerance to sugar (300 g/L) and ethanol (10% v/v) concentration, temperature (36ºC) and pH (3.0) of three non-*Saccharomyces* yeasts (*Wickerhamomyces anomalus*, *Meyerozyma guilliermondii* and *Schefersomyces stipitis*) compared to two industrial strains of *S. cerevisiae* (PE-2, CAT-1), as part of the feasibility approach of these unconventional yeasts in the 1G ethanol production. Growth in microplates in YPD under the stressful conditions was carried out to calculate the maximum specific growth rate in relation to the standard condition (YPD without stressful condition). Spot assay was also performed in the same conditions varying the cell concentration. *W. anomalus* and *M. guilliermondii* had comparable tolerance profile to the industrial strain of *S. cerevisiae* PE-2, one of the most important and employed selected strain for 1G ethanol production. Further studies regarding the growth and fermentative capabilities of these unconventional yeasts in sugarcane musts should be carried out to have a more complete frame of their potentiality for 1G ethanol industry.

Keywords: Acid stress. Osmotic stress. Thermal stress. Ethanol stress. Unconventional yeasts.

1 INTRODUCTION

Saccharomyces is the main genus of yeast used in various industrial processes due to its genetic diversity, which allows its use both in the form of cells and metabolites in biotechnological processes.[1,2]. *S. cerevisiae* species presents high fermentative and sugar metabolizing efficiency, rapid growth, ability to produce ethanol, tolerance to high concentrations of ethanol and to large temperature variations, and cellular activity in acidic environments, characteristics that are fundamental in industrial processes.[3].

Although *S. cerevisiae* species is the only one currently used in the production of ethanol from sugarcane musts, known as 1G ethanol, non-*Saccharomyces* yeasts may have some advantages over them, with greater adaptation to the environment and greater genetic diversity, which can result in better fermentation efficiency.[4]. The search for new isolates or selected/personalized yeasts can be a safe and viable alternative to increase the number of yeast strains that have fermentative efficiency with high potential for ethanol production in industrial conditions.[5]. Also, tolerance to stresses commonly found in 1G ethanol production such as osmotic, acidic, ethanol and thermal stresses may indicate the potentiality of employing non-*Saccharomyces* yeasts replacing *S. cerevisiae*.[4]. Therefore, the aim here was to evaluate the response to sugar concentration, temperature, pH, ethanol in stressful conditions of three non-*Saccharomyces* yeasts (*Wickerhamomyces anomalus*, *Meyerozyma guilliermondii* and *Schefersomyces stipitis*) in comparison with two industrial strains of *S. cerevisiae* (PE-2, CAT-1). This work is part of a project to evaluate the feasibility of using these non-*Saccharomyces* yeasts in the context of 1G ethanol including the fermentative and growth efficiencies in sugarcane musts such as sugarcane juice and molasses.

2 MATERIAL & METHODS

Three non-*Saccharomyces* (*S. stipitis* NRRL-7124, *W. anomalus* T1 – isolated from decaying wood, and *M. guilliermondiii* 311/CCT7783), and two industrial *S. cerevisiae* (PE-2, CAT-1) yeasts were utilized. All yeast strains were maintained in YPD slants at 4ºC and reactivated in fresh medium when necessary for the assays.

First experiment: Yeasts were cultivated in liquid YPD medium overnight at 30ºC, standardized at 10^7 cells/mL, and maintained in saline solution. Assays were performed in Corning® Costar sterile 96-well polystyrene plates with transparent flat bottom. Each well contained 20 µL of the standardized inoculum and 180 µL of sterile liquid YPD medium, in the conditions described in Table 1. The standard conditions were: YPD with 20 g/L glucose, pH 6-7, 0% ethanol, 30ºC. The assays were performed in triplicate, shaking the microplates for 24 h. Yeast growth was monitored by measuring absorbance at 600 nm every 15 min using a microplate reader (Tecan Infinite M200, Mannedorf, Switzerland). Maximum specific growth rate (μ_{max}, h^{-1}) was calculated from the plot 'in absorbance' versus 'time' in the exponential growth phase (μ_{max} = slope of the linear regression). The results were expressed as relative growth (%), which is the ratio in percentage of the μ_{max} in the stressful condition in relation to the μ_{max} in standard condition.

Second experiment: A spot assay test was utilized to verify the yeast growth in the stressful conditions in solid medium as in Table 1. Assays were carried out in duplicate inoculating 5 µL of each cell concentration (10^7, 10^6, 10^5, 10^4 cells/mL) for each yeast. The Petri dishes were incubated for 72 h.

Table 1 Composition of YPD medium and cultivation parameters for each stressful condition.

Condition	Glucose concentration (g/L)	Temperature (ºC)	pH	Ethanol concentration (% v/v)
Low pH	20	30	3.0	0
High sugar concentration	300	30	6-7	0
High ethanol concentration	20	30	6-7	10
High temperature	20	36	6-7	0

3 RESULTS & DISCUSSION

At a concentration of 300 g/L of glucose (about 2 times higher than that used in the industry in terms of reducing sugar), the yeast *S. cerevisiae* PE-2 was the only one that showed a growth rate higher than that shown in the standard medium. For the CAT-1 strain, *W. anomalus* and *M. guilliermondii*, the growth rate was between 80-90% of that presented in standard medium, without stress. The yeast *S. stipitis* showed only 40% of the growth rate of the standard medium in the presence of this stress (Figure 1b). The results of the spot assay confirm the low growth of this last yeast in medium with 300 g/L glucose (Figure 1a). *W. anomalus* is a yeast species capable of growing at low pH (<3.0) and high osmotic pressure (160 g/L glucose), remarkable characteristics to be exploited for biotechnological applications.[6].

As for the stress of low pH (3.0), there was a reduction in the growth rate for all yeasts, but the most sensitive were *S. cerevisiae* CAT-1 and *M. guilliermondii*. The other yeasts showed growth rates in the range of 50 to 75% of the rate in the medium without low pH stress (Figure 1d). The spot assay results show the growth of all yeasts in the medium with pH 3.0 (Figure 1c), however, when compared to the growth in the medium without stress (data not shown), there is a reduction in the size of the spot. Tolerance to pH 3.0 is a common characteristic among industrial strains used in the bioethanol industries and acts as an important selective pressure.[7,8,9].

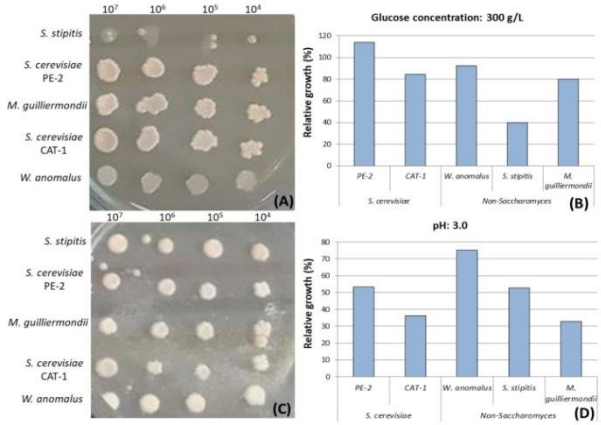

Figure 1 Spot assay (A, C) and relative growth (B, D) of the yeast strains under high glucose concentration (300 g/L) and low pH (3.0) in YPD medium. The number above the photos indicates the yeast cell concentration (cells/mL).

In the Brazilian 1G ethanol process, an ethanol concentration of up to 12% can be achieved[10], and it is important to have ethanol-resistant yeast. The yeasts that showed little affected growth rate (80 to 90% of the rate presented in the standard medium) by the addition of 10% ethanol (v/v) were *S. cerevisiae* PE-2, *W. anomalus* and *M. guilliermondii* (Figure 2b). The most sensitive was *S. stipitis*, also confirmed by the spot assay (Figure 2a). A low tolerance to ethanol is found in *S. stipitis* besides a tendency to assimilate ethanol even when sugar is present. Another drawback of this yeast species is the inability to grow anaerobically. Despite these facts, *S. stipitis* is one of the most efficient microorganisms for xylose fermentation, notably 2G ethanol.[11]. A strain of *S. stipitis* did not grow in medium with 35% glucose or 8% ethanol in spot assay.[12].

Under high temperature stress conditions (36°C) applied in this study, it was observed that the industrial yeasts PE-2 and CAT-1 had little affected growth rate (around 90% of the rate in standard medium), while for the yeasts *S. stipitis* and *W. anomalus*, a relative growth rate of less than 40% was observed (Figure 2d). The yeast *M. guillermondii* showed the highest thermotolerance among the yeasts tested, without the growth rate being affected by thermal stress. The maximum temperature tolerated by this species is 42ºC.[13]. In the spot assay, the effect of temperature on the growth of the yeasts *S. stipitis* and *W. anomalus* is evident in the lowest concentration of cells, 10^4 cells/mL (Figure 2c). In an industrial environment, where fermenter cooling is not sufficiently efficient in removing heat, and temperatures can reach up to 40°C[7], especially in summer, this ability to grow at high temperatures is an important characteristic for ethanol production processes.

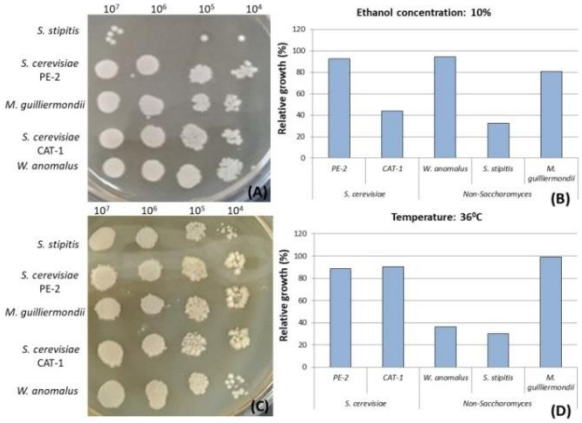

Figure 2 Spot assay (A, C) and relative growth (B, D) of the yeast strains under high ethanol concentration (10% v/v) and high temperature (36°C) in YPD medium. The number above the photos indicates the yeast cell concentration (cells/mL).

The stress tolerance to conditions commonly present in the bioethanol industry should be taken in account along with the efficient ethanol production. Among the non-*Saccharomyces* evaluated here, the species *W. anomalus* and *M. guilliermondii* had comparable tolerance profile than the industrial strain of *S. cerevisiae* PE-2. Further studies regarding the growth and fermentative capabilities of these unconventional yeasts in sugarcane musts should be carried out to have a more complete frame of their potentiality for 1G ethanol industry.

4 CONCLUSION

Regarding the stress tolerance to low pH, high temperature and high glucose and ethanol concentrations, the species *W. anomalus* and *M. guilliermondii* had comparable profile to the industrial strain of *S. cerevisiae* PE-2, one of the most important and employed selected strain for 1G ethanol production.

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