

**\*\* Isolation And Evaluation Of Thermotolerant Strains Of  
*Saccharomyces Cerevisiae* For Aguardiente And Rum Production \*\***

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**Abstract**

Thermotolerant strains of *Saccharomyces cerevisiae* were isolated from the residual yeast resulting from industrial fermentations subjected to thermal stress. The isolated strains were purified and characterised morphologically and biochemically. The thermal tolerance of one of the selected strains was evaluated in laboratory-scale batch fermentations at temperatures between 30 and 45 °C. The high ethanol yield (0.41 g g<sup>-1</sup>) at 40 °C demonstrated the thermotolerance of the selected strain. In six industrial-scale experimental runs the selected strain yielded a mean ethanol concentration of 5.2 % (v/v), which was higher than the 4.8 % (v/v) achieved with a reference industrial strain. Furthermore, a more complete substrate utilisation was achieved and the fermentation time was shortened from 16 to 12 h. The use of the thermotolerant strain resulted favorable for rum and aguardiente production since the formation of by-products, such as acetaldehyde, ethylacetate, methanol, n-propanol, isobutanol and isoamyl alcohol was reduced.

Rum is a drink that is produced from sugarcane molasses. To this end, the molasses is diluted and fermented wines and distillates were obtained with the formation of spirits, which, once aged, are used in the formulation of rums (Gonzalez et al., 2003). The strict control of fermentation temperature is essential in the production of rum to keep the quality of spirits is affected by the presence of side products formed as a result of heat stress and cell lysis. Tolerance to high temperature is a desired feature in the yeast used in tropical countries, in some seasons where the temperature exceeds the optimum values for growth mesophilic microorganisms.

For example, in Cuba during the summer the temperature reaches values of 32-35 °C, which, combined with the heating of the fermentation due to the exothermic metabolic reactions (Hernandez et al., 1986) makes the temperature in fermentors than 40 °C, causing heat stress and declining productivity of ethanol. The use of thermotolerant yeast or other organisms can reduce the thermophilic process as it avoids the use of expensive cooling systems (Laluce, 1991). In addition, avoids stops due to overheating, reducing the chance of infection and minimizes the volume of wastewater generated in the distilleries (Banat et al., 1998).

The phenomena associated with thermal tolerance (Benschoter and Ingram, 1986; Laluce, 1991) and tolerance to ethanol (Ingram and Butke 1984, D'Amore et al., 1990) of microorganisms have been investigated thoroughly. It has been reported that tolerance to ethanol and high temperatures are interactive: high concentrations of ethanol decreased the optimum temperature for growth and the

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increase in temperature increases the inhibitory effect of ethanol (Sa-Correia and van Uden, 1983; van Uden, 1989; Laluze, 1991, Banat et al., 1998). Toxic effects of temperature and ethanol are related to alterations in the permeability of cell membranes, intracellular protein denaturation and inhibition of transport

nutrients (D'Amore et al., 1990, Banat et al., 1998). The mechanism of cellular response to temperature stress and ethanol is essentially similar (Piper, 1995) and consists of changes in the composition of lipid membranes and protein synthesis transient heatshock (Thomas and Rose, 1979; Benschoter and Ingram, 1986). Among the strategies to improve thermal tolerance are the selection of strains (D'Amore et al., 1989, Ballesteros et al. 1991; Laluze et al., 1991), the adaptation of strains with increasing incubation temperature (Suutari et al., 1990), protoplast fusion (Sakanaka et al., 1996) and mutagenesis (Wati et al., 1996, Alvarez et al., 2003).

In the present work were isolated thermotolerant strains of *S. cerevisiae* yeast from the fermentation of the residual molasses at 40 °C. Was assumed that the viable cells that have survived the conditions of heat stress have increased their ability to grow and produce ethanol under these conditions. This research is aimed at solving the problem arisen by the temporary service the cooling system of the fermenters in a rum distillery. The objective was to isolate thermotolerant strains that allows the fermentation conditions of heat stress without affecting the quality of the spirits produced. This study complements previous investigations of the group on tolerance of yeast inhibitory compounds (Björklund et al. 2003; Martin Jönsson, 2003, Martin et al., 2007).

## MATERIALS AND METHODS

### Media

For the isolation of the strains used malta reinforced broth, consisting of 30 g L<sup>-1</sup> extract malta (biocides, Havana, Cuba), 1.5 g L<sup>-1</sup> of (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> (Merck, Darmstadt, Germany) with a pH of 4.5. The means employed for the evaluation of isolates were prepared from sugarcane molasses. For the spread of cultivation, molasses was diluted to a concentration of total sugars 120 g L<sup>-1</sup> and the pH was adjusted to 5.0 with H<sub>2</sub>SO<sub>4</sub> (Rayonitro, Matanzas, Cuba). For the fermentations, the concentration of total sugars in the medium was 160 g L<sup>-1</sup> and pH 4.0. The media were enriched with 1.5 g L<sup>-1</sup> of (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>.

### Isolation and purification of yeast thermotolerant

From the cream of *S. cerevisiae* obtained as residual from the fermentation stage of ethanol production

under conditions of heat stress in the distillery Arechavala (Cuba Ron, SA, Cardenas, Cuba) samples, which were kept at 4 °C until use.

Aliquots were inoculated in 1 mL of cream delevadura in 150 mL Erlenmeyer flasks containing 50 mL broth malta strengthened and incubated at 45 °C with shaking (120 rpm) for 15-20 hours until the turbidity measurements and optical density showed evidence of cell growth. Samples were taken from the flasks containing the results of positive growth and serial dilutions were 10<sup>-6</sup> up in physiological saline (9 g L<sup>-1</sup> NaCl). Since the crops were sown diluted by extension Drigalsky surface with spatula into Petri dishes with agar-malta. The plates were incubated at 35 °C for 48 h. After incubation colonies were selected characteristics of *S. cerevisiae*, which were in resemebradas wedges agarmaltapara subsequent purification by striations and exhaustion. Most of the colonies were isolated on agar-resiembras again malta for preservation at 4 °C.

### Evaluation of strains in the laboratory.

A roast of the strains under study were inoculated in 250 mL of medium contained in bottles of 1 L. The cultivation was spread to 35 °C for 12-14 h (until an optical density at 620 nm between 8-11) at 120 rpm in a heated chamber (ZWG Mytron, Germany) coupled with a shaker inside. Portions were transferred to 45 mL culture to 500 mL flasks containing 455 mL of fermentation medium. Jars with lids with rubber needle to remove the CO<sub>2</sub> produced, were incubated at 30, 35, 40 and 45 °C for 18 h. Samples were taken every 2 h to analyze the consumption of sugars and formation of ethanol. Fermentations were performed with the reference strain of *S. cerevisiae* used regularly in the distillery Arechavala. There were three replications of all fermentations.

### Evaluation of the strains on an industrial scale

Starting from the pure culture, the selected strain was inoculated into 300 mL of the medium itself for successive crops in increasing amounts up to half of 20 L. Subsequently, the culture obtained was inoculated into 100 L of sprouts at a nóculo medium of 1 / 10 and pH 4,0-4,2. When the sugar content was reduced by half was transferred workload growers L. 1500 Following the same criteria will be transferred to successive pre 35000 L fermenters as 360,000 L. Except in the fermenters, in all other cases provided air to maintain aerobic conditions that guarantee the biomass needed for fermentation. Fermenters were used without cooling system, so that the temperature

during fermentation exceeded 40 ° C. In all fermentation experiments were performed with a reference strain of *S. cerevisiae* Industrial.

### Analysis

The cell growth was determined by measuring the optical density with a spectrophotometer (Zuzi UV-4200, Spain) using a calibration curve relating the optical density with dry yeast biomass. To determine the dry biomass, 5 mL culture were filtered through filter paper of 45 m (Double Rings, Xinhua Paper Mill, PR China) previously weighed. The filters were dried at 105 ° C (MLW Labortechnik, Ilmenau, Germany) until constant weight and then weighed on an analytical balance (VEB Nagem, Gro vaagen, Berlin, Germany).

The ethanol content was determined by HPLC with a cationic resin column (LG KS-0802, Prague, Czech Republic) in the form hidrogeniónica operating at 45 ° C. The mobile phase was 5 mM H<sub>2</sub>SO<sub>4</sub> supplied 0.6 mL min<sup>-1</sup> with a high pressure pump (HPP 4001, Laboratorní Pøístroje, Prague, Czech Republic). Ethanol was detected with a differential refractometer (RID 101 Laboratorní Pøístroje). The yield of ethanol was calculated by dividing the final concentration of ethanol (g L<sup>-1</sup>) between the initial concentration of total sugars (g L<sup>-1</sup>). In the experiments to industrial scale, ethanol was analyzed by in situ methods areométricos, which express the percentage of ethanol in terms of a mass displacement of liquid equivalent to the mass of a Gay-Lussac areómetro immersed in a hydro-alcoholic solution (Carrazana, 1987).

The concentration of total sugars in the experiments conducted in the test was quantified by the method of phenol-sulfuric acid (Dubois et al., 1956). In the experiments to industrial-scale sugar and soluble solids were determined by refractometry Brix scale as in the classical methodology of the sugar industry (Spencer and Meade, 1945). We used an Abbe refractometer (Jena, Germany) in stages to spread the inoculum and methods areométricos in stages during the germination of growers, pre and fermenters.

The concentration of aldehydes, esters and total higher alcohols, as well as the acidity and susceptibility to oxidation, expressed as time permanganate were determined according to the quality standards of alcohol and spirits (Gonzalez and Porto, 1997). Minority component of both products, acetaldehyde, ethyl acetate, methanol, n-propanol, isobutanol and Isoamyl alcohol was analyzed by gas chromatography (Chrom 5, Laboratorní Pøístroje).

The statistical processing of results was performed using the statistical package Statgraphics Plus 2.1 for Windows.

## RESULTS AND DISCUSSION

Evaluated the strain was isolated from the residual cream following the procedure shown in Figure 1. During incubation in broth at 45 ° C malta in some jars were a cell growth was detected visually by increased turbidity and confirmed by the high optical density values in the spectrophotometric measurements. Crops with positive growth were observed with the aid of optical microscope and detected the presence of round and ovoid cells, typical of *S. cerevisiae* Pelczar et al., 1981). These results demonstrate that the isolated strains can be classified as *S. cerevisiae* that have improved thermal tolerance due to exposure to an environmental stress characterized by temperatures above their optimum growth, which ranges between 27 and 30 ° C.

Incubation at 35 ° C-malta agar plates, which previously had been performed sowing crops with lots of growth, isolated colonies that had helped form rosettes with elevation in the center and edge characteristic redoubled *S. cerevisiae*. Cologne was selected a well-defined, which was subsequently purified by depletion and designated as thermotolerant strain UM-11. The pure culture was resembrado tubes to preserve them in the bank and its characterization for morphological, biochemical and physiological.

The morphological characterization was performed by microscopic observations. For the biochemical characterization was assessed the ability of the strain to ferment sugars to ethanol. Physiological characterization was done taking into account the formation of products in fermentations carried out at different temperatures.

The concept of thermotolerant yeasts laramente is not defined in the literature (Laluce, 1991). According to some researchers (D'Amore et al., 1989, Lee et al., 1993) thermotolerant yeasts are those capable of growing at temperatures above 40 ° C. In other reports are considered a thermotolerant yeast growth in the range of 33-35 ° C (Morimura et al., 1997). Furthermore, yeast can grow at temperatures above 48 ° C are considered thermophilic (Watson, 1987, Banat et al., 1998). According to the above,

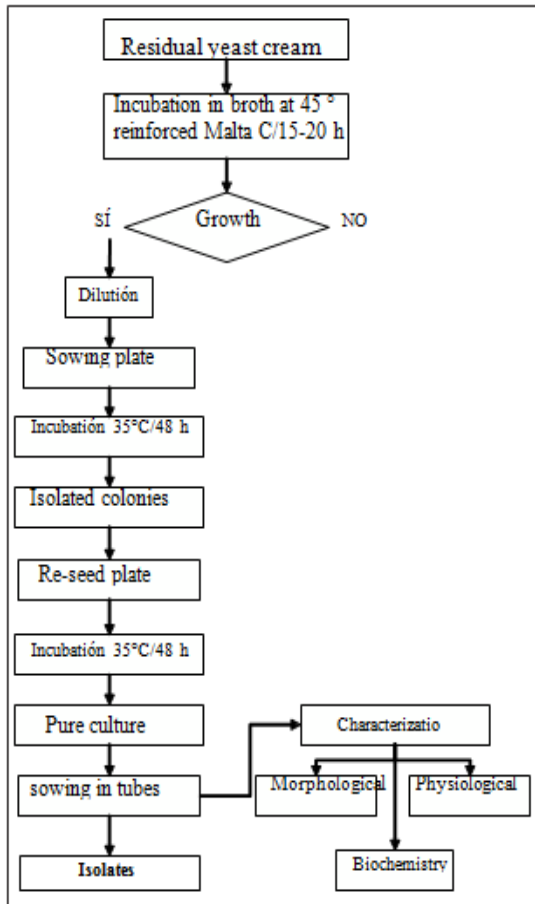


Figure 1. Flowchart for isolation and characterization of *S. cerevisiae* thermotolerant strains.

Table 1. Ethanol yield ( $Y_{E/AT}$ ) obtained in fermentations in lab scale of both strains.

Temperature (°C)	$Y_{E/AT}$ (g g <sup>-1</sup> )	
	Reference strain	Cepa UM-11
30	0,42	0,34
35	0,37	0,39
40	0,30	0,41
45	0,17	0,33

strains isolated in this work, which were able to grow at 45 °C may be considered as thermotolerant. To evaluate the thermal tolerance of thermotolerant strain UM-11, we examined the growing consumption of sugars and formation of ethanol fermentation at temperatures between 30 and 45 °C. The results were compared with those obtained with the reference strain

in similar conditions. Strain UM-11 presented a more favorable than the reference strain. In fermentations carried out with the thermotolerant strain, the final concentration of ethanol was 5.9% (v/v) at 35 °C and 6.2% at 40 °C (Figure 2), equivalent to yields of 0,39 and 0.41 g of ethanol per g of total sugars (Table 1). For its part, the best reference strain fermented at 30 °C and fermentation efficiency decreased continuously with increasing temperature. In the fermentations at 40 °C the yield of ethanol with the reference strain was 26.8% lower than that achieved with the strain UM-11. The difference was greater at 45 °C, where the yield of ethanol with the reference strain was reduced by 48.5% compared to the value reached with the thermotolerant strain.

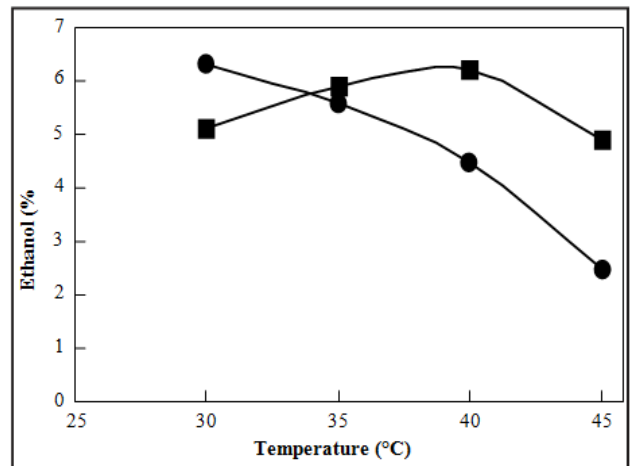


Figure 2. Final ethanol concentration produced during the fermentation of molasses in laboratory scale by UM-11 strain (squares) and the reference strain (circles) at different temperatures.

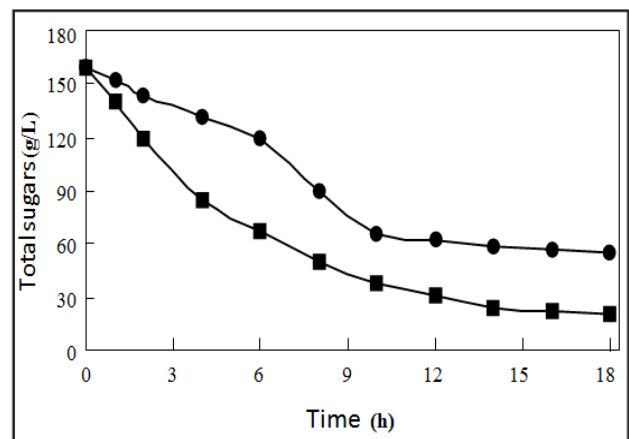


Figure 3. Sugar consumption during the fermentation of molasses in laboratory scale by UM-11 strain (square) and the reference strain (circles) at 40 °C.

**Table 2.** Ethanol and residual sugars content in fermented must obtained in six experimental runs at industrial scale.

Corrida	Reference strain		Thermotolerant strain	
	Ethanol (%, v/v)	Residual sugar (g L <sup>-1</sup> )	Ethanol (%, v/v)	Residual sugar (g L <sup>-1</sup> )
1	3,8	78,1	4,9	53,8
2	4,6	69,0	5,1	51,6
3	5,4	63,2	5,6	46,8
4	4,8	70,4	5,2	50,5
5	4,7	77,0	5,2	51,3
6	5,2	62,5	5,4	48,5
Average	4,8	70,0	5,2	50,4
Standard deviation	0,56	6,61	0,24	2,46

**Table 3.** Characteristics of the alcohol A produced by both strains.**Table 3.** Characteristics of the alcohol A produced by both strains.

Strain	Time of Permanganate (min)	Acidity (mg L <sup>-1</sup> )	Aldehydes (mg L <sup>-1</sup> )	Esters (mg L <sup>-1</sup> )	Polyalcohols (mg L <sup>-1</sup> )
Referencia	31,6	12,6	9,4	30,0	52,9
UM-11	30,6	10,9	5,4	26,8	47,7

The dynamics of consumption of sugars is another evidence of the superiority of the thermotolerant strain. In experiments conducted at 40 ° C with a UM-11 strain showed a rapid consumption of sugars from the start of fermentation (Figure 3). For its part, with the reference strain showed a delay in the start of fermentation, and only did a significant consumption of sugar between 6 and 9 h of incubation. The statistical significance of these results was confirmed using the software Statgraphics Plus 2.1 for Windows.

These results revealed the potential of the strain UM-11 for use in the industrial process of ethanol production in Cuba. However, taking into account the high efficiency of the industrial strain at temperatures around 30 ° C, it would be interesting to study mixed cultures in which the strain is the main industrial fermentative organism at the start of fermentation, and play a thermotolerant strain role in the hours when temperatures are higher.

To confirm the potential of the strain UM-11 evaluation was performed in industrial fermenters under heat stress (without cooling system) where the temperature exceeds 40 ° C. The evaluation revealed significant differences ( $p = 0.01$ ) with respect to the strain in terms of industrial ethanol production, depletion of the substrate and fermentation time. On average, the final concentration of ethanol obtained from six experimental runs the distillery was 5.2% (v / v) with the thermotolerant strain and 4.8% (v / v) with strain industry (Table 2). The exhaustion of molasses was higher

with the strain UM-11, with which the residual sugar concentration was approximately 50 g L<sup>-1</sup>, whereas the industrial strain was higher than 70 g L<sup>-1</sup>. Although the concentrations reported correspond to the values obtained after 16 h, it should be noted that the strain UM-11 fermentation practically ended at 12 h. These results confirm the thermotolerancia of strain UM-11 bearing in mind that industrial-scale fermentations were performed in fermenters refrigerated. Strain UM-11 was able to withstand the severe conditions to which it was submitted and was able to metabolize sugars achieving greater conversion to ethanol and further depletion of the substrate at a lesser time, which is extremely important from an economic point of view.

To check the feasibility of using strain UM-11 in the industrial production of rum, analyzed different parameters of quality of products obtained from the distillation of fermented grape molasses (Gonzalez and Porto, 1997). In particular, it investigated the alcohol A, which consists of high quality ethanol for beverage and liquor, which is a basic component in the formulation of rums. The results shown in Table 3 indicate that the acidity and concentrations of aldehydes, esters and higher alcohols to produce alcohol with the thermotolerant strain were lower than the values of the alcohol produced by the reference strain. Differences in these parameters were statistically significant at a significance level equal to 0.01. The differences in time of permanganate were not significant.

**Table 4.** Minor components in alcohol A and aguardiente (g L<sup>-1</sup>).

Strains	Alcohol A		Aguardiente	
	Reference strain	strain UM-11	Reference strain	strain UM-11
Acetaldehído	0,007	0,004	0,026	0,017
Acetato de etilo	0,009	0,007	0,067	0,052
Metanol	0,008	0,004	0,010	0,003
n-Propanol	0,059	0,051	0,185	0,141
Isobutanol	0,014	0,011	0,249	0,196
Alcohol isoamílico	0,000	0,000	0,909	0,660

Chromatographic identification of the components revealed that minority spirits obtained with thermotolerant strain showed concentrations of acetaldehyde, ethyl acetate and methanol than in 0009, 0015 and 0007 g L<sup>-1</sup>, respectively, the concentrations of these components in the liquors obtained with the reference strain (Table 4). For the n-propyl alcohol, isobutyl Isoamyl and the difference was more marked. The same trend was observed for all compounds analyzed in alcohol A. These results indicate that the replacement of the yeast strain used regularly at the distillery by thermotolerant strain selected to obtain liquor and alcohol at a higher quality, which in turn should affect the quality of the rums produced.

The best results in terms of product composition are a consequence of the increased thermal tolerance of the strain UM-11, which can survive the fermentation process at temperatures above 40 °C without cell lysis, preventing the dumping of material intracellular environment. In addition, shorter time and the adaptation of the strain at high temperature decreases the formation of byproducts that are formed due to side effects in an environment unfavorable to the cells.

## CONCLUSIONS

This work demonstrated a procedure for the selection of thermotolerant yeast strains from the fermentation of sugarcane molasses for the production of spirits and rums.

The introduction into the industry from a strain selected for its thermal tolerance fermentations to achieve more efficient with a faster production of ethanol, a further depletion of the substrate and a better quality of the products obtained.

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