Brazilian Journal of Microbiology (2002) 33:202-208
ISSN 1517-8382

TREHALOSE ACCUMULATION, INVERTASE ACTIVITY AND PHYSIOLOGICAL CHARACTERISTICS OF YEASTS ISOLATED FROM 24 H FERMENTATIVE CYCLES DURING THE PRODUCTION OF ARTISANAL BRAZILIAN *CACHAÇA*

Carla Pataro[1]; Juliana B. Guerra[2]; Fátima C.O. Gomes[2]; Maria J. Neves[3]; Patrícia F. Pimentel[1]; Carlos A. Rosa[2]*

[1]Setor de Biotecnologia e Tecnologia Química, Fundação Centro Tecnológico de Minas Gerais (CETEC), Belo Horizonte, MG, Brazil. [2]Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil. [3]Comissão Nacional de Energia Nuclear (CNEN), Belo Horizonte, MG, Brazil.

Submitted: August 21, 2001; Returned to authors for corrections: January 09, 2002; Approved: July 26, 2002

ABSTRACT

Trehalose accumulation, invertase activity and physiological characteristics of 86 yeast isolates from short fermentative cycles during the production of *cachaça* in three artisanal distilleries of the State of Minas Gerais were studied. Among these isolates, 70% were able to grow at temperatures between 40 and 42ºC. Only *Saccharomyces cerevisiae* isolates were able to grow over 40ºC. Lower temperatures (<40ºC) favoured the growth of yeasts such as *Candida parapsilosis*-like, *C. maltosa*-like, *Kloeckera japonica, S. exiguus* and *C. bombicola*-like. The isolates from all three distilleries were ethanol tolerant, produced invertase, and accumulate trehalose in the presence of glucose. The strains isolated from distillery A presented more resistance to ethanol (around 84.2% of the strains were able to grow in the presence of 12% ethanol) when compared to the ones from distilleries C and B (9.5% and no strain, respectively). The strains of *S. cerevisiae* isolated from the three distilleries presented a higher capacity to produce invertase and accumulate trehalose in the presence of glucose. Based on the results of thermal and ethanol stress experiments, it was possible to identify strong relationship between intracellular trehalose accumulation and cell viability. The increase in cell viability was even more pronounced when the strains were subjected to a pre-treatment at sublethal temperatures.

Key words: *Saccharomyces cerevisiae, cachaça,* trehalose, invertase, fermentation.

INTRODUCTION

The production of many different alcolholic beverages involves spontaneous fermentations caused by microorganisms present in the raw materials and/or the equipments (10). Such a natural fermentation process takes place in the production of sugar cane *cachaça* (*aguardente*) in Minas Gerais State. *Cachaça* production in Minas Gerais amounts to approximatelly 210 milion liters per year, through the distillation of the fermented sugar cane must. This production occurs from May to December, during the sugar cane cropping period. One of the main peculiarity of the artisanal production of *cachaça* is the preparation of the starter ferment, which consists of the propagation of the fermentative microbiota through a mix of sugar cane juice with corn, rice and/or soya flour. The process occurs inside the fermentation vat and can last from 5 to 20 days, until the yeast population is sufficient to initiate the fermentative cycle. The fermentative cycle results in the consumption of the total sugar present in the sugar cane juice and occurs in a period that can vary between 18 to 48 hours. The yeast communities present in such fermentations are in constant succession, and the species present in the fresh sugar cane juice are constantly introduced in the microenvironment of the fermentation. *Saccharomyces cerevisiae* is the predominant species at the end of starter preparation and during the fermentative cycle (14,18).

* Corresponding author. Mailing address: Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Caixa Postal 486, Pampulha, Belo Horizonte, MG, Brasil. Fax: (+5531) 3499 2730; E-mail: carlrosa@icb.ufmg.br.

The physiological abilities of the yeast strains isolated from fermented must have a special relevance in understanding the mechanisms involved in must colonization, and determining the optimum conditions to maintain healthy fermentations (17). In *S. cerevisiae*, a strong correlation between trehalose content and stress resistence has been demonstrated in different strains, in a variety of growth conditions, during sublethal heat treatment and other stress conditions (4,24). Some of the unique features of *cachaça* fermentation, namely a short fermentative cycle with the daily addition of sugar cane juice, a high ethanol concentration and a high fermentation temperature may be responsible for the selection of highly adapted yeast strains. In this work, yeasts isolated during 24 h-fermentation cycles of three *cachaça* distilleries in the State of Minas Gerais were studied for their osmotolerance, ability to grow and ferment at high temperatures, alcohol tolerance, invertase activity, and trehalose accumulation under normal and stressful conditions.

MATERIALS AND METHODS

Eighty-six yeasts were isolated from three *cachaça* distilleries in State of Minas Gerais as described in Pataro *et al*. (18). The samplings were made at 4 h intervals during the 24 h fermentative cycle of *cachaça* production. The strains were characterized according to standard methods (8,23), and identified by the keys of Barnett *et al*. (2) and Kurtzman and Fell (9).

The yeast isolates were grown on modified Sabouraud agar (D-glucose 2%, peptone 1%, yeast extract 0.5%, and agar 2%) at room temperatures for 24 h, and 0.1 ml of a suspension containing $1x10^7$ cells was inoculated in 5 ml of the following culture media: Sabouraud broth with 15, 20, 25, 40 and 50% of glucose to test osmotolerance; YM broth (yeast extract 0.3%, malt extract 0.3%, peptone 0.5% and glucose 1%) with 8, 10, 12 and 13 g/l of ethanol (ethanol was added after sterilization and the tubes were covered with a parafilm to avoid ethanol evaporation); and in Sabouraud broth incubated in water bath at 35 to 43ºC to evaluate the maximum growth temperature. Osmotolerance and ethanol resistance were determined at room temperature (25 ± 3ºC). Yeast growth was evaluated from the turbidity of the liquid medium. Fermentation at high temperature was tested in Durham tubes containing fermentation basal medium (peptone 0.75%, yeast extract 0.45% and 2% glucose) and incubated at temperatures from 37 to 45ºC at one degree intervals. The maximum temperature of fermentation was taken to be the highest temperature at which gas production occurred after 24 hours of incubation (13).

The invertase activity was measured by the dinitrosalicilic (DNS) colorimetric test as described in Ekunsanmi and Odunfa (5). Each yeast was grown on Sabouraud agar for 48 hours, the cells were diluted in sterile water, washed by centrifugation, and 0.1 g wet weight of each was resuspended in 10 ml of acetate buffer, pH 5.0. One mililiter of each cell suspension was added to 2 ml of 4% sucrose solution in the same buffer and incubated for 5 min. at 30ºC. One unit of invertase activity was defined as the amount of enzyme which liberated one µmol/min of reducing sugars under these conditions.

For trehalose accumulation tests, *S. cerevisiae* cells were grown in 50 ml YEPD (1% yeast extract, 2% peptone and 2% D-glucose) at room temperature for approximately 24h until glucose was consumed. The cells were filtered through 0.45 µM Millipore membranes and the cell pellet was scraped off the membranes and immediately frozen in a vessel with liquid nitrogen. Trehalose was determined enzymatically with a trehalase preparation extracted from *Humicola grisea* essentially as described by Neves *et al.* (15,16), and glucose liberated was assayed by the glucose-oxidase method (7). A trehalase unit was defined as the amount of enzyme which released one mmol glucose per min under the assay conditions.

Two strains of *Saccharomyces cerevisiae* were selected from each studied distillery, on the basis of the highest and lowest trehalose content, to be subjected to the thermal and ethanol stress tests. The heat shock test and ethanol stress test were performed as described in Ribeiro *et al*. (19). For heat shock experiments, the cultures harvested in the presence of glucose were aseptically divided in two aliquots. The first aliquot was given a 50ºC treatment in a water bath for 8 min. The second portion was first preconditioned at 40ºC for 60 min and then immediately exposed to the 50ºC heat shock for 8 min. For ethanol stress experiments, an aliquot containing 100 mg of cells (dry wt) in stationary phase was transferred to a sterile erlenmeyer flask and absolute ethanol was added to a final concentration of 10% (v/v) – modified from Mansure *et al*. (12) – and incubated at 28ºC at 160 rpm for 24 h. Trehalose accumulation was measured as described previously. Samples for trehalose measurements and cell viability were taken just before and immediately after the treatments for ethanol stress and heat shock. Cell suspensions were appropriately diluted, and spread onto plates containing YEPD agar in triplicate. Tolerance to each treatment was expressed as the percentage of cell survival calculated from the difference in colony forming units (CFU) before and after the treatment.

RESULTS AND DISCUSSION

Most of the isolates (90.7%) were able to grow in the presence of 40% glucose, but none were able to grow in 50% glucose media. The yeasts that were not able to grow in 40% glucose were isolated only in distillery B and correspond to the following species: *C. maltosa*-like, *C. parapsilosis*-like, *C. rugopelliculosa*, *C. azyma* and *K. japonica*. Table 1 presents the results obtained from the physiological characterization tests. Most of the strains from distilleries A and B were able to grow at temperatures between 40 and 42ºC but only 18.4% (distillery A) and 25.9% (distillery B) were able to grow at temperatures under 40ºC, representing mainly non-*Saccharomyces* yeast

Table 1. Physiological characteristics of yeast isolated during the fermentative cycle (24 hours) in *cachaça* distilleries A, B and C.

Time[a] (h)	Species/strains (UFMG97 A)	Log[b] (cell numbers)	MGT[c]	MFT[d]	Ethanol tolerance (%etanol)	Invertase production[e]	Trehalose accumulation[f]
	Distillery A						
T0	1607- *Saccharomyces cerevisiae*	9.23	39	43	12	60.0 ± 2.47[g]	47.5 ± 0.02
	1608- *S. cerevisiae*	7.6	41	43	12	20.0 ± 0.70	44.9 ± 0.11
	1609- *C. bombicola*-like[h]	8.2	40	43	12	58.6 ± 1.77	67.9 ± 0.01
	1610- *S. cerevisiae*	9.11	39	43	12	77.2 ± 0.00	55.4 ± 0.01
	1611- *S. cerevisiae*	8.64	41	45	12	40.7 ± 0.14	59.6 ± 0.01
	1612- *S. cerevisiae* gly+	7.57	41	45	12	27.2 ± 0.51	57.1 ± 0.05
T1	1613- *S. cerevisiae*	8.08	41	43	12	44.8 ± 0.42	67.7 ± 0.03
	1614- *S. cerevisiae*	6.52	41	45	12	26.4 ± 1.70	8.6 ± 0.03
	1615- *C. bombicola*- like	7.4	40	45	12	62.0 ± 0.92	32.2 ± 0.01
	1616- *S. cerevisiae*	8.11	41	43	12	49.2 ± 0.28	55.6 ± 0.10
	1617- *S. cerevisiae* gly+	7.61	41	45	12	43.4 ± 0.14	5.8 ± 0.51
	1618- *S. cerevisiae*	6.23	41	45	12	3.6 ± 1.05	27.7 ± 0.25
T2	1619- *S. cerevisiae*	8.20	41	45	12	69.2 ± 1.30	49.2 ± 0.04
	1620- *S. cerevisiae*	6.70	41	41	12	46.2 ± 2.61	15.6 ± 0.33
	1621- *C. bombicola*-like	7.30	40	45	12	52.0 ± 0.84	29.6 ± 0.10
	1622- *S. cerevisiae*	8.18	39	41	12	73.2 ± 0.28	20.7 ± 0.01
	1623- *S. cerevisiae*	7.64	41	45	12	62.0 ± 1.20	30.4 ± 0.15
	1624- *S. cerevisiae*	6.36	40	45	8	18.6 ± 1.90	43.7 ± 0.11
T3	1626- *S. cerevisiae*	8.41	39	40	12	69.2 ± 1.76	60.0 ± 0.12
	1627- *S. cerevisiae* sal+	6.36	39	45	12	71.2 ± 0.07	45.3 ± 0.02
	1628- *C. bombicola*- like	7.52	39	42	12	69.2 ± 0.77	63.9 ± 0.75
	1629- *S. cerevisiae* gly+	8.15	39	42	12	52.0 ± 0.54	64.4 ± 0.01
	1630- *S. cerevisiae* sal+	7.54	41	45	10	15.4 ± 0.85	50.5 ± 0.35
	1631- *S. cerevisiae* sal+	6.30	40	45	12	11.4 ±	66.5 ± 0.21
T4	1632- *S. cerevisiae* sal+	7.46	39	41	12	77.2 ± 0.30	46.3 ± 0.51
	1633- *S. cerevisiae* sal+	5.48	39	42	12	17.7 ± 1.41	26.1 ± 0.01
	1634- *S. cerevisiae* sal+	7.32	41	42	12	57.0 ± 1.34	8.2 ± 0.01
	1635- *S. cerevisiae* gly+	6.89	39	45	8	45.3 ± 0.35	7.6 ± 0.20
	1636- *S. cerevisiae* sal+	6.36	41	45	12	15.0 ± 1.48	3.0 ± 0.12
T5	1637- *S. cerevisiae* gly+	7.20	39	45	12	62.0 ± 1.83	2.1 ± 0.01
	1638- *C. bombicola*- like	6.43	39	45	12	69.2 ± 1.83	2.4 ± 0.15
	1639- *S. cerevisiae* sal+	7.11	39	42	12	71.2 ± 1.69	2.6 ± 0.02
	1640- *S. cerevisiae* sal+	6.60	41	45	8	14.2 ± 0.42	3.2 ± 0.15
T6	1641- *S. cerevisiae*	7.11	39	42	12	57.0 ± 0.28	0.35 ± 0.01
	1642- *S. cerevisiae*	6.11	39	45	8	21.2 ± 0.78	1.4 ± 0.10
	1643- *S. cerevisiae*	7.04	39	43	12	31.9 ± 0.92	1.7 ± 0.01
	1644- *S. cerevisiae*	6.36	39	45	12	60.0 ± 1.2	8.5 ± 0.00
	1645 *S. cerevisiae*	5.48	39	45	10	5.3 ± 0.77	1.8 ± 0.01
	Distillery B						
T0	1661- *S. cerevisiae*	7.41	41	42	10	28.7 ± 0.70	2.2 ± 0.01
	1662- *C. maltosa*- like	5.48	40	40	6	0.8 ± 1.15	0.4 ± 0.01
	1663- *S. cerevisiae*	7.30	39	42	10	19.1 ± 0.00	0.0 ± 0.00
	1665- *C. parapsilosis* -like	5.48	40	40	6	0.9 ± 0.03	0.0 ± 0.00
	1666- *C. rugopelliculosa*	5.48	40	42	6	1.3 ± 1.15	0.3 ± 0.01

Table 1. continuation...

T1	1667- *S. cerevisiae*	8.53	42	45	8	60.2 ± 1.13	0.7 ± 0.02
	1668- *C. guilliermondii*	6.48	37	40	6	2.1 ± 0.00	5.3 ± 0.04
	1669- *S. exiguus*	8.76	40	43	8	52.0 ± 0.70	15.4 ± 0.03
	1670- *C. azyma*	6.48	41	45	8	38.6 ± 1.34	0.0 ± 0.00
	1671- *S. cerevisiae*	5.48	40	42	8	17.0 ± 1.41	0.0 ± 0.00
	1672- *C. azyma*	4.78	37	40	6	1.0 ± 0.56	0.0 ± 0.00
T2	1673- *S. cerevisiae* ma-tr-	8.72	41	45	8	25.3 ± 1.49	10.0 ± 0.33
	1674- *S. cerevisiae*	8.53	41	42	8	17.5 ± 1.83	5.3 ± 0.02
	1675- *C. parapsilosis*- like	4.48	37	40	6	1.1 ± 1.41	0.8 ± 0.15
T3	1656- *S. cerevisiae*	9.04	42	41	10	17.6 ± 0.85	7.8 ± 0.22
	1657- *S. cerevisiae* ma-	8.98	42	45	10	28.7 ± 0.65	5.3 ± 0.01
	1658- *S. cerevisiae* tr-	5.48	41	45	10	26.4 ± 0.22	0.3 ± 0.01
	1659- *C. maltosa*- like	4.48	37	40	6	5.0 ± 1.27	0.2 ± 0.00
	1660- *C. maltosa*- like	4.48	37	40	6	7.1 ± 0.63	20.7 ± 0.01
T4	1676- *S. cerevisiae*	9.30	41	43	8	73.2 ± 2.12	9.7 ± 0.11
	1677- *S. cerevisiae* ma-	9.30	40	42	10	31.9 ± 1.87	0.3 ± 0.01
	1678- *K. japonica*	4.78	39	37	6	0.8 ± 0.42	0.6 ± 0.01
T5	1680- *S. cerevisiae* gly+	9.18	40	45	8	46.2 ± 1.34	10.3 ± 0.03
	1681- *S. cerevisiae* gly+	9.41	41	45	10	21.0 ± 0.43	25.8 ± 0.88
T6	1682- *S. cerevisiae* ra-	9.26	41	45	8	26.9 ± 1.48	9.5 ± 0.01
	1683- *S. cerevisiae*	9.89	41	43	8	24.6 ± 0.64	15.8 ± 0.27
	1684- *S. cerevisiae*	5.48	42	43	8	49.2 ± 0.99	5.2 ± 0.31
	Distillery C						
T0	1685- *S. kluyveri*	8.15	41	43	8	40.7 ± 0.92	7.3 ± 0.02
	1686- *S. cerevisiae* rb+gly+	8.90	41	45	8	27.2 ± 0.65	10.0 ± 0.31
	1687- *S. cerevisiae* rb+gly+	7.65	41	45	8	31.9 ± 0.76	14.1 ± 0.20
	1688- *S. cerevisiae* rb+gly+	7.85	41	45	8	21.7 ± 0.77	4.8 ± 0.01
T1	1689- *S. cerevisiae* rb+gly+	7.48	41	45	8	38.6 ± 2.26	13.1 ± 0.23
	1690- *S. cerevisiae* rb+gly+	7.70	41	45	8	24.6 ± 0.14	5.4 ± 0.22
	1691- *S. cerevisiae* rb+gly+	7.54	41	45	8	47.8 ± 0.35	21.5 ± 0.02
	1692- *S. cerevisiae* rb+gly+	7.72	41	45	10	19.1 ± 1.98	21.9 ± 1.01
T2	1694- *S. cerevisiae* rb+gly+	7.46	42	45	8	29.3 ± 0.65	25.2 ± 1.01
	1695- *S. cerevisiae* rb+gly+	7.82	41	45	8	9.5 ± 1.70	28.6 ± 0.98
	1696- *S. cerevisiae* rb+gly+	7.86	41	42	10	60.2 ± 1.70	24.3 ± 1.70
T3	1697- *S. cerevisiae* ra+tr-	8.32	41	45	8	32.8 ± 0.28	19.4 ± 1.21
	1698- *S. cerevisiae* ra+tr-	9.04	40	43	8	22.4 ± 0.42	9.1 ±1.31
	1699- *S. cerevisiae* tr-gly+	8.36	41	42	10	42.9 ± 0.21	10.2 ± 0.21
	1700- *S. cerevisiae* tr-gly+	9.40	42	45	8	31.9 ± 1.90	19.6 ± 0.97
	1701- *S. cerevisiae* tr-gly+	6.78	40	42	8	26.9 ± 0.07	4.2 ± 0.21
T4	1702- *S. cerevisiae* tr-gly+	8.70	41	45	8	18.3 ± 1.90	22.6 ± 0.04
	1703- *S. cerevisiae* tr-gly+	9.48	42	45	12	19.0 ± 1.34	9.1 ± 0.01
	1704- *S. cerevisiae* tr-gly+	8.70	42	45	12	44.8 ± 1.69	12.2 ± 0.02
	1705- *S. cerevisiae* tr-gly+	9.48	41	42	8	45.3 ± 1.20	7.4 ± 0.12

a-T0-addition of juice; T1-4hours, T2-8hours, T3-12hours, T4-16hours, T5-20hours, T6-24hours after addition of juice; **b**- $\log_{10}$ number of cells; **c**- MGT- Maximum growth temperature; **d**- MFT- Maximum fermentation temperature; **e**- Invertase activity was expressed in µmol reducing sugar/mg cell/min. at 30ºC; **f**- Trehalose accumulation was expressed in µmol glucose/g wet weight; **g**- Mean ± standard error; **h**- Probable new yeast species physiologically similar to the species indicated.

strains. The maximum fermentation temperature was normally greater than the maximum growth temperature. The highest value for fermentation was 45ºC. Most of the isolates from distillery A (84.2%) grew in the presence of 12% ethanol whereas in distillery C only 9.5% from the isolates were able to grow in that ethanol concentration. In contrast, none of the distillery B isolates could grow in the presence of 12% ethanol. The *cachaça* fermentation microenvironment therefore appeared to select in favour of osmotolerant and ethanol resistent yeasts. The non-*Saccharomyces* yeasts that were unable to grow at high sugar and ethanol concentrations were similar to those isolated from sugar cane plantation soils by Corrêa (3). These data suggest that the non-*Saccharomyces* species are allochthonous members of the fermentative process, and these yeasts can be introduced daily with the addition of sugar cane juice. However, they disappear in the early phases of the fermentative cycle, being supplanted by *S. cerevisiae*. The majority of isolates had a maximum growth temperature of 40ºC. The average temperature of *cachaça* fermentations in Minas Gerais is *ca*. 30ºC, reaching as much as 42ºC in the hottest regions of the state. Consequently, the presence of the thermotolerant strains in this environment is fundamental to the progress of the fermentative process.

The capacity to produce invertase (Table 1) was greatest in *S. cerevisiae*, with a maximum activity of 77.2 µmol reducing sugar/mg cell/min. The lowest invertase activity value (0.8 µmol reducing sugar/mg cell/min.) was detected in a *C. maltosa*-like strain and a *K. japonica* strain. Our *S. cerevisiae* isolates showed greater invertase activity than that of a strain of *S. cerevisiae* isolated from the cassava tuber by Ekunsanmi and Odunfa (5), which had an invertase activity of 30.4 mmol reducing sugar/mg cell/min. Pataro *et al*. (17) isolated *S. cerevisiae* strains in other *cachaça* fermentations with an invertase activity greater than 100 µmol reducing sugar/mg cell/min. The daily addition, to the fermentation vats, of sugar cane juice with aproximatelly 16% sugar makes this environment selective for yeast that are efficient in the conversion of non-reducing sugars.

Most of the *S. cerevisiae* strains exhibited a great capacity to accumulate trehalose during the stationary growth phase (Table 1). *S. cerevisiae* strains from Distillery A had the highests levels of trehalose accumulation compared to those from distilleries B and C. Yeasts colonizing the fermentation microenvironment of artisanal *cachaça* production are always under thermal, osmotic and ethanol stress. Recently, it has been shown that intracellular trehalose levels can determine survival potential of yeasts in extreme environmental conditions (1,15). Trehalose affords protection against stress *in vitro* and *in vivo* when cells are in stationary state or under stress (22). Many *Saccharomyces* strains exhibit a strong connection between trehalose accumulation and resistence to stress (5,6,11,20). Around 40% of the isolated strains accumulated more than 40 mmol glucose/g wet weight in the stationary growth state. This trehalose accumulation capacity may be involved in the survival of strains in *cachaça* fermentation environments.

Tables 2 and 3 show the intracelular trehalose accumulation values and the viability of the *S. cerevisiae* strains under thermal and ethanol stress, respectively. The strains studied showed significant levels of trehalose accumulation when shifted to a preconditioning treatment at 40ºC for 60 min. A strong relationship between high viability and high trehalose accumulation was observed for all strains tested, specially when shifted to a sublethal temperature preconditioning treatment. Yeast strains submitted to an initial heat shock at 50ºC for 8 min. showed little resistence to severe heat shock and a very low trehalose level. All the yeast tested showed low viability when transferred directly from 28ºC to 50ºC for 8 min (Table 2). Hottiger *et al.* (6) reported that a temperature change from 27 to 40ºC in growing cells of *S. cerevisiae* caused a trehalose accumulation from 0.01 g g^{-1} protein to 1 g g^{-1} protein over a period of 60 min. Also a six-fold increase of intracellular trehalose concentration was found by Lewis *et al*. (11) when *S. cerevisiae* cells were exposed to a heat shock of 37ºC lasting 45 min. The strong relationship between the trehalose accumulation and strain

Table 2. Intracellular trehalose accumulation and cell viability of *Saccharomyces cerevisiae* strains submitted to heat stress.

Strains	µ mol de glucose/g wet weight			Cell viability[d]		
S. cerevisiae	Control[a]	Pre-treatment[b]	Heat shock[c]	Control	Pre-treatment[e]	Heat shock[f]
UFMG-A1613	2.07 ± 0.88[g]	84.04 ± 1.75	10.28 ± 0.29	75.0 ± 0.54	0.63 ± 0.26 (0.84)	0.97 ± 0.20 (1.29)[h]
UFMG-A1641	1.78 ± 0.20	59.63 ± 5.61	7.65 ± 0.25	36.0 ± 0.55	1.40 ± 0.05 (3.88)	2.00 ± 0.71 (5.55)
UFMG-A1671	1.46 ± 0.00	54.61 ± 0.47	7.27 ± 0.13	0.4 ± 0.14	0.095 ± 0.01 (23.75)	0.12 ± 0.00 (30.0)
UFMG-A1681	1.06 ± 0.11	34.56 ± 0.85	15.58 ± 0.73	31.5 ± 0.00	1.30 ± 0.05 (4.12)	1.30 ± 0.02 (4.12)
UFMG-A1695	2.50 ± 0.22	87.68 ± 2.00	19.61 ± 0.87	1.0 ± 0.00	0.70 ± 0.00 (70.0)	0.99 ± 0.00 (99.0)
UFMG-A1701	0.21 ± 0.00	47.28 ± 2.60	3.10 ± 0.66	1.0 ± 0.01	0.07 ± 0.00 (7.0)	0.087 ± 0.00 (8.7)

a- Exponential growth cells at 28ºC; **b**- Pre-treatment: exponential phase cells incubated at 40ºC for 1 h and immediately transferred to 50ºC for 8 min; **c,f**- Heat shock: exponential phase cells incubated directly at 50ºC for 8 min; **d**- Values in 10^8 cfu ml^{-1}; **e**- Viability of exponential cells first incubated at 28ºC, and then at 40ºC for 1 h; **g**- Mean ± standard error; **h**- Survival percentage in relation to no stress control.

Table 3. Trehalose accumulation and cell viability in *Saccharomyces cerevisiae* strains isolated from *cachaça* distilleries after ethanol stress.

S. cerevisiae strains	Trehalose accumulation[a]		Cell viability[b]	
	Control[c]	Ethanol stress[d]	Control	Etanol stress
UFMG-A 1613	30.86 ± 1.23[e]	26.0 ± 2.26	22.60 ± 0.50	0.04 ± 0.00 (0.15)[f]
UFMG-A 1441	39.35 ± 0.57	31.83 ± 0.61	280.00 ± 1.01	1.50 ± 0.03 (0.5)
UFMG-A 1671	28.24 ± 0.75	17.35 ± 1.27	2.00 ± 0.00	0.03 ± 0.00 (1.4)
UFMG-A 1681	44.75 ± 0.35	18.51 ± 0.27	4.91 ± 0.12	0.43 ± 0.01 (8.7)
UFMG-A 1695	40.68 ± 1.80	23.64 ± 3.69	39.01 ± 1.35	0.03 ± 0.00 (0.07)
UFMG-A 1701	14.93 ± 1.40	9.60 ± 1.53	0.50 ± 0.00	$<10^5$ (<0.2)

a- µmol glucose/g wet weight; **b**- Values in 10^8 cfu ml^{-1}; **c**- Cells in stationary phase; **d**- Cells mantained for 24 hs in 10% ethanol; **e**- Mean ± standard error; **f**- Survival percentage in relation to no stress control.

survival under thermal stress conditions observed in our studies may play an important role in the predominance of certain strains the fermentation processes. During *cachaça* fermentation the temperarture can reach 42ºC inside the vats, making this environment selective to yeasts that possess good survival mechanisms under these conditions.

The intracellular trehalose content decreased after an ethanol stress in all of the strains tested (Table 3). Strain UFMG97-A1681 had the highest capacity to accumulate trehalose and the greater cell viability after ethanol addition. This strain showed a trehalose decrease of approximately 60% after the stress. This yeast was probably able to mobilize the intracelular trehalose to protect its proteins, leading to a greater cell viability (21). At the end of the fermentative cycle, the fermented sugar cane juice must reach an ethanol concentration near 7% (14), such that the strains with a higher trehalose accumulation capacity may have a greater survival probability in this environment.

The high adaptation capacity demonstrated by the yeasts isolated from the fermentation enviroment, and the strong relationship between the trehalose accumulation and the cell viability indicate that this could be one of the mecanisms involved in the survival and predominance of the strains in the fermentation vats. This environment may be a source of yeasts that are potentially useful for the biothechnological applications in the production of food and beverages.

ACKNOWLEDGMENTS

We are grateful for financial support from Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and PADCT (process number 62.0477/98-9). We thank the *cachaça* producers João Paulo Araújo, Acácio de Paula and Walter Caetano for allowing us to use their plant facilities, and Dr. Marc-André Lachance's suggestions to improve the manuscript.

RESUMO

Acúmulo de trealose, atividade invertásica e características fisiológicas de leveduras isoladas do ciclo fermentativo de 24 horas durante a produção de cachaça artesanal brasileira

As características fisiológicas, acúmulo de trealose e produção de invertase de 86 leveduras isoladas do ciclo fermentativo durante a produção artesanal de cachaça em três destilarias no Estado de Minas Gerais foram estudadas. Destes isolados 70% foram capazes de crescer acima de 40 e 42ºC. Somente os isolados de *Saccharomyces cerevisiae* foram capazes de crescer acima de 40ºC. Baixas temperaturas (<40ºC) favoreceram o crescimento de leveduras como *Candida parapsilosis*-like, *C. maltosa*-like, *Kloeckera japonica*, *S. exiguus* and *C. bombicola*-like. Os isolados das três destilarias foram tolerantes ao etanol, produziram invertase e acumularam trealose na presença de glicose. As linhagens isoladas da destilaria A apresentaram maior resistência ao etanol (cerca de 84.2% das linhagens foram capazes de crescer na presença de 12% de etanol) quando comparadas àquelas das destilarias B e C (nenhuma linhagem e 9,5%, respectivamente). As linhagens de *S. cerevisiae* isoladas das três destilarias apresentaram uma alta capacidade para produzir invertase e acumular trealose na presença de glicose. Com base nos resultados dos experimentos de estresse térmico e de etanol, foi possível observar uma forte relação entre acúmulo de trealose intracelular e viabilidade celular. O aumento da viabilidade celular foi mais pronunciado quando as linhagens foram submetidas a um pré-tratamento em temperaturas sub-letais

Palavras-chave: *Saccharomyces cerevisiae*, cachaça, trealose, invertase, fermentação.

REFERENCES

1. Banat, I.M.; Nigam, P.; Singh, D.; Marchant, R.; McHale, A.P. Ethanol production at elevated temperatures and alcohol concentrations: Part 1 – yeasts in general. *World J. Microbiol. Biotech.*, 14: 809-821, 1998.
2. Barnett, J.A.; Payne, R.W.; Yarrow, D. *Yeasts: Characteristics and identification*. Cambridge University Press, Cambridge, 1980.
3. Corrêa, S.R. *Microhabitats ocupados por* Saccharomyces *cerevisiae durante os períodos de entressafra e produção em três destilarias de aguardente artesanal*. Belo Horizonte, 1999, 74p. *(Dissertação de Mestrado* Instituto de Ciências Biológicas, Depto. Microbiologia da UFMG).
4. D'Amore, T.; Crumplen, R.; Stewart G.G. The involvement of threalose in yeast stress tolerance. *J. Ind. Microbiol.* 7: 191-196, 1991.
5. Ekunsanmi, T.J.; Odunfa, S.A. Ethanol tolerance, sugar tolerance and invertase activities of some yeast strains isolated from steep water of fermenting cassava tubers. *J. Appl. Bacteriol*., 69: 672-675, 1990.
6. Hottiger, T.; Schmutz, P.; Wiemken, A. Heat induced accumulation and futile cycling of trehalose in *Saccharomyces cerevisiae*. *J. Bacteriol*., 169: 5518-5522, 1987.
7. Hugget, A.S.G.; Nixon, P.A. Enzimic determination of blood glucose. *Biochem. J.* 66: 12-15, 1957.
8. Kreger-van Rij, N.J.W. *The yeasts: a taxonomic study*. Elsevier Science Publishers BV, Amsterdam, 1984.
9. Kurtzman, C.P.; Fell, J.W. *The yeast: a Taxonomic Study*. Amsterdam: Elsevier Science Plublisher, 1998.
10. Lachance, M.A. Yeast communities in a natural tequila fermentation. *Antonie van Leeuweehoek*, 68: 151-160, 1995.
11. Lewis, J.G.; Learmonth, R.P.; Watson, K. Induction of heat, freezing and salt tolerance by heat and shock in *Saccharomyces cerevisiae. Microbiology*, 141: 687-694, 1995.
12. Mansure, J.J.C.; Panek, A.D.; Crowe, L.M.; Crowe, J.H. Trehalose inhibits ethanol effects on intact yeast cells and liposomes. *Bioch. Bioph. Acta*, 1191: 309-316, 1994.
13. Morais, P.B.; Rosa, C.A.; Linardi, V.R.; Carazza, F.; Nonato, E.A. Production of fuel alcohol by *Saccharomyces* strains from tropical habitats. *Biotech. Lett.*, 18: 1351-1356, 1996.
14. Morais, P.B.; Rosa, C.A.; Linardi, V.R.; Pataro, C.; Maia, A.B.R.A. Characterization and sucession of yeast populations associated with spontaneous fermentation during the production of the brazilian sugar-cane "aguardente". *World J. Microbiol. Biotechn.*, 13: 241-243, 1997.
15. Neves, M..J.; François, J. On the mechanism by which a heat shock induces trehalose accumulation in *Saccharomyces cerevisiae*. *Biochem. J.*, 288: 859-864, 1992.
16. Neves, M.J.; Terenzi, H.F.; Leone, F.A.; Jorge, J.A. Quantification of trehalose in biological samples with a conodial trehalase from the thermophilic fungus *Humicola grisea* var. thermoidea. *World. J. Microbiol. Biotech*., 10: 17-19, 1994
17. Pataro, C.; Santos, A.; Corrêa, S.R.; Morais, P.B.; Linardi, V.R.; Rosa, C.A. Physiological characterization of yeasts isolated from artisanal fermentation in an *aguardente* distillery. *Rev. Microbiol.*, 29:104-108, 1998.
18. Pataro, C.; Guerra, J. B.; Petrillo-Peixoto, M.L.; Mendonça-Hagler, L.C.; Linardi, V.R.; Rosa, C.A. Yeast communities and genetic polymorphism of *Saccharomyces cerevisiae* strains associated with artisanal fermentations in Brazil. *J. Appl. Microbiol*., 89: 24-31, 2000.
19. Ribeiro, M.J.S.; Leão, L.S.C.; Morais, P.B.; Rosa, C.A.; Panek, A.D. Trehalose accumulation by tropical yeast strains submitted to stress conditions. *Antonie van Leeuwenhoek*, 75: 245-251, 1999.
20. Ribeiro, M.J.S.; Silva, J.T.; Panek, A.D. Trehalose metabolism in *Saccharomyces cerevisiae* during heat-shock. *Bioch. Bioph. Acta*, 1200: 139-147, 1994.
21. Singer, M.A.; Lindquist, S. Thermotolerance in *Saccharomyces cerevisiae*: the Yin and Yang of trehalose. *Tibtech*, 16: 460-468, 1998.
22. Soto, T.; Fernandez, J.; Vicente-Soler, J.; Cansado, J.; Gacto, M. Accumulation of Trehalose by overexpression of *tps1*, coding for trehalose-6-phosphate synthase, causes increased resistance to multiple stresses in the fission yeasts *Schizosaccharomyces pombe*. *Appl. Environ. Microbiol.*, 65: 2020-2024, 1999.
23. van der Walt, J.P.; Yarrow, D. Methods for the isolation, maintenance, classification and identification of yeasts. In: *The yeasts: a taxonomic study*. Elsevier Publishers BV, Amsterdam. 1984. pp.45-104.
24. Van Dijck, P.; Colavizza, D.; Smet, P.; Thevelein, J.M. Differential importance of trehalose in stress resistance in fermenting and nonfermenting *Saccharomyces cerevisiae* cells. *Appl. Environ. Microbiol*., 61: 109-115, 1995.