Physiological characterization of a new thermotolerant yeast strain isolated during Brazilian ethanol production and its application in high temperature fermentation

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Abstract

Background

The use of thermotolerant yeast strains can improve the efficiency of ethanol fermentation, allowing fermentation to occur at temperatures higher than 40 °C. This increment in temperature could benefit traditional bio-ethanol production and allow simultaneous saccharification and fermentation (SSF) of starch or lignocellulosic biomass.

Results

We identified and characterized the physiology of a new thermotolerant strain able to fermentate at 40 °C while producing high yields of ethanol. Our results showed that, in comparison to the industrial yeast CAT-1, our strain was more resistant to various stressors generated during the production of first- and second-generation ethanol, and it also was able to change the pattern of genes involved in sucrose assimilation (SUC2 and AGT1). The formation of secondary products of fermentation was different at 40ºC, with reduced expression of genes involved in the formation of glycerol (GPD2), acetate (ALD6 and ALD4), and acetyl-CoA (ACS2).

Conclusion

The LBGA-01 strain is a thermotolerant strain that modulates the production of key genes, changing metabolic pathways during high-temperature fermentation, and increasing its tolerance to the high concentration of ethanol, sugar, acetic lactic, acetic acid, furfural and HMF. This indicates that this strain can be used to improve first- and second-generation ethanol production in Brazil.

Background

Currently, Brazil is the second ethanol producer worldwide using sugar cane as a fermentative substrate since the high sucrose concentration is suitable for sugar and ethanol production. [1; 2; 3; 4]. During ethanol production, the wort, composed by molasses from sugar production and sugar cane juice, is mixed with yeasts in high cell densities (8–22% v/v). The fermentation occurs in approximately 6–8 hours with high ethanol yield at the end of the fermentation (90–92%) [3; 4]. An important particularity within the Brazilian process is the recycling process of yeast achieved by centrifugation followed by acid treatment in sulfuric acid (pH 2-2.25 for 2–3 hours) used to reduce bacteria contamination during the harvest. The fermentation occurs between 28–35 ºC and, during the summer, the maintenance of this temperature is only possible through water cooler use installed in fermentation tanks. This process is crucial to maintain the temperature within an ideal range, not exceeding 35 ºC, but is costly and requires large quantities of water [5].
In addition to temperature, several other stressing conditions such as pH variation, osmotic stress, contamination by bacteria, and wild yeasts can affect the fermentative process [5]. Therefore, the production process should be performed using strains able to survive all of these conditions in the fermentation tank. Brazil has a consolidated process, and for a long time, a significant number of ethanol producers have employed mainly two industrial yeast strains known as CAT-1 and PE-2. These yeasts were isolated from Brazilian industries in 1990s directly from the ethanol process, and their genomes were sequenced in 2009 [2; 6; 7; 8; 9].

Although these strains have had a high performance for several years, the constitution regarding Brazilian fermentation substrates has been changing throughout the last years, especially after the sugar cane burn was prohibited by law. As a result, there was a lower persistence of these strains in the fermentation tanks during the whole harvest that were then replaced by wild yeasts in several mills in Brazil [5]. Otherwise CAT-1 and PE-2 were not able to fermentate appropriately above 35 ºC, reducing their viability [10].

Aiming to maintain productivity or to improve the Brazilian fermentation process, our research group has been isolating new strains from distilleries since 2009 to identify new \textit{Saccharomyces cerevisiae} strains that can adapt to the new conditions or that have new characteristics as ethanol, sugar and thermotolerance [5].

Here, we demonstrate the identification, and physiological and molecular characterization of a new thermotolerant \textit{S. cerevisiae} strain that is able to grow at 40ºC and generates high ethanol yields under the aforementioned conditions. Also, this strain showed important changes in the gene expression pattern in pathways involved in fermentation efficiency, what can provide information about its thermotolerant phenotype and associated fermentative performance. In addition, this strain is resistant to the stressors produced during the first- and second-generation ethanol production, highlighting the attributes of this strain for employment in high-temperature fermentation, endeavoring improvements in the Brazilian ethanol production chain.

**Results And Discussion**

**Isolation, identification and molecular genotyping of a thermotolerant yeast strain for use in high temperature fermentation for ethanol production**

To obtain personalized yeast for ethanol production in Brazil, several attributes need to be considered and evaluated, such as high yields of ethanol cell viability tolerance to stressors produced during ethanol production. Another important attribute is growth temperature. In Brazilian ethanol production, the strains currently used ferment at temperatures between 28 and 33ºC, and to maintain this temperature constant, the mills need water coolers. The use of thermotolerant yeasts could circumvent this problem improving ethanol production. The Laboratory of Biochemistry and Applied Genetics of the Federal University of São Carlos (LBGA-UFSCar) has been isolating yeasts from ethanol production since 2009, which have been deposited in the LBGA strain collection. We used this collection to screen for isolates that can grow at
higher temperatures, above those used in the ethanol fermentation plants in Brazil. Within the approximately 300 strains, four isolates have been identified as thermotolerant since they are able to grow and display similar fitness at both 30 and 40°C, unlike the industrial strain CAT-1 that is unable to growth at high temperatures [10]. These yeasts were identified as LBGA-01, LBGA-69, LBGA-157, and LBGA-175, respectively (Fig. 1)

To identify these strains, the amplification of ITS [11] and a genotyping test were carried out. The results showed that the four strains were genetically different among them and also from the industrial strain CAT-1. The ITS analysis indicated that only LBGA-01 and LBGA-69 presented the amplification of a 900 bp, expected to identify the strain as a possible *Saccharomyces* [11; 12]. The LBGA-157 and LBGA-175 showed a different pattern of amplification suggesting that these yeasts are non-*Saccharomyces* strains, but possible wild contaminating strains (see Additional File 1: Figure S1). To confirm these results, the ITS amplicons were sequenced, and we confirmed through the BLAST analyzes that LBGA-01 and LBGA-69 strains are *S. cerevisiae* isolates, while LBGA-157 and LBGA-175 strains are *Kluyveromyces marxianus* yeasts.

The thermotolerant LBGA strains present superior fermentation performance at 40°C in comparison to the industrial strain at 30°C

Although only strains LBGA-01 and LBGA-69 were identified as *S. cerevisiae*, LBGA-157 and LBGA-175 were also included in the cell growth and fermentation tests at 30 and 40°C since it is already known that strains of *Kluyveromyces* also ferment at high temperatures [13]. The results of cell proliferation growth at 30°C show that the strains LBGA-01 and LBGA-69 have the same growth profile as the industrial strain CAT-1. However, when subjected to growth at 40°C, these strains have higher growth rates than the industrial strain. This result was already expected since it is largely reported that CAT-1 does not show good performance when subjected to high temperatures [9; 10; 14]. The strains LBGA-157 and LBGA-175 showed slower growth rates at both temperatures. In fact, the strains LBGA-01 and LBGA-69 showed a similar growth profile in both temperatures indicating a strong thermotolerant phenotype in these strains (Fig. 2a and 2b). As mentioned above, to be a yeast applicable in industrial use, strains have to present excellent fermentation characteristics and a good conversion of sugar to ethanol in addition to thermotolerant growth. It is known that in the ethanol production process, industrial yeasts perform this conversion in the first hours of fermentation, thus converting all available sugar to ethanol after approximately 4–6 hours of fermentation. To evaluate the fermentative potential of isolated yeasts, we first performed a fermentation experiment using 4% of glucose and all the isolates. The results showed that LBGA-01 and LBGA-69 had a pattern similar to the industrial strain CAT-1 in fermentation conducted at 30°C, and had superior performance at 40°C. Strains LBGA-157 and 175 presented a low performance in both temperatures (Fig. 2c and 2d). The yeasts used during ethanol fermentation are subjected to high concentrations of sugar and therefore are constantly exposed to osmotic stress. Since LBGA-01 showed good fermentation performance and presented a slight advantage over LBGA-69 in the glucose fermentation (Fig. 2c and 2d), we also conducted fermentative tests using 8% of sucrose concentration to simulate the conditions widely used in the standard ethanol production in Brazil, in which sucrose is the
fermentable sugar used. In comparison to the CAT-1 industrial strain, LBGA-01 showed a better performance in both temperatures with a clear superiority at 40ºC (Fig. 2e and 2f). In the fermentation assays conducted at 30ºC, both strains had a similar pattern. However, at 40ºC, LBGA-01 strain converted 57.5% of the initial sugar while the industrial strain converted 45% (Fig. 2f). From an economic point of view regarding the expected yields for a sugar cane plant, these results are expressive, since the LBGA-01 strain converted 12.5% more sugar under stress conditions than the CAT-1 industrial strain. It is worth mentioning that the use of a thermotolerant yeast operating under stress conditions with good fermentation rates can represent a significant increase in ethanol production in the plant. Other advantages are a decrease in the contamination with wild yeasts and bacteria, and in water use to control the temperature in the fermentation tanks, consequently reducing energy costs, collaborating with environmental issues related to water usage and mitigating the use of natural resources.

LBGA-01 is resistant to stressors produced during 1G and 2G ethanol production process.

Yeasts undergo constant stressing conditions during the Brazilian fermentation process to produce first (1G) and second (2G)-generation ethanol, directly resulting in a decrease of final yield in the process. To evaluate the tolerance of LBGA-01 strain, we analyzed its growth and survival under different concentrations of stressors such as ethanol, sugar, lactic acid, acetic acid, HMF and furfural (the latter three inhibitors of the 2G ethanol production process) and compared them to the industrial strain CAT-1 and the laboratory haploid strain Sc9721. The concentration of each stressor was established according to the literature, as described in the methodology section. The results obtained through the dropout analysis showed that the LBGA-01 strain is more resistant in all tested stressors (Fig. 3).

The overall positive results exceeded the initial expectations, especially in the presence of 2% acetic acid, where we observed that the LBGA-01 strain was more resistant than both the CAT-1 strain and the laboratory strain. When subjected to 4% acetic acid, all of the strains suffered stress and had their growth inhibited. For the lactic acid test, the LBGA-01 strain was also more resistant in the two tested concentrations (2% and 4%), reveling that under conditions of contamination by Bacillus spp or other lactic acid-producing bacteria [15], this yeast would be resistant, and not affected during alcoholic fermentation. However, a wider range of concentration needs to be tested to determine in which concentration of lactic acid the LBGA-01 strain can survive. The high ethanol stress test showed that LBGA-01 has a similar resistance profile to that of the industrial strain for all the used ethanol concentrations: 12, 14 and 16%. We observed higher resistance of LBGA-01 in comparison to CAT-1 strain at 16% of ethanol. In this trial, the control laboratory strain was drastically affected by ethanol stress. When subjected to different concentrations of sucrose, the LBGA-01 strain was also more resistant than CAT-1 and SC9721 strains in the three tested sugar concentrations (20, 25 and 30%), corroborating once more the results obtained during the fermentative tests. For the HMF test, the LBGA-01 had a slight increase in resistance at the lowest evaluated concentration (40 mM). Growth tests with furfural showed that the LBGA-01 strain is more resistant than the industrial and the laboratory strain at both tested concentrations of 0.3 mM and 0.9 mM. Furfural are potent inhibitors of Saccharomyces during lignocellulosic fermentation [16]. For this reason, the thermotolerance conjugated to furfural resistance,
as described for LBGA-01, could be an important feature for the production and improvement of 2G ethanol. Taken together, these results demonstrate the robustness of the LBGA-01 strain in the presence of several stressors during the fermentation process, thus becoming a potential strain for the production of 1G ethanol production. Industrial strains have shown to be more robust towards the main lignocellulosic inhibitors produced during biomass pre-treatment processes [17]. The present results are in accordance to these findings, in which the laboratory strain (Sc-9721) was more affected by the presence of HMF, furfural and acetic acid than the industrial LBGA-01 and CAT-1 strains. Regarding the stressors of 2G ethanol production, further tests need to be carried out to confirm the resistance to the same stressors. Nonetheless, our initial results point out that the LBGA-01 strain is more resistant than the CAT-1 industrial strain when submitted to HMF, furfural and acetic acid concentrations, thus calling attention to its potential use in the 2G ethanol production.

Transcriptional responses of LBGA-01 under high temperature fermentation conditions.

To better understand the metabolic changes in LBGA-01 strain during high temperature fermentation, the expression of genes involved in efficiency of fermentation and membrane biosynthesis and sucrose assimilation were evaluated using qPCR, and compared with the industrial yeast CAT-1 at both fermentation temperatures (30°C and 40°C). The mRNA levels for genes involved in the efficiency of fermentation are summarized in Fig. 4.

During the fermentation assays, in both glucose-limited chemostats, and in fed-batch fermentation, increase in glycerol production rate and glycerol titer were observed at 40°C, respectively, when compared to control condition (30°C). Since GPD1 and GPD2 are key enzymes in glycerol synthesis, we hypothesized that the expression of GPD1 and GPD2 would increase during the fermentations. However, our results indicate that the expression of these genes did not change (Fig. 4). GPD2 and GPD1 are paralog genes encoding the isoenzyme NAD-dependent glycerol-3-phosphate dehydrogenase, which has an important role in osmoadaptation (GPD1) and anoxic growth conditions (GPD2) [18; 19; 20]. Mutants lacking both GPD1 and GPD2 do not produce detectable glycerol, leading to the accumulation of dihydroxyacetone phosphate (DHAP). This DHAP can be converted to methylglyoxal, a cytotoxic compound that can inhibit the yeast growth [21]. Differently, the growth of the LBGA-01 was not affected in either temperature. Interestingly, our results show that under high temperature (40 ºC), expression of SNF1 was highly increased in fermentations using the LBGA-01 strain (Fig. 4). The kinase SNF1 was described as an repressor of GPD2 via phosphorylation to halt glycerol production when nutrients are limited [22]. Therefore, we hypnotize that the unchanged GPD2 abundance is because its repression is exacerbated in the LBGA-01 strain due to the increase of SNF-1 expression (Fig. 5).

As expected, a decrease in SUC2 (invertase) expression was observed in all the strains after 4 hours of fermentation (Fig. 4), what was due to the inhibition of SUC2 expression caused by the accumulation of glucose and fructose during the first hours of fermentation, as a result of the invertase activity [23]. However, a different pattern was found between CAT-1 and LBGA-01 after 8 hours of fermentation. During CAT1 fermentation, the SUC2 gene is reactivated as glucose concentration decreases and the invertase
resumes the metabolism of the residual sucrose. The metabolic shift caused by glucose concentration and by inactivation and reactivation of \textit{SUC2} is accompanied by the expression of \textit{SNF1}. The activation of this kinase is glucose-dependent and directly related to the inactivation of glucose transporters and activation of genes involved in the utilization of alternative carbon sources [23; 24; 25]. As previously described, the \textit{SNF1} expression in LBGA-01 strain is maintained at high levels during the fermentation. Meanwhile, the \textit{SUC2} expression decreases, as mentioned above, and the sucrose consumption remains unchanged (Fig. 2e and 2f). In fact, the ethanol production rate is higher than at 40ºC than at 30ºC in this strain, accompanying the \textit{SNF1} expression that is increased in this temperature. We suggest that this process happens because there is an augment in the internalization of sucrose by MAL31 and AGT1 transporters since both proteins are able to actively transport sucrose, maltose, and maltotriose, although this process naturally occurs in absence of glucose [26; 27]. Our hypothesis is also supported by the expression of the \textit{SNF1} gene, that is highly expressed in LBGA-01 strain during the whole fermentation process, even in the presence of glucose, thus possibly activating the receptors [28; 29; 30]. Interestingly the expression of SNF1 was higher in the middle and in the end of the fermentation conducted with LBGA-01 in both temperatures, when sucrose was used as carbon. Therefore, we argue that LBGA-01 can be used in higher levels of this sugar since sucrose would be inverted by SUC2 and transported by AGT1 at the same time, and later inverted by intracellular SUC2.

When the expression of genes involved in the formation of secondary products of fermentation such as glycerol (\textit{GPD2}), acetate (\textit{ALD6} and \textit{ALD4}), and acetyl-CoA (\textit{ACS2}) was evaluated, we found repression of all of these genes at 40 ºC (Fig. 5a-c). These results suggest that the alternative pathways for glucose utilization are inhibited at high temperatures in the LBGA-01 strain, which preferentially uses the available carbon source for the ethanol production pathways.

\textbf{Quantitative physiological parameters of LBGA-01 during anaerobic glucose-limited chemostat at high temperature}

Chemostat cultivations have been broadly applied on quantitative study of physiological parameters in \textit{S. cerevisiae}. We deemed to investigate the impact of a high temperature (40 ºC) on the anaerobic physiology of LGBA-01 in comparison to a control temperature (30ºC) and to the experiment conducted by Della-Bianca et al. [31] that used the industrial strain PE-2, largely used for Brazilian ethanol production, using glucose-limited chemostat cultures (Table 1). An advantage of studying microbial cells under continuous culture instead of batch culture is that in the former, the specific growth rate can be held constant under different stressful conditions [32].
Table 1
– Physiology of *S. cerevisiae* strains in glucose-limited.

| *S. cerevisiae* strain | LBGA-01 (This work) | *Saccharomyces cerevisiae* PE-2 [31] |
|------------------------|---------------------|-------------------------------------|
| Temperature            | 30 °C               | 40 °C                               |
|                        |                     | 30 °C                               |
| q glucose              | -5.28 ± 0.50        | -7.22 ± 0.93                        |
|                        |                     | -5.06 ± 0.15                        |
| q CO2                  | 7.98 ± 0.69         | 12.02 ± 1.04                       |
|                        |                     | 8.51 ± 0.28                        |
| q Ethanol              | 8.79 ± 1.03         | 11.50 ± 1.72                       |
|                        |                     | 7.70 ± 0.26                        |
| q Glycerol             | 0.89 ± 0.22         | 1.38 ± 0.32                        |
|                        |                     | 0.89 ± 0.04                        |
| q Lactate              | 0.05 ± 0.03         | 0.11 ± 0.02                        |
|                        |                     | 0.05 ± 0.00                        |
| q Pyruvate             | 0.01 ± 0.00         | 0.08 ± 0.03                        |
|                        |                     | Not reported                        |
| q Acetate              | 0.00 ± 0.00         | 0.01 ± 0.02                        |
|                        |                     | 0.00 ± 0.00                        |
| X (g DW L\(^{-1}\))   | 2.64 ± 0.30         | 2.09 ± 0.26                        |
|                        |                     | 2.63 ± 0.01                        |
| \(Y_{X/S}\) (g DW g glucose\(^{-1}\)) | 0.11 ± 0.01        | 0.08 ± 0.01                        |
|                        |                     | 0.11 ± 0.00                        |
| \(Y_{ETH/S}\) (g ethanol g glucose\(^{-1}\)) | 0.43 ± 0.12        | 0.41 ± 0.01                        |
|                        |                     | Not reported                        |
| \(Y_{G/S}\) (g glycerol g glucose\(^{-1}\)) | 0.09 ± 0.01        | 0.10 ± 0.01                        |
|                        |                     | Not reported                        |
| Residual glucose (mM) | 0.17 ± 0.23         | 2.4 ± 0.58                         |
|                        |                     | Not reported                        |
| C recovery (%)         | 101.97 ± 1.73       | 101.28 ± 1.35                      |
|                        |                     | 100.9 ± 0.7                         |

The assays were conducted using anaerobic chemostats with synthetic medium at a dilution rate of 0.1 h\(^{-1}\). Specific \(q\) rates are given in mmol g\(^{-1}\) h\(^{-1}\). Data are the average value from duplicate or triplicate experiments ± deviation of the mean.

In anaerobic glucose-limited chemostat cultures of the LGBA-01 strains, carbon was mainly diverted to ethanol and CO\(_2\), and minor amounts of glycerol and lactic acid with a concomitant formation of yeast biomass were produced. When comparing the data obtained from LGBA-01 strain cultivated at 40ºC and 30ºC (control), we observed an increase in consumption of glucose (38%) as well as in the production rates of CO\(_2\) (51%), glycerol (54%) and ethanol (36%). On the contrary, we observed a substantial decrease in biomass yield (25%), and no effect on glycerol yield or on the maximum specific growth rate during the batch phase (Table 1). Interestingly, we did not observe difference between 40 ºC and the control condition in ethanol yield during steady-state in cultures of the strain LGBA-01. In contrast, we observed that glucose concentration was higher at 40ºC the during steady-state, suggesting a possible inhibition of glucose uptake.
Stressing conditions such as high temperature can generate perturbations in the redox balance inside the cells [33]. The increase in the rate of synthesis of by-products (such as acetate and lactate), which are involved in the reoxidation of NADH, are indicative of how cells are responding to this stressful condition, as well as the differential gene expression of ACS2 gene reported above.

A similar experimental setup was performed by Bianca et al. (2014) [31] using the industrial S. cerevisiae strain PE-2, known as highly stress-tolerant [8]. The results obtained in the present study showed that LGBA-01 presents higher ethanol and glycerol production rates than S. cerevisiae PE-2 under similar conditions, i.e., at 30ºC. These data suggest an advantage on the industrial process. Moreover, as mentioned by Bianca et al. (2014), the absence of acetic acid in all cultivations is a remarkable phenotypic characteristic found in a strain that grows in acidic environments, such as those found in the industrial ethanol process.

A previous report analyzed the thermotolerance of industrial S. cerevisiae strains isolated from Brazilian ethanol plants, such as CAT-1, PE-2, BG-1, and JP-1 in synthetic media with glucose as the sole carbon and energy source [14]. Although the authors have used different conditions from those reported in this study during the batch phase, i.e., oxygen-limited shake-flask cultures as opposed to anaerobic bioreactors, the growth rates of some strains (JP-1 and CAT-1) were higher at 37 ºC than at 30ºC (0.39 and 0.38 h⁻¹, respectively). Furthermore, they were lower than the growth rates obtained for the LBGA-01 strain at both 30 ºC and 40ºC. In respect to ethanol yield, JP-1 and BG-1 presented an increase in cultivations at 37ºC when compared to 30ºC, differently from our results. Instead, PE-2 presented a small increase in ethanol yield at 37 ºC than at 30 ºC.

In terms of the specific ethanol production rate, our results revealed that LBGA-01 strain has a higher rate at 40ºC than at 30ºC, although reaching similar values of ethanol yield under both conditions. Similarly, increased specific rates of glycerol production were also observed under such conditions, although glycerol yield was not affected. The deviation of carbon away from biomass formation at 40ºC seems to be due to pyruvate and lactate production. This result can be explained by the reduced expression of genes encoding the enzymes responsible for the production of secondary products (Fig. 5).

**Fermentative performance of LBGA-01 in conditions mimicking the Brazilian industrial ethanol process under high temperature**

Different aspects of S. cerevisiae strains as specific growth, yields in ethanol, glycerol, and cell productivity are commonly investigated under laboratory conditions, using a batch mode operation and synthetic defined culture media offering conditions containing all nutrients in adequate amount, to enable maximum growth rate. In the synthetic medium, the carbon source is usually a limiting nutrient [34].

Industrial conditions are not reproducible and vary from batch to batch. There is insufficient data reported for conditions that reproduce the different characteristics found in industrial environments. Specifically in Brazilian 1G ethanol production, sugarcane juice and molasses are often used as a lower cost carbon source for fermentation [35]. Its composition and quality also vary among different batches and
harvesting periods; therefore, synthetic laboratory medium replicates of conditions are poor and may lead to misinterpreted conclusions [36]. Also, other stresses are associated with industrial production, such as toxicity of products, non-aseptic conditions, substrate inhibition, cell recycle, acid treatment, bacterial contamination, and temperature stress [34]. Thus, high tolerance for such a great variety of stressful conditions is a desirable feature for a yeast strain in the fuel ethanol industry [34; 37; 38].

To assess and study physiologic aspects and performance of LBGA-01 under highly stressful conditions, we scaled down the Brazilian 1G ethanol production with sugarcane molasses as carbon source using protocol described by Raghavendran et al. [34]. Thermotolerance was investigated by submitting cells to 34 °C and 40 °C that are unusual laboratory temperatures, commonly found in Brazilian sugarcane mills and inside reactors due to the exothermic reactions of ethanol production [36].

Fermentation capacity was monitored by plotting the produced CO$_2$ per gram of wet biomass as a function of fermentation time. As shown in other studies, there could be an increase or decrease in biomass weight, and the plot of the total amount of CO$_2$ against time could not represent well the fermentation capacity [34]. In this case, the normalization of the specific mass is necessary. The fermentation at 34 °C showed a slightly lower fermentation capacity (Fig. 6a) compared to fermentation at 40 °C in the first cycle. At both temperatures, the yeast started with virtually the same viability of approximately 80%. The fermentation capacity of the first cycle at 34 °C was kept in the other sequential cycles, so with a viability of about 100% (Fig. 6b). The fermentation capacity decreased cycle to cycle at 40 °C, as it can be seen from the reduction of the experimental data slope, probably associated with a reduction of viability. At 40 °C, LBGA-01 viability slightly decreased after the first cycle (54.9%), reaching 28.7% of viability after the fourth cycle.

During the experiments, both fermentations started with similar weights. (4 ± 0.06 g). Corroborating constant viability, the biomass at 34 °C had a negligible decrease at first, followed by a small increase (less than 5%) in all subsequent cycles (Fig. 6c), thus resulting in a total weight increase of 7%. At the high temperature, biomass decreased, similarly to viability, with a mean decrease of 6.2% for each cycle and a total reduction of 24% (Fig. 6c).

Ethanol yield and glycerol production were also assessed during fermentation. As mentioned above, glycerol production is associated with a part of the physiological response of cells to osmotic shock. Thus, its formation occurs in many kinds of stressful situations. As a cell response, intracellular glycerol is thought to decrease water activity in the cytosol, leading to higher water uptake [39]. Glycerol levels were monitored and showed an increase with cycle in both temperatures, and were higher at 40 °C than at 34 °C. As a protection mechanism, increased glycerol levels are caused by the high-temperature stressful condition that cells are submitted to (Fig. 6d).

Ethanol yield for each cycle was calculated as described elsewhere [35]. A correction factor for high cell density was applied as previously reported, and a specific volume of 0.7 mL g$^{-1}$ (wet basis) was considered for yeast cells. Thus, the ethanol yield accounts for ethanol from centrifuged wine and
pelleted yeast biomass. A mass balance for ethanol is applied as a difference between ethanol content at
the end of the cycle and ethanol in the beginning (returned wine plus pelleted yeast biomass from the
previous cycle). The ethanol yield is expressed as a percentage of the maximum theoretical ethanol that
could be produced by the total sugar content (Eq. 1):

\[
\text{Ethanol yield} (\%) = \left( \frac{10000}{51.11 \times V_s \times \text{TRS}} \right) \times \left\{ (V_w + 0.7 \times P) \times \text{ET} - (V_v + 0.7 \times P_p \times \text{ET}_p) \right\}
\]

(1)

where \( V_s \) is the total amount of substrate (mL) of concentration TRS (g.100 mL\(^{-1} \)), 51.11 g\text{ethanol}\*(100 g\text{TRS})\(^{-1}\). \( V_w \) is the volume of centrifuged wine (mL), \( P \) is the pelleted yeast biomass (g), \( \text{ET} \) is the ethanol concentration in centrifuged wine (% w * v\(^{-1} \)). \( P_p \) is the pelleted yeast biomass from the previous cycle, \( \text{ET}_p \) is the ethanol concentration from centrifuged wine from the previous cycle, and \( V_v \) is the volume of wine from the previous cycle.

The ethanol yields during the four cycles at 34 °C were similar, with a median value of 86.03 ± 1.56%. It is
noteworthy that the results obtained for LBGA-01 are similar to those obtained for CEN.PK 113-7D, Baker's
yeast, and S288c, that ranged from 86 to 92% [34]. Moreover, the results are also comparable to those
obtained for the two main industrial strains PE-2 (87.2 ± 3.9%) and Ethanol Red™ (87.6 ± 5.1%), both
employed in Brazilian 1G production [14; 35]. As previously discussed, fermentation capacity was
reduced after cycles at 40 °C, even though the ethanol yield after cycles were slightly lower than those
obtained at 34 °C, with a mean value of 76.9 ± 2.72% (Fig. 6e). This reduction could be explained by the
deviation for the production of other compounds in the fermentative metabolism, such as glycerol
production, and also by a higher loss of ethanol attributed to the temperature elevation.

Collectively, these results showed that LBGA-01 has a good fermentative performance. However, this
yeast needs to improve its viability along cell recycles to be used in the Brazilian ethanol production.
Efforts using adaptive evolution will be attempted to fix the genetic characteristics and increase the
viability of this yeast in the recycle of high temperature fermentation.

Conclusions

We have reported the characterization of a new S. cerevisiae strain resident with important fermentative
characteristics. In addition, these isolated strains may have important biotechnological characteristics,
such as the ability to grow under stress conditions, such as high concentrations of ethanol and sugar as
well as high temperature. Our results showed that although there was a reducing viability throughout
yeast recycles, the LBGA-01 strain is a potential thermotolerant strain producing a high yield of ethanol at
40 °C. Furthermore, this strain changes its metabolic pathways to resist several stressors produced in 1G
and 2G ethanol production, including high ethanol and sugar concentrations, generating better results for
acetic acid, lactic acid, furfural, and higher HMF concentrations. These results contribute to the
development of production processes using higher temperatures, reducing the use of cooling water during the process, and facilitating the persistence of these strains throughout fermentation, since few wild yeast strains can grow under these conditions. In addition, we hope to encourage new discoveries for the application of LBGA-01 in sugar cane mills with this manuscript.

**Material And Methods**

**Yeast Isolation And Identification Of Thermotolerants**

The yeast strains used in this study were obtained from fermentation tanks after acid treatment, at the São Luiz sugar cane plant located in the city of Ourinhos–SP–Brazil. Samples were collected and stored in sterile conical flasks, taken to the laboratory and centrifuged at 3,000 rpm for 5 minutes. The precipitate was washed 3 times with sterile water, and after the last wash, five grams of the precipitate was eluted to a volume of 50 mL. Serial dilution (1:50; 1: 2500; 1: 12500) was performed and yeast was isolated using solid YPD (1% yeast extract, 2% peptone, 2% glucose and 2% agar) incubated for approximately 48 hours at 30 °C. After this period, 10 colonies were randomized for analyses.

For cultivation and storage of the strains, a pre-inoculation in 2% YPD (1% Yeast extract, 2% Peptone and 2% dextrose) liquid medium was performed, followed by growth for 16 hours in shaker at 30 °C, 180 rpm. After growth, 500 uL of culture medium containing *S. cerevisiae* was transferred to flasks containing 500 uL of glycerol 30% (v/v), and kept in a freezer at -80 °C. All strains were genotyped as described below, and evaluated for growth at high temperatures using the dropout analysis with serial dilutions from $10^6$ to $10^3$ cells mL$^{-1}$.

**Dna Extraction**

Yeast DNA of all thermotolerant strains found in this study was extracted using the phenol-chloroform protocol. In summary, cells were grown overnight in 2% liquid YPD. After this period, cells were centrifuged and lysed with glass beads in 500 µL extraction buffer (200 mM Tris-HCl, 25 mM EDTA and 0.5% SDS), following Malavazi and Goldman [40]. Subsequently, 400 µL of phenol chloroform (50–50%) was added, and sample was centrifuged for 10 minutes at 13,000 rpm. DNA was precipitated using 600 µL of isopropanol and washed with 300 µL of 70% ethanol. DNA was eluted in 80 µL of water and stored at -20 °C for subsequent analyses.

*Molecular identification for individual characterization of isolated strains and classification by species.*

In order to uniquely identify thermotolerant strains, genotyping analysis were performed by PCR using specific primers that were developed based on the amplification of polymorphic regions [41]. To identify the yeast species, PCR experiments were performed to amplify the DNA fragment between intergenic regions ITS-1 and ITS4, using specific primers as described by Uranská et al and other authors [11; 42].
The amplification product (PCR) was sent out for sequencing and results were compared using BLASTN tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi), as well as matching fragment size results amplified.

**Fermentation Assays Using Thermotolerant Strains**

To investigate the fermentative ability of thermotolerant cells, isolated yeasts were submitted to fermentation tests using glucose 4 or 8% as fermentative substrate, at two distinct temperatures, 30 and 40 °C. Yeasts were inoculated in fermentative medium (50 mL) in 250 mL conical flasks with final optical density at 600 nm (OD$_{600nm}$) of 0.1 to ensure standardized inoculations, both temperatures without agitation. Samples were collected at 2-hour intervals for glucose consumption analyses. Sugar was measured by the glucose oxidase colorimetric enzymatic method (Glucose GOD-PAP), following the manufacturer's recommendations.

**Characterization of yeast cell stress resistance (Ethanol, Sugar, Acetic Acid, Acid Lactic, Furfural, HMF)**

For each stress, two or three concentrations were established, according to the literature. Concentrations higher than those described were used to evaluate the resistance of the thermotolerant yeast LBGA-01 in comparison to the industrial yeast CAT-1 and the laboratory haploid yeast SC9721. (Table 2)

| Stressor     | Concentration       | Reference |
|--------------|---------------------|-----------|
| Ethanol      | 12, 14 e 16%        | [43]      |
| Sucrose      | 20, 25 e 30%*       | [44]      |
| acetic Acid  | 2 e 4%              | [45]      |
| Lactic Acid  | 2 e 4%              | [46]      |
| HMF          | 40 e 80 mM          | [47]      |
| Furfural     | 0,1 e 0,9 mM        | [47]      |

* in this case, the used medium was only composed of YP2 × (2% Yeast ex tract and 4% Peptone), adding the desired glucose concentration.

Strains were maintained in logarithmic proliferation phase overnight, at 30ºC with agitation of 180 rpm, then diluted to an OD$_{600}$ of 0.1 in 200 µL of YPD medium (1% Yeast extract, 2% Peptone and 2% dextrose) containing the evaluated stressor at the desired concentration. The assay was performed in triplicate in 96-well plates and incubated at 30 ºC for 10 hours. Then, each strain was ten-fold diluted ($10^{-1}$, $10^{-2}$ and $10^{-3}$) and spotted to YPD solid medium (1% Yeast extract, 2% Peptone, 2% dextrose and 2% agar) containing the evaluated stressor. Figures are generated from the plates photographed at the incubation time that best demonstrated differences between control and experimental samples.
Qpcr Analysis

Total RNA was extracted using Trizol reagent (Invitrogen, Rockville, MS, USA), according to the manufacturers’ protocol. Samples were quantified using a Nano Vue ND-1000 spectrophotometer (GE Healthcare, Chicago, Illinois, EUA).

RNA samples (1 µg) were subjected to DNAsel treatment (Invitrogen, Rockville, MS, USA) and reverse transcribed with High Capacity cDNA Reverse Transcription kit using oligo dTV and random primers blend (Thermo Scientific, Waltham, Massachusetts, EUA). Primers were designed using the PrimerExpress™ program (Applied Biosystems, Foster City, CA, USA) (see Additional file 2: Table S1). The concentration of each primer was determined (the best concentration was 150 nM for all primers used in this study) and the amplification efficiency was calculated according to the equation $E = (-1/slope)$ to confirm the accuracy and reproducibility of the reactions. Amplification specificity was verified by running a dissociation protocol. qPCRs were performed in a StepOne Plus Real-time PCR System (Thermo Scientific Waltham, Massachusetts, EUA). The fold change in mRNA abundance was calculated using $2^{-\Delta\Delta Ct}$ [28] and all values were normalized as the expression of the beta actin (ACT1) gene.

Chemostat Cultivations

Chemostat cultivations with *S. cerevisiae* LBGA-01 strain were carried out in a 2.0 L water jacketed model Labfors 5 (Infors AG, Switzerland) with 1.0 L working volume kept constant by a mechanical drain controlled by a peristaltic pump. The culture medium composition for all cultivations was the one described by Verduyn et al. [48], containing glucose as carbon source, ammonium sulphate as nitrogen source, and supplemented with ergosterol and unsaturated fatty acids in the form of Tween 80, which were dissolved in boiling 96% (v/v) ethanol to final concentrations of 0.01 and 0.42 g L$^{-1}$, respectively [49; 50]. All chemostat cultivations were carried out under anaerobic condition, which was controlled by constant flush of industrial nitrogen gas.

Agitation frequency was set to 800 rpm, temperature was controlled at 30 °C, and pH was controlled at 5.0 via controlled addition of 2 M KOH solution. Precultures for batch bioreactor cultivations were grown overnight in an orbital shaker at 30 °C and 200 rpm in 2500 mL shake flasks containing 30 mL of the defined medium, with 20 g L$^{-1}$ initial glucose. After carbon source exhaustion (that was monitored by a sharp drop in the CO$_2$ concentration in the off-gas), batch cultivation was switched to continuous mode with a fresh medium containing inhibitor compounds, either isolated or combined, in a feeding of 100 mL h$^{-1}$, which corresponded to a dilution rate of 0.10 h$^{-1}$, assuming a working volume of 1.0 L. Chemostate cultures were performed for at least five residence times prior to sampling.

Fermentation Assays Mimicking The Brazilian Industrial Ethanol Process
The bench-scale assays of the industrial fermentation were carried out in triplicate using the protocol described to scale down the Brazilian 1G ethanol production [34]. Pre-culture was prepared with one colony of LBGA-01 inoculated overnight in YPD medium (4% glucose, 1% yeast extract, and 2% peptone) under sterile conditions at 30 °C and 200 rpm. Propagation medium (1 L, 10° Brix, sugarcane molasses from São Luiz sugar cane industry was inoculated with 100 mL of YPD pre-culture under static conditions for approximately 36 h at ambient temperature. The flask was carefully agitated from time to time to release trapped CO$_2$. This step was performed under non-sterile conditions. After 36 hours, the cells decanted and the content of the flask was carefully transferred to a vessel, so that cells did not re-suspend. The content was reserved and used in the fermentation step. In the end, 150 mL of the propagation medium concentrated in cells was maintained. The volume to achieve approximately 4 g of cells was centrifuged in 50-mL centrifuge tubes (2000 g, 4 °C, 15 min). The cell-free supernatant (“vinho”) was stored. Fermentation started with the addition of 6 mL of water and 2 mL of the “vinho” to the 50-mL centrifuge tubes containing 4 g of cells. The cells corresponded to 10% w.v$^{-1}$ and the remaining “vinho” from the previous step simulated the efficiency of the industrial centrifuge [35]. The fermentation cycle started with the addition of 9.25 mL of fermentation medium (19% TRS sugarcane molasses from usina SJ) to the tubes. The initial mass of the tubes was marked to monitor CO$_2$ loss. Tubes were stored in an incubator at 34 °C under static conditions and were weighted hourly until 10 hours. After two and four hours, 9.25 mL of fermentation medium was added, and tubes were weighed again. On the following day, the final weights were measured during the morning. The tubes were centrifuged (200 g, 4 °C, 15 min), and the supernatant was transferred to a different vessel and then stored. The 50 mL centrifuge tubes were weighed, to account for the biomass increase from cycle to cycle. Acid treatment was carried out after the tubes were weighted after the addition of 2 mL of “vinho” and 6 mL of water to the wet cells. The final pH was adjusted to 2-2.5 with 1 N H$_2$SO$_4$, and the tubes were left for one hour before the first addition of fermentation medium to start a new cycle. The simulation was assessed in four cycles, representing four days. The same procedure was performed to 40 °C in the static incubator, following the same described procedure.

Abbreviations

LBGA: Laboratory of Biochemistry and Applied Genetics; UFSCar:Federal University of São Carlos; USP:University of São Paulo; SSF:Simultaneous Saccharification and Fermentation; SUC2:Sucrose transport protein; AGT1:Alpha-glucoside permease; CAT-1:Industrial Yeast Strain; GPD2:Glycerol-3-Phosphate Dehydrogenase 2; ALD6:Aldehyde dehydrogenase-6 ; ALD4:Aldehyde dehydrogenase-4; ACS2:Acetyl CoA Synthetase; HMF:Hydroxymethylfurfural; ITS:Internal Transcribed Spacer; 1G:First Generation; 2G:Second Generation; SNF1:Non-specific serine/threonine protein kinase; MAL31:Maltose permease; ACTB:beta actin; Eq:Equation; YPD:Yeast extract, Peptone and Dextrose; DNA:Deoxyribonucleic Acid; EDTA:Ethane-1,2-diyldinitrilo) tetraacetic acid; SDS:Sodium lauryl sulfate; PCR:Polymerase chain reaction OD:Optical Density; GOD-PAP:Glucose oxidase-phenol amino phenazone RNA:Ribonucleic acid; cDNA:qPCR:Real-time polymerase chain reaction; KOH:Potassium hydroxide; CO$_2$:Carbom dioxide; TRS:Total Reducing Sugars; H$_2$SO$_4$:Sulfuric Acid.
Declarations

Availability of data and materials
Not applicable

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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Authors' contributions
CDP an AFC participated in the design of the study. CDP and FBS performed the experimental work relating to yeast strains growth, fermentation and stressors analyses, and CDP drafted the manuscript. GLM, JPS, HRN, and MHRA performed the genomic DNA extraction and viability analyses of the yeast strain library. GLM and JPMOS performed qPCR analysis. KPE, GCGC, RG, DPP, and TOB performed the chemostat cultivation and bioreactor fermentation assays, as well as drafted the specific results for this portion of the manuscript. AFC and IM supported and supervised the study, as well as helped discuss and analyze the results. All authors read and approved the final version of the manuscript.

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**Figures**

**Figure 1**

Isolation of thermotolerant yeasts from Brazilian ethanol production. Four thermotolerant yeasts have been identified as LBGA-01, LBGA-69, LBGA-157, and LBGA-175. Industrial strain CAT-1 was used as a non-thermotolerant strain control. Cells were grown overnight in YPD medium and cell concentration was adjusted to 10^6 cells/ml. 10-fold serial dilutions were spotted in YEPD solid media. Cells were incubated at 30 and 40°C for 2 days. CAT-1 showed poor growth at 40°C and the four strains had the same pattern of growth at 30 and 40°C.
Figure 2

Comparison of growth and fermentation of isolated yeasts. The growth of yeasts (at 30°C and 40°C) was obtained by optical density at 600 nm (A and B). The fermentation rate at 30°C and at 40°C was evaluated at 4% of glucose (C and D) or 8% of sucrose (E and F) consumed throughout time.
Figure 3

Viability analysis of thermotolerant yeast LBGA-01 compared to industrial (CAT-01) and haploid (Sc-9721) strains under different concentrations of 1G and 2G ethanol production stressors. A 10-fold dilution range was spotted on the YPD medium supplemented with different concentrations of each stressor. Cells were incubated at 30°C for 2 days.
Figure 4

Gene expression profile of genes involved in fermentation efficiency in LBGA-01 fermented at high temperature. The LBGA-01 and the industrial strain CAT-1 fermentation was performed at 30°C were used by comparison using Glucose (G) and Sucrose (S) as carbon sources. The bar colors represent the gene expression profile with green representing low expression and red high expression.
Figure 5

Expression of genes involved in secondary product formation during the fermentative process. mRNA values of Aldehyde dehydrogenase-6 (ALD6) (A), Aldehyde dehydrogenase-4 (ALD4) (B), Acetyl CoA Synthetase (ACS2) (C), and Glycerol-3-Phosphate Dehydrogenase 2 (GPD2) (D) were normalized using the beta actin (ACT1) expression. Fermentation assays were performed in duplicate with LBGA-01 at 30 and 40°C.
Figure 6

Physiological aspects of LBGA-01 strain in bioreactor fermentation under highly stressful conditions using sugarcane molasses as carbon source. The CO2 profiles were normalized with the wet biomass. The cell viability was evaluated over four cycles. Biomass increase were calculated between two consecutive cycles for LBGA-01 at 34 °C and 40°C. Glycerol production (% w.v-1) was evaluated at 34 °C and 40°C and ethanol yield is showed as a percentage of theoretical maximum (%)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile2.pdf
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