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ORIGINAL PAPER

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Isolation by genetic and physiological characteristics of a fuel-ethanol fermentative *Saccharomyces cerevisiae* strain with potential for genetic manipulation

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Abstract Fuel ethanol fermentation process is a complex environment with an intensive succession of yeast strains. The population stability depends on the use of a well-adapted strain that can fit to a particular industrial plant. This stability helps to keep high level of ethanol yield and it is absolutely required when intending to use recombinant strains. Yeast strains have been previously isolated from different distilleries in Northeast Brazil and clustered in genetic strains by PCR-fingerprinting. In this report we present the isolation and selection of a novel *Saccharomyces cerevisiae* strain by its high dominance in the yeast population. The new strain, JP1 strain, presented practically the same fermentative capacity and stress tolerance like the most used commercial strains, with advantages of being highly adapted to different industrial units in Northeast Brazil that used sugar cane juice as substrate. Moreover, it presented higher transformation efficiency that pointed out its potential for genetic manipulations. The importance of this strain selection programme for ethanol production is discussed.

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Introduction

Bioethanol is now considered a profitable commodity by its increasing use as renewable energy source and car fuel. Brazil is the biggest world producer, with over 15 billions liters per year produced by more than 300 distilleries concentrated in southern and northeast regions. This last region presents over 70 distilleries and represents one of the most important ethanol-producing region the world. Sugar cane juice and sugar cane molasses are the substrates for yeast fermentation in Brazil that can last for 6 months [1]. An important characteristic of the process is its non-sterile condition, which makes it susceptible to contamination by non-*Saccharomyces cerevisiae* yeasts. Moreover, the incoming of wild strains of *S. cerevisiae* produces a succession in the yeast population, where high genetically and physiologically adapted strains tend to dominate [2].

The most important aspect of the fuel-ethanol fermentation is ethanol yield, or more generally the industrial yield. It is dependent, among many factors, on the fermentative capacity of the yeast population [3] and the resistance of those industrial cells to stress conditions [4, 5]. After being exposed to different types of stress, yeast cells build up a rapid cellular response that aims to protect cell components against damages, which involves production of protective proteins, like heat shock proteins, and synthesis or degradation of metabolites, such as glutathione and threalose [6–8]. Thermotolerant yeasts have being identified and their potential for fermentation process reported, although a few of them have been selected for industrial use [4, 9].

The isolation and selection of yeast strains takes advantage of the many DNA-based typing methods already available [10]. Microsatellite-based primers are

wide spread on this subject due to their potential to generate fingerprinting patterns with well-accepted intraspecific discriminatory power. The $(GTG)_5$ primer was successfully used to discriminate wine and brew [11] and fuel-ethanol [2] isolates of *S. cerevisiae*, while primers like $(GACA)_4$ failed in this field [11, 12].

In this work, we report the selection of a yeast strain with high dominance in yeast population of industrial fuel-ethanol production and its molecular identity by the use of three PCR-fingerprinting markers. This strain, named JP1, showed the same stress resistance than the most used commercial strain in Brazil, and high stability to the process. Together with its potential for genetic manipulation, the physiological characteristics of JP1 accredit it to be used as a new industrial strain for bio-ethanol production.

Material and methods

Strains and cell maintenance

Commercial *S. cerevisiae* strains PE-2, SA-1 and BG-1 were kindly provided by Lallemand, Inc. (Canada) through its representative in Brazil. *S. cerevisiae* IA1238 strain was kindly provided by Department of Antibiotics Culture Collection (UFPE) and the industrial strains were isolated from distillery Japungu Agroindustrial (Santa Rita-PB, Brazil). Must samples were plated onto WLN medium containing nalidixic acid and ampicillin (both at 50 μg ml^{-1}) after appropriate dilutions. Individual colonies were typed by their morphology and genotyped by PCR-fingerprinting as recently described [2]. The strains were maintained in YPD plates. *E. coli* strain DH5α was used as host for genetic manipulations [13].

Physiological analysis

Specific growth rates were calculated from cell growth in mineral medium containing 2% glucose [14] or in diluted molasses medium containing glucose to 69 g l^{-1} and ammonium sulphate to 5 g l^{-1}. Cells from late log phase cultures were diluted to fresh medium to $OD_{660\ nm}$ of 0.1 and incubated at 30°C. Samples were withdrawn each 60 min to measure OD at 660 nm. The slope of the exponential curve defined maximum specific growth rate (μ, h^{-1}). Each experiment was performed twice and the mean values were shown.

To test the fermentative capacity of the *S. cerevisiae* strains, cells from late log phase cultures were collected by centrifugation, washed with sterile distilled water and suspended to 20% (w/v) in saline. The cell suspensions were used to inoculate mineral medium containing 12% sucrose (final concentration) to a final biomass concentration of 10% (w/v). The suspensions were incubated at 34°C under slow agitation. The brix was measured with a manual refratometer and the fermentation was considered finished by repeating the brix between samples. At the end of the fermentation, the cell suspensions were harvested and the supernatants were used for further analysis. Ethanol was determined by gas chromatography at Japungu distillery, while glycerol and sucrose were determined by triglycerides analysis kit (Biobras, Brazil) and by DNSA method [15], respectively. Each experiment was performed twice and the mean values were shown.

To test for stress resistance, late log phase yeast cells were suspended at 1×10^8 cell ml^{-1} in water or 10 mM H_2SO_4 solution (acidic water at pH 2). Cell suspensions were incubated at 37 or 42°C in the presence or absence of ethanol to 7% (v/v) final, in different combinations. At defined periods, samples were withdrawn and yeast cells spread onto YPD-containing plates after appropriated dilutions. The number of colony forming units was determined and the percentage of cell survivor was calculated by using untreated cells as control (100%). Each experiment was performed twice and the mean values were shown.

Molecular methods

DNA extraction and PCR-fingerprinting analysis were performed as described by Silva-Filho et al. [2]. Plasmids pUG6 and pNatCre have been described elsewhere [16, 17]. The primers EDPf1 and EDPrev1 contained a 40 bp sequences downstream of the 5′-ATG and upstream of the 3′-TAA termini of the *S. cerevisiae GDH*1 gene, respectively, followed by 20 bp nucleotide sequences used to amplify the 1.6 kb integrative cassette *loxP*-Kan^r-*loxP* from pUG6, similar to that described by Steensma and Ter Linde [17]. Yeast cell transformations were performed by lithium acetate method [18], with some modifications. The transformed cells were suspended in YPD and cultivated for 2 h at 30°C before platting onto YPD supplemented with Geneticin (200 μg ml^{-1}) for integrative transformation or Clonat (50 μg ml^{-1}) for pNatCre. The plates were incubated at 30°C until colony appearance. Each experiment was performed three times and the mean values were shown.

Results and discussion

Samples were collected from the fermentation must at Japungu Agroindustrial distillery during the harvesting period 2000–2002 and platted onto WLN medium. Cells from individual colonies were typed by PCR-fingerprinting [2] and for molecular karyotyping for strain discrimination (data not shown). One of the most frequent microsatellite primer $(GTG)_5$ amplification patterns was classified as P18 [2] and one of its isolate, MF114(2), was named JP1 strain. Further fingerprinting analysis was done to compare the genetic background of JP1 strain to the commercial *S. cerevisiae* strains used for industrial ethanol

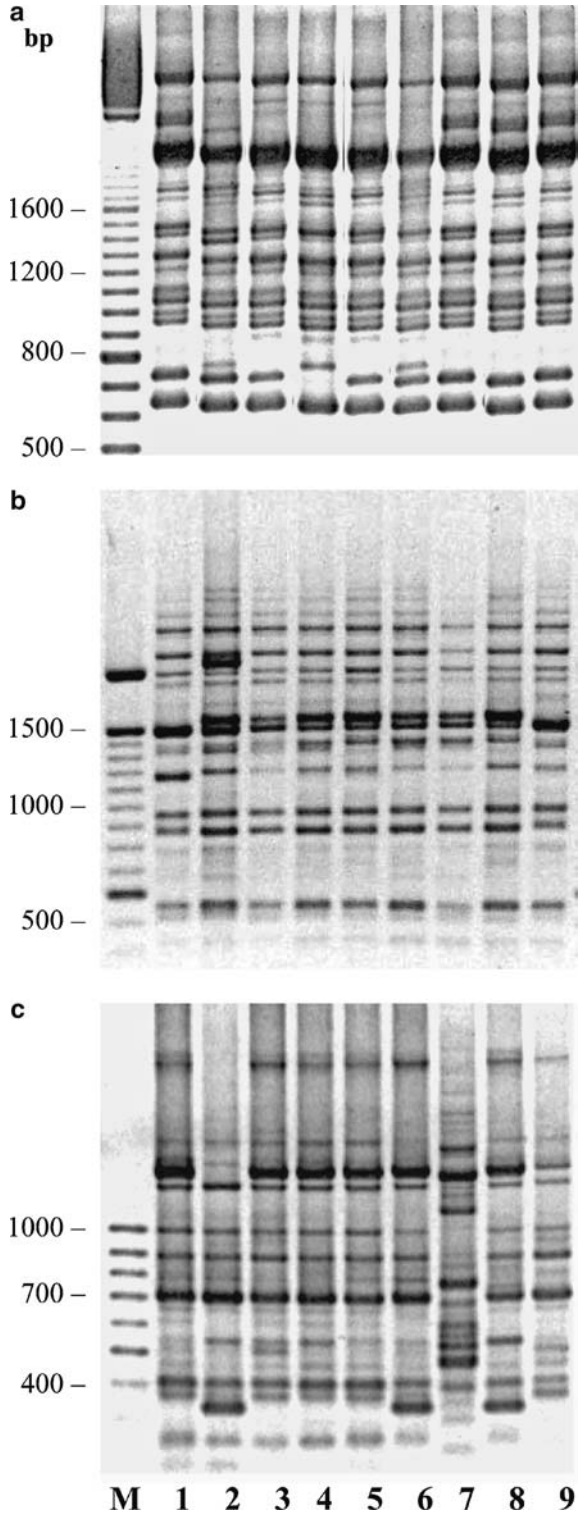

Fig. 1 PCR-fingerprinting with primers $(GTG)_5$ (**a**), intron E-1 (**b**) and $(GACA)_4$ (**c**) of the industrial yeast strains IA1238 (lane 1), JP1 (lane 2), VR-1 (lane 3), CAT-1 (lane 4), CR-1 (lane 5), SA-1 (lane 6), PE-2 (lane 7), BG-1 (lane 8) and FT-001 (lane 9). Molecular weight (*M*) of commercial standards is given at the left side in base pairs (bp)

production to date (Fig. 1). The concomitant use of the three PCR primers $(GTG)_5$, intron E1 and $(GACA)_4$ was able to unequivocally discriminate all yeast strains. It was especially true for VR-1 and CR-1 and for JP1 and FT-001 industrial strains. Both VR-1 and CR-1 strains showed the same $(GTG)_5$ classified as P28A [2], but differed in their amplification patterns by the use of intron E1 and $(GACA)_4$ primers (Fig. 1, lines 3 and 5). In the second case, JP1 and FT-001 were discriminated by all three primers (Fig. 1, lines 2 and 9). The microsatellite primer $(GTG)_5$ has been successfully used to discriminate yeast strains [2], while the microsatelite primer $(GACA)_4$ did not succeed in this subject. This last primer showed lower discriminatory power than $(GTG)_5$ when applied to *S. cerevisiae* [11] and to *Cryptococcus neoformans* [12]. The intron splice site primer EI1 produced species-specific amplification patterns useful for species but not strain discrimination, due to its low degree of polymorphism [19]. However, in this work both the primers were important to differentiate industrial yeast strains when $(GTG)_5$ failed to do so. It is in accordance with the necessity of using more than one molecular marker to a complete differentiation of yeast strains [10, 20].

We have previously shown that different strains are adapted to a particular industrial process [2]. For example, our previous work on microbiological control of the fermentation process revealed that some baker's strains and the PE-2 strain, the most used commercial strain in Southern Brazil, are not adapted to industrial conditions in Northeast Brazil (unpublished results). In the harvesting period 2001–2002, JP1 strain accounted for 12.5% of the yeast population in the whole fermentation process (Fig. 2a). Thus, Japungu distillery used this strain as starter in the crop harvesting period 2002–2003 for bioethanol production and industrial samples were typed to follow the fate of the yeast population. As it can be seen from Fig. 2b, the industrial yeast population started with 100% JP1 cells, which remained unaltered for over 3 months. Even after a severe contamination episode, JP1 sub-population recovered to dominate the whole population at the end of fermentation. The overall sugar-conversion efficiency was around 93% at the distillery, which is normally considered high for the fermentation conditions used in Northeast Brazil distilleries. In the following harvesting period 2003–2004, again the distillery used JP1 as started strain. JP1 cells, contaminant cells and new-coming *S. cerevisiae* strains composed the yeast population at the end of the first 30 days. However, JP1 strain recovered its dominance in the course of the fermentation process (Fig. 2c), with the overall sugar-conversion efficiency around 92% at that period. That surveillance of the yeast population over three consecutive harvesting periods showed the robustness of JP1 strain in the Japungu fermentation plant. In another distillery nearby, Miriri distillery, JP1 cells were detected at high frequency in the fermentation must (Fig. 3a). Both distilleries are less than 20 km faraway and share crop fields, which may explain the common origin of JP1 strain. On the other hand, this strain was not found at

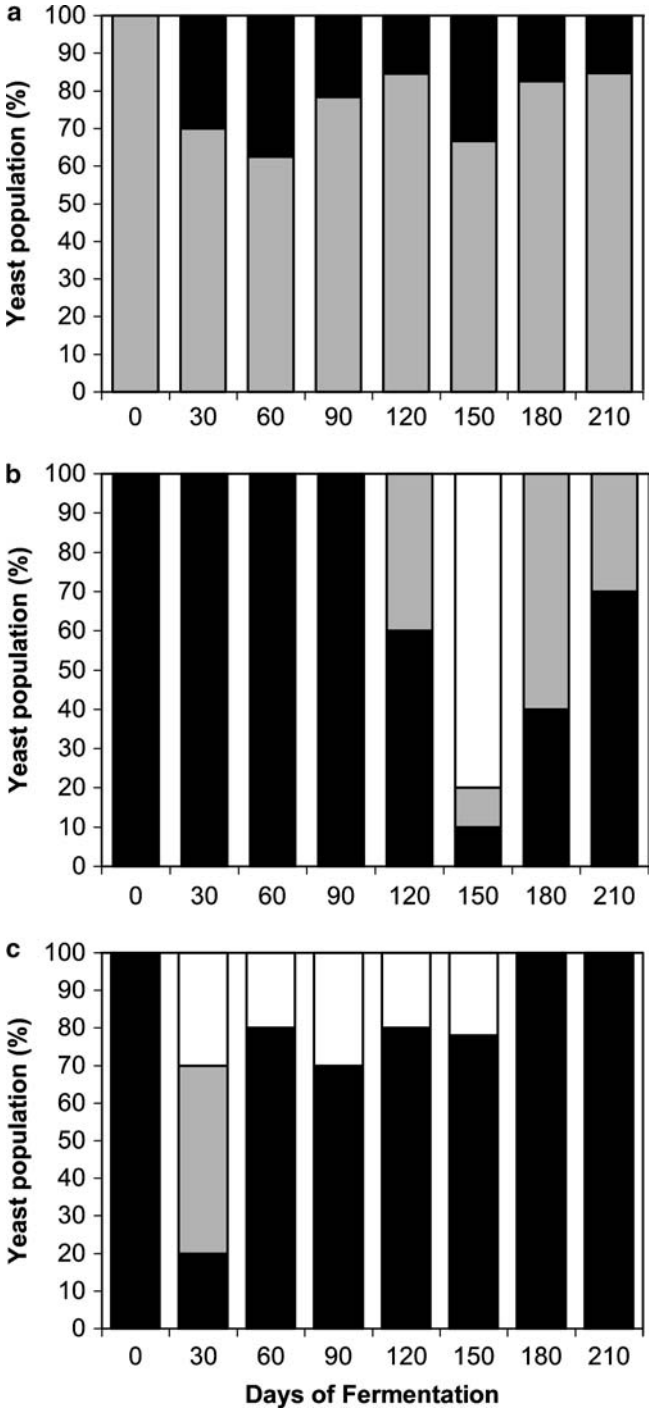

Fig. 2 Frequency of JP1 cells (*black columns*) in the yeast population of Distillery Japungu in the course of fermentation process at 2001–2002 (**a**), 2002–2003 (**b**) and 2003–2004 (**c**) harvesting periods. The sum of other *S. cerevisiae* strains (*grey columns*) and the presence of non-*S. cerevisiae* contaminant yeasts (*white columns*) were also plotted

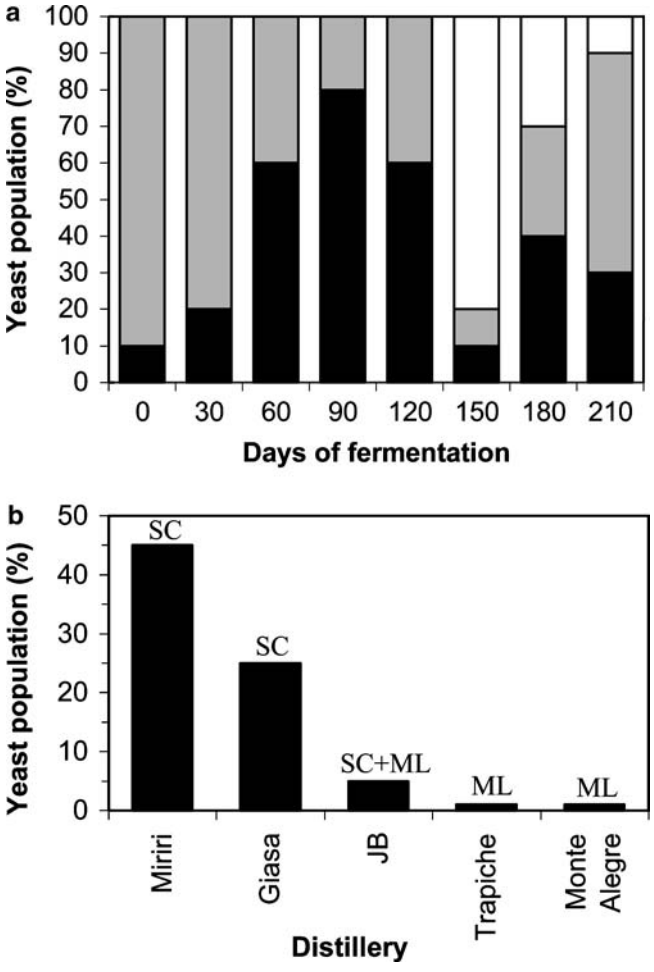

Fig. 3 Frequency of JP1 cells in the yeast population of different distilleries in the course of fermentation process of the crop harvesting period 2002–2003. **a** Frequency of JP1 cells at Distillery Miriri. **b** Average of JP1 cells in the whole fermentation process in the crop harvesting period 2002–2003 at distilleries that uses sugar cane juice (*SC*), molasses (*ML*) or a mixture of both substrates (*SC* + *ML*)

high frequency in distilleries that used molasses as fermenting substrate (Fig. 3b). That substrate-specificity has been previously shown by our group [2], and may be a consequence of differences in yeast tolerance to toxic compound found in molasses, formed during sugar production that generates molasses as by-product. Growing yeast cells on molasses medium tested this characteristic and the results showed that JP1 strain growth (0.16 h^{-1}) was half of that observed for PE-2 (0.23 h^{-1}) (Fig. 4), a strain originally isolated from molasses-using distillery in São Paulo state. However, they showed almost the same growth rate when using diluted sugar cane juice containing 5% sucrose (c.a. 0.3 h^{-1}). It reinforced the specificity of JP1 for sugar cane using distilleries.

Thermotolerant yeast strains have been selected from an enrichment and isolation program in sugar cane molasses distillery in Egypt [21]. The heat-resistant isolate was used for bioethanol production with economical advantages for the distillery. Together, those results demonstrate that continuous selection program to isolate more adapted autochthones strains may be an efficient way to achieve higher ethanol production. Such dominant strains are natural target for future genetic

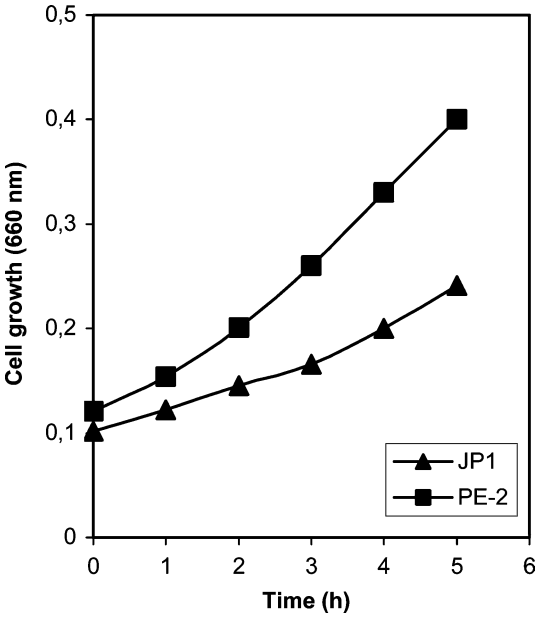

Fig. 4 Growth curve of JP1 and PE-2 strains in shake flask containing molasses diluted to 5% sugar concentration

manipulations, which ensures that the recombinant strain will stay in the process. Further experiments were done to test the resistance of JP1 cells to industrial stressing conditions. Almost no difference was detected between JP1 and PE-2 strains to heat, ethanol and acid tolerance (Fig. 4). Only a slight higher resistance of JP1 was observed when combining low pH with high temperature (Fig. 4), without major significance. This behaviour is especially important to fermentation processes at hot areas such as Northeast Brazil and Central America [1]. The results showed that the toxic effect of low pH or the presence of ethanol was magnified by increasing the temperature from 37 to 42°C. The cell viability dropped to 90% after incubation at 42°C for both the strains and practically no killing affect was observed after incubation at 37°C (data not shown).

By its performance at the industrial yeast population, JP1 was considered a promising strain to be used as starter yeast for industrial processes that use sugar cane juice as substrate and it has been used by some distilleries in Northeast Brazil thenceforward. The most used fuel-ethanol commercial yeast strain in Brazil to date, PE-2 strain, has been used as starter strain in some distilleries in Northeast Brazil, two of which (Monte Alegre and Giasa) have been accompanied by our work on industrial microbiological control by PCR-fingerprinting. In both distilleries, the population of PE-2 cells decreased from 100% to less than 10% in 30 days of fermentation, without recovering later on. This phenomenon was observed for two consecutive harvesting periods (data not shown).

The most important aspect at industrial level, ethanol yield, was tested for industrial and commercial strains in laboratory conditions. The yeast strain IA1238 was also analysed because it was used for a long time as the starter strain in Japungu Distillery (Table 1). All the strains tested presented fermentation time around 3 h, leaving residual sugar at 0.3–0.5 g l^{-1}. JP1 strain did not show the highest ethanol production and yield, probably due to its high growth rate that led to high glycerol production (Table 1). Anyway, its sugar–ethanol conversion-efficiency in laboratory medium was 92%. However, its high fitness–industrial process pointed it as a good platform for future genetic manipulation to increase ethanol production.

Recent experimental evidences showed that genetic manipulation of the nitrogen assimilation pathway in *S. cerevisiae* has consequences to increase ethanol production while decreasing glycerol formation [22]. It involved both cell transformation with episomal plasmid and genetic integration of linear DNA for gene disruption. Additionally, *S. cerevisiae* cells can be modified to ferment xylose to ethanol by expressing plasmid-based or genome integrated heterologous genes [23]. To test JP1 for genetic manipulations, yeast cells were assayed for cell transformability. The results showed JP-1 strain with transformation efficiency with both episomal plasmid and integrative DNA higher than all commercial strains tested (Table 1). The transformation efficiency shown by JP-1 strain was over 10^4 and 10^2 transfor-

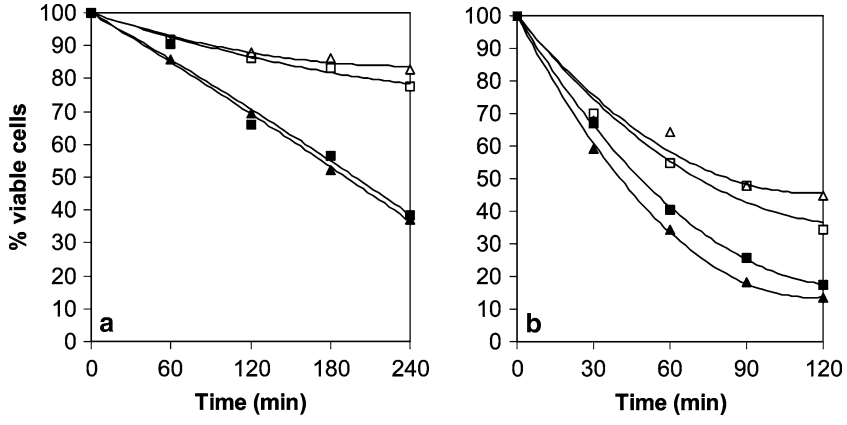

Fig. 5 Yeast cell resistance to ethanolic (**a**) and acidic (**b**) stresses. Late log phase cells of JP1 (*square*) and PE-2 (*triangle*) strains were suspended in 7% ethanol or 10 mM H_2SO_4 solutions and incubated at 37°C (*open symbols*) or 42°C (*closed symbols*) as described in Materials and Methods

Table 1 Physiological parameters and genetic transformation efficiency of industrial *S. cerevisiae* strains

Strain	μ_{max} (h^{-1})	Ethanol ($g\ l^{-1}$)	Glycerol ($g\ l^{-1}$)	Ethanol yield $Y_{Et/suc}$	CloNat^r transf.[a] (μg DNA)$^{-1}$	G-418^r transf.[b] (μg DNA)$^{-1}$
BG-1	0.44	59.3	7.19	0.49	0.18×10^4	0.01×10^2
CR-1	0.47	60.3	7.76	0.50	0.64×10^4	ND
PE-2	0.45	56.4	6.76	0.47	ND	ND
SA-1	0.45	60.3	6.40	0.50	0.25×10^4	0.33×10^2
IA1238	0.45	54.8	7.22	0.46	0.39×10^4	ND
JP1	0.49	56.2	7.30	0.47	1.37×10^4	2.85×10^2

[a]Cell transformation with the episomal plasmid pNatCre [17]
[b]Cell transformed with the integrative cassette *loxP*-KanMX-*loxP* [16]*ND* Not done

mants per microgram of plasmid and linear DNA, respectively, which is enough for the majority of the desired genetic manipulation procedures [18].

The strain JP1 is now being commercialised as a new fermentative strain for bioethanol production in Brazil in addition to the up-to-date six strains PE-2, CAT-1, BG-1, VR-1, SA-1 and CR-1 used so far for that purpose. Moreover, this strain has been deposited at the Department of Mycology Culture Collection (URM-Recife), Federal University of Pernambuco, which is part of the World Directory of Collections of Culture of Microorganisms (WFCC) under the nr.604, and can be released for research purpose upon request.

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