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Higher thermal and ethanol tolerance of a yeast strain isolated from oral cavity

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Efficient bioethanol producing microorganisms must be endowed with peculiar physiological and technological traits, such as, higher thermal and ethanol tolerance. This encompasses a strain thermotolerance and an ability to grow at elevated sugar and ethanol concentration or ability to sustain dehydration process such as freeze drying. In this study, we characterized a thermotolerant yeast strain isolated from the human oral cavity regarding the above mentioned parameters. Such an uncommon niche was considered as the great potential reservoir, to isolate strains endowed with metabolic aptitudes requested for ethanol production. In the process, we have defined the YTerm-1strain ability to sustain high sugar and ethanol concentration that are two technological constrains in bioethanol production. Finally, we highlighted that the strain YTerm-1 was able to accumulate, at a high level, trehalose and β-glucan, two compounds conferring the cells an increased resistance to freeze drying process and osmotic stress. Our results suggest that the Yterm-1 strain showed a better growth ability and higher ethanol yield as compared to the industrial strain. Other metabolic traits, such as resistance to dehydration stress, tolerance to ethanol, accumulation of intracellular trehalose or membrane β-glucan confer to that isolate all the characteristics requested in industrial production of ethanol.

Keywords: beta-Glucan, Biofuel, *Saccharomyces cerevisiae*, Thermotolerant, Trehalose

The yeast *Saccharomyces cerevisiae* has been isolated from different environmental niches, domesticated and used in many biotechnological applications based, notably, on its ability to convert sugars into alcohol. Actually, the bio-based production of ethanol has been considered as one alternative to petroleum[1]. Although *S. cerevisiae* has been used for decades for this purpose, there are several challenges to face such as a rise in temperature during aerobic sugar fermentation[2,3]. Therefore, thermotolerant strains may contribute to reduce the cost of bioreactor cooling at an industrial scale. Yeasts isolated from a given environmental niche present metabolic adaptations acquired overtime to sustain the specific environmental pressure[4-6]. In our previous study, an uncommon niche, namely the human oral cavity, has been considered. Several strains have been identified as *S. cerevisiae* through analysis of ITS-16S-rDNA region and characterized as thermotolerant[7]. Among them, strain Yterm-1 showed a significantly increased specific growth rate at 37°C (i.e. 0.489 h^{-1}) as compared to 30°C (i.e. 0.304 h^{-1}), demonstrating thus its adaptation to higher temperatures. Moreover, its specific growth rate was also found significantly higher (43% on average) than that of several commercial thermotolerant strains used for ethanol production.

Thermotolerance is not the only requested physiological trait to consider for ethanol production. In the 'so-called' high gravity fermentation, the initial sugar concentration is around 150 g/L increasing, thus the osmolarity of the culture medium[8,9]. During the process, this sugar is converted into ethanol that accumulates over time. Therefore, the ability for cells to sustain a high osmotic pressure and high ethanol concentration must be also considered. Finally, the ability of cell to resist to dehydration must be also considered as yeast starters are usually commercialized as a dry product[10,11].

Several works have reported isolation of *S. cerevisiae* strains in clinical samples from patients[12,13]. However, further characterizations of these strains for industrial applications have not been explored yet. In our previous studies, five *S. cerevisiae* clinical isolates able to growth at elevated temperature have been identified. Based on the thermotolerance, we have made an attempt to further characterize the most promising isolate, named

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YTerm-1, and to produce ethanol and to tolerate high concentration of ethanol and glucose. We also investigated the ability of strain YTerm-1 to accumulate trehalose and β-glucan, as these two compounds are well known to confer cells an increased resistance to dehydration and osmotic stress induced at high sugar concentration[14,15]. To be of practical use, modern industries mainly consider dehydrated microorganisms in order to avoid any change in the physiological and morphological characteristics of cells during storage[10]. Freeze drying is a technique of dehydration widely used in industry for formulation of dry starters which could affect considerably the viability of cells[11]. Hence, we compared the resistance of YTerm-1 and Quickferm Super strains to drying process as well.

Materials and Methods

Media, strains and culture conditions

All chemicals and media were from Sigma-Aldrich or Biocorp (Poland). In the study, *S. cerevisiae* strains YTerm-1[16] and Quickferm Super (SternEnzym GmbH & Co) were used. They were grown at 30°C or 37°C as stipulated in the text in media YPD (20 g/L glucose, 10 g/L peptone, 10g/L yeast extract) or YPDE (20 g/L to 200 g/L of glucose, 50 g/L to 200 g/L of ethanol, 10 g/L peptone, 10g/L yeast extract). Cultures were performed for 48 h in shake-flasks (50 mL medium in 100 mL) or in 15 mL sealed tube (Falcon, Greiner). For ethanol production, cultures were performed in 50 mL sealed tube (Falcon, Greiner) containing 45 mL of modified YPD medium containing 20 or 100 g/L glucose. Cultures were seeded at an initial optical density at 600 nm (OD600) of 2.

Analytical methods

Biomass was monitored by OD600 measurement using a TECAN Infinite M200 spectrophotometer (Thermofisher). β-glucan concentration was performed using the β-Glucan Assay Kit (K-YBGL, Megazyme, USA) according the manufacturer's instructions. Trehalose was extracted as previously described[16] and its concentrations were determined by corona CAD-HPLC (integrated UltiMateDionex system: 3000 RSLC, Sunnyvale, CA, USA) using a Sugar-D column (4.6×250 mm, 5 mm, Cosmosil). The mobile face was 7:3 acetonitrile/water mixture (v/v) operated at flow rate of 1.0 mL/min, temperature 30°C. Trehalose concentration was expressed in mg/g of dry cell weight (CDW). Ethanol concentrations were determined by gas chromatography using a Trace GC Ultra (Termo, Italy) instrument equipped with a split/splitless injector, a flame ionization detector (FID) and a TG-ALC1 column (30 m×0.32 mm ID×1.8 μm). The oven temperature was 65°C. Helium was used as a carrier gas at a flow-rate of 3.0 mL/min. The temperature at detector was set at 280°C. Split injection (1:150) was carried out at 210°C. For each analysis one μL of the sample was injected on the column. The results were recorded and processed using Chrom-Card version 2.4.1software (Termo, Italy).

Freeze drying process

Yeast cells from a 48 h culture (150 mL) in YPD were collected by centrifugation at 5,000×g for 15 min at 4°C. The harvested cells were then washed twice with 0.9% NaCl solution before being resuspended in 3 mL of a cryoprotectant mixture solution (10% w/v skimmed milk and 5% w/v sucrose) as previously described[33]. The cell suspensions were poured as a thin layer in a Petri dishes and frozen at −80°C for 24 h. Samples were then freeze-dried using a Christ LSC Plus freeze-dryer (Grosseron, France) operating at a condenser plate temperature of −70°C and a chamber pressure less than 1.3 mbar for 72 h. The yeast survival ratio was calculated after a cell count on YPD plates after serial 10-fold dilutions before and after freeze-drying.

Statistical analysis

The statistical analyses were performed using the GraphPad Prism 6. Statistical significance was assessed by Dunnett's or Tukey's multiple comparisons test.

Results and Discussion

Alongside thermotolerance, the ability of *S. cerevisiae* to sustain high ethanol concentration and to grow at high sugar content (i.e. high osmolarity) directly influence ethanol productivity[17]. Therefore, the growth ability of strains YTerm-1 and the commercial QuickFerm Super used here as a reference was monitored in regard to ethanol and sugar concentrations. According to Nuñez-Guerrero *et al*.[18] a good ethanol tolerance, up to 8%, is one of the main desirable characteristics for yeast fermentation. Thus, strains were first grown in rich YPDE medium containing ethanol at different concentrations (0; 5 and 10% v/v). After 48 h of culture, the biomass values were used to compare cell growth ability. As shown in Fig. 1A, cell growth of

YTerm-1 was slightly affected by the presence of 5% ethanol in the culture medium as compared to the non-supplemented medium. However, no further significant difference in biomass could be observed for higher ethanol concentrations (i.e. 10%). This demonstrates that cell growth ability was not altered for strain YTerm-1 in those conditions. By contrast, cell growth of the reference strain QuickFerm Super was inhibited at high ethanol concentration (i.e. 10%, Fig 1A). These observations made for YTerm-1 are in accordance with previous researches reporting an ethanol tolerance ranging between 7-10%[19]. In another study, authors have screened for ethanol tolerance several yeast strains belonging to *S. cerevisiae*, *Kluyveromyces marxianus* and *Torulaspora. delbrueckii*[18]. As a good candidate for ethanol production, wild type *S. cerevisiae* strain ITD-00185 which exhibited ethanol resistance up to 8%, has been selected. Divate *et al*.[20] compared the ethanol resistance of the wild type (SC) and engineered derivative (SCTΔN) of *S. cerevisiae*. It has been shown that the growth of SCTΔN was inhibited by ethanol concentrations greater than 10%, whereas 6% of ethanol slowed down the growth of SC[20].

One of the factors which affect the cell growth is the sugar concentration since it affects the osmotic pressure of the culture medium. Therefore, a high initial glucose level in the fermentative media may induce in cell an osmotic stress that could lower or inhibit ethanol production[21-23]. Indeed, Zhang *et al*.[21] reported on the effect of substrate inhibition on performance of ethanol fermentation using *S. cerevisiae* BY4742. Within the tested concentrations, ranging between 40-280 g/L, a glucose concentration of 80 g/L was found the optimal value in regards to ethanol yield. Exposure to hypertonic solution may decrease the cell membrane fluidity and cause the organelle dehydration while an excessive glucose concentration could lead to some metabolic disorder and thus to a lower ethanol productivity[21]. In another study, the highest ethanol production rate and glucose consumption rate occurred in the presence of 10% of sugar in the medium[23]. Thus, strains YTerm-1 and Quickferm Super were grown in YPD medium supplemented with different amount of glucose (2-200 g/L) and the corresponding biomasses were determined after 48 h of culture. As shown in Fig. 1B, the biomass obtained, and thus the cell growth ability, were not significantly different for YTerm-1 in regard to glucose concentration. The biomass obtained in all the experimental conditions tested were also slightly higher than those of reference strain. This demonstrate that YTerm-1 could be used in high gravity fermentation process.

Among the different constituents of the fungal cell wall, β-glucan, a polysaccharide composed of D-glucose units linked by β-glycosidic bonds, is involved in maintaining cell integrity notably at elevated osmotic pressure[24-27]. Therefore, the potential of YTerm-1 and Quickferm Super strains to accumulate β-glucans in their cell wall was measured at 30°C and 37°C. As shown in Table 1, β-glucan content was higher at 37°C as compared to 30°C (2-fold and 1.4-fold, respectively). Moreover, at 37°C β-glucan content was 30% higher for strain YTerm-1 as compared to Quickferm Super strain. These values

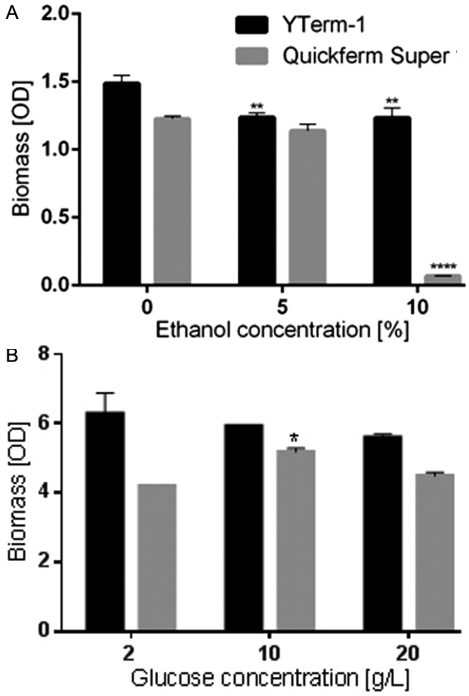

Fig. 1 — Biomass (OD600) of strains YTerm-1 and QuickFerm Super after 48 h of growth in (A) YPD medium (glucose 20 g/L) supplemented with 0, 5 and 10% of ethanol; and (B) YPD medium containing 2, 10 and 20% of glucose. [Statistical analyses were performed using Dunnett's multiple comparisons test, asterisks indicate significant differences (**P <0.01) between control (0%) and ethanol stress conditions]

Table 1 — β-glucans and trehalose content of the YTerm-1 and Quickferm Super strain

	β-glucan [%]		Trehalose [mg/gCDW]	
	30°C	37°C	30°C	37°C
YTerm-1	12.56±0.19	25.21±0.03	6.67±0.08	7.61±0.04
Quickferm Super	14.76±0.68	17.73±0.01	1.75±0.05	4.43±0.29

of β-glucan content are also higher than those reported in the literature[24]. For instance, Varelas *et al*.[24] reported a β-glucan content of 6.3% for *S. cerevisiae* strain VIN 13 while Mongkontanawat *et al*.[28] reported a value of 15, 8.3 and 12.7%, respectively for *S. cerevisiae* strains TISTR 5919, 5020 and Angel. The higher content of the β-glucans obtained for strain YTerm-1 could be related to its aptitude to grow at high sugar concentration (i.e. high osmolarity). In summary, this set of experiments highlighted that the thermotolerant strain YTerm-1 is compatible with ethanol process conditions, as it is able to sustain high sugar and ethanol concentration.

As high gravity fermentation processes are operated at a starting sugar concentration in the range of 100 to 150 g/L[29], ethanol production of strains YTerm-1 and Quickferm Super was determined after 24 h and 48 h of growth at 37°C in YPD medium containing 10% of glucose. As shown in Fig. 2, YTerm-1 produced ethanol with titer of 41 g/L while QuickFerm Super yielded to a significantly lower value (12.9 g/L). For strain *S. cerevisiae* BCRC21812 grown in batch bioreactor for 30 h at an initial glucose concentration of 100 g/L, an ethanol titer of 48.7 g/L was reported[23]. Other studies reported the similar results. For instance, for *S. cerevisiae* wild type strain SC and engineered derivative SCTΔN grown in fermentation broth containing 10% of glucose, a maximal ethanol titer of 40 g/L was obtained after 96 h[20]. Similarly, other authors pointed out that among various genetically improved *S. cerevisiae* strains, the highest concentration of ethanol (34.6 g/L) was assessed for the hap4-OE strain (*S. cerevisiae* BY4741 overexpressing the transcription factor HAP4) after 26 h of fermentation of 100 g/L glucose[30]. Zhang *et al*.[21] highlighted that for *S. cerevisiae* BY4742 maximum ethanol yield was observed after 72 h and in the presence of 80 g/L glucose in the medium, 39 g/L of ethanol was produced. This demonstrates that the ability of YTerm-1 to produced ethanol is in the same range of other reported strains.

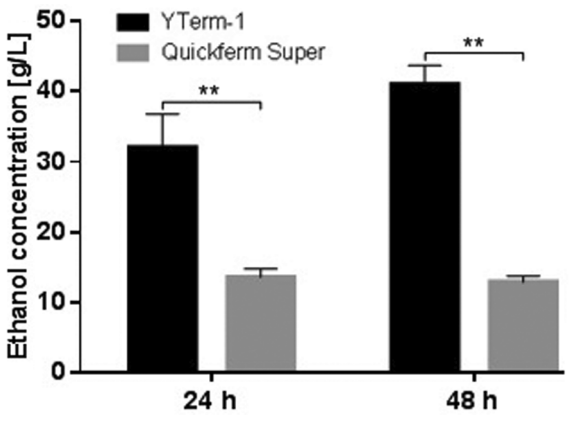

Fig. 2 — Ethanol production [g/L] by thermotolerant yeast YTerm-1 and Quickferm Super after 24 h and 48 h of fermentation at 37°C in YPD medium containing 10% of glucose. [Statistical analyses were performed using Tukey's multiple comparisons test (**P <0.01)]

For many biotechnological applications, dehydrated yeast starters are preferred due to their ease of commercialization, storage and handling. In yeast, resistance to dehydration is related, among other, to the presence of intracellular metabolites such as trehalose[31]. Indeed, the disaccharide avoids fusion of membranes by replacing water molecules in the lipid bilayer[32]. Intracellular accumulation of trehalose was thus quantified in strains YTerm-1 and Quickferm Super, at 30 and 37°C, as its accumulation is triggered by heat stress. As shown in Table 1, the trehalose content increased for both strains as the growth temperature was increased. Trehalose content was equal to 7.61 mg/g_{CDW} and 4.43 mg/g_{CDW} for YTerm-1 and Quickferm Super strains, respectively. Moreover, the quantity of trehalose in Quickferm Super strain was notably increased at 37°C (more than 2.5-fold) while, in strain YTerm-1, trehalose content was only slightly increased in those conditions. Based on these observations, a higher resistance of YTerm-1 to dehydration process could be hypothesized.

As in Fig. 3, strain YTerm-1 showed a higher resistance to freeze-drying process as compared to Quickferm Super. Indeed, the viability of YTerm-1 strain cultivated at 30 and 37°C was equal to 53 and 55%, respectively. By contrast, Quickferm Super showed a viability of 37.8 and 40%, which is in the same range than the viability obtained for strain *S. cerevisiae* MUCCL 28359 (i.e. 38%) in the same experimental conditions[33]. The remarkable difference (i. e. 29% and 27%, respectively) in cell viability for strains YTerm-1 and Quickferm super grown at 30 and 37°C, respectively, could be correlated to the difference of their intracellular concentrations of trehalose. Indeed, Nakamura *et al*.[34] showed that the survival of yeast cells subjected to freezing depends on the intracellular trehalose content. This was also demonstrated in other studies[20,35,36]. Based on these

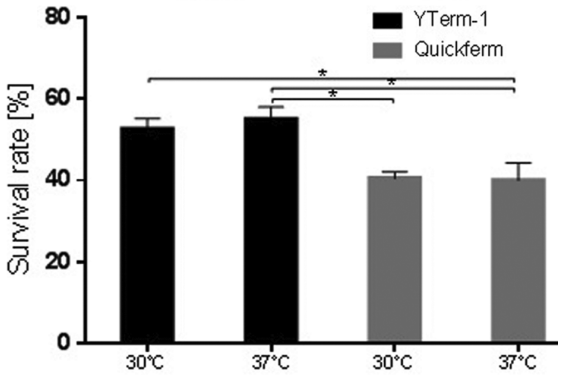

Fig. 3 — Effect of freeze-drying process on yeast cells viability after cultivattion at 30 and 37°C. [Statistical analyses were performed using Tukey's multiple comparisons test (*P <0.05)]

findings, higher content of trehalose and higher survival rate after freeze-drying process, have shown a good cryotolerance of YTerm-1 strain which makes it very usable in areas of applied microbiology.

Conclusion

From an industrial point of view, there is a great interest to isolate yeast strains from specific niches since these strains could be endowed with the desired specific metabolic aptitudes. In the process of bioethanol production, thermotolerance, ability to sustain high sugar and ethanol concentrations, together with the ability to convert sugars into ethanol and a good resistance to dehydration are the key parameters to consider. In this study, we demonstrated that uncommon niches, such as the human oral cavity could constitute a good reservoir to isolate such a strain. The Yterm-1strain isolated from the human oral cavity was found to perform equal or even better regarding these parameters than the commercial strain Quickferm Super or other strains reported in the literature for ethanol production.

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Conflict of Interest

Authors declare no competing interests.

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