International Journal of Food Microbiology 268 (2018) 27–34

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Expression of heterologous transporters in *Saccharomyces kudriavzevii*: A strategy for improving yeast salt tolerance and fermentation performance

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ARTICLE INFO

Keywords:
Fermentation
Salt tolerance
Alkali-metal-cation exporter
Non-conventional yeasts
Nha1 antiporter
Ena ATPase

ABSTRACT

S. kudriavzevii has potential for fermentations and other biotechnological applications, but is sensitive to many types of stress. We tried to increase its tolerance and performance via the expression of various transporters from different yeast species. Whereas the overexpression of *Z. rouxii* fructose uptake systems (*Zr*Ffz1 and *Zr*Fsy1) or a glycerol importer (*Zr*Stl1) did not improve the ability of *S. kudriavzevii* to consume fructose and survive osmotic stress, the expression of alkali-metal-cation exporters (*Sc*Ena1, *Sc*Nha1, *Yl*Nha2) improved *S. kudriavzevii* salt tolerance, and that of *Sc*Nha1 also the fermentation performance. The level of improvement depended on the type and activity of the transporter suggesting that the natural sensitivity of *S. kudriavzevii* cells to salts is based on a non-optimal functioning of its own transporters.

1. Introduction

The demand for products of high quality, with novel taste properties and good effects on human health has been steadily increasing in recent years. Thus a lot of effort has been spent on studying non-conventional yeast species, including sequencing their genomes, on characterizing their specific properties related to fermentation processes, such as the production of ethanol and aroma compounds or the use of various sugars as carbon sources (Borneman and Pretorius, 2015; Johnson, 2013; Jolly et al., 2014; Kurtzman et al., 2010).

Recently, non-*cerevisiae Saccharomyces* species have attracted attention as a barely exploited resource of yeast biodiversity with interesting traits not present in *S. cerevisiae*, the dominant species in alcoholic fermentation (Jolly et al., 2014; Marsit and Dequin, 2015; Radecka et al., 2015). *Saccharomyces kudriavzevii* is one of them. Besides being one of the parental strains of several interesting natural hybrids (Gonzalez et al., 2006), it has interesting oenological properties itself, including a higher glycerol and lower ethanol production than *S. cerevisiae* during wine must fermentation (Gamero et al., 2013; Gonzalez et al., 2007). Its production of aroma-active compounds, which surpasses *S. cerevisiae* (Stribny et al., 2015; Stribny et al., 2016a; Stribny et al., 2016b), is highly interesting and can be exploited e.g. in the biotechnological production of aroma compounds for the food industry. Though *S. kudriavzevii* has a better growth profile and fructose consumption rate than *S. cerevisiae* at low temperatures (Salvado et al., 2011; Tronchoni et al., 2009), it is highly sensitive to various stresses (Zemancikova et al., 2017) and is probably less adapted to stressful fermentation conditions (Perez-Torrado et al., 2016). Although *S. kudriavzevii* exhibits very similar basic physiological parameters (such as membrane potential, intracellular pH and the degree of quick Pma1 H^+-ATPase activation upon glucose addition) to the other *Saccharomyces* species (*S. cerevisiae*, *S. bayanus*, *S. paradoxus*), it has a low ability to proliferate in media with a limited concentration of potassium, very low osmotolerance and tolerance to toxic cations and cationic drugs, and minimal capacity to survive anhydrobiosis (Zemancikova et al., 2017). The difference in stress-survival among the *Saccharomyces* species seems to be based on their ability to quickly accommodate their cell size and metabolism to changing environmental conditions, and to adjust their portfolio of available transporters.

S. kudriavzevii also lacks competitiveness at higher fermentation temperatures (Arroyo-Lopez et al., 2011). Although it has been defined as a psychrotrophic yeast exhibiting a growth temperature range of 6–32 °C, its optimal growth temperature is 23–26 °C (Arroyo-Lopez et al., 2009; Arroyo-Lopez et al., 2011; Perez-Torrado et al., 2016; Salvado et al., 2011; Sampaio and Goncalves, 2008).

The aim of our work was to improve the fitness and performance of *S. kudriavzevii* under various stresses as it might help in using this species in biotechnology applications, e.g. the production of aroma compounds. We used the approach of the heterologous expression of various transporter genes from other, more stress-tolerant yeast species

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https://doi.org/10.1016/j.ijfoodmicro.2018.01.002
Received 23 August 2017; Received in revised form 28 December 2017; Accepted 2 January 2018
Available online 05 January 2018
0168-1605/

Table 1
Strains used in this study.

Strain	Genotype	Source/Reference
S. kudriavzevii		
IFO1802 *ura3Δ*	*ura3Δ*	A. Querol (IATA-CSIC)
S. cerevisiae		
FL100	wt; MATa	ATCC 28383
W303-1A	MATa *ura3-1 leu2-3/112 trp1–1 his3-11/15 ade2-1 can1-100 GAL SUC2 mal0*	Thomas and Rothstein (1989)
BW31a	W303-1A *nha1Δ::LEU2 ena1::HIS3::ena4Δ*	Kinclova-Zimmermannova et al. (2005)
AB11c	W303-1A *nha1Δ::LEU2 ena1::HIS3::ena4Δ nhx1Δ::TRP1*	Maresova and Sychrova (2005)

in order to improve various properties of *S. kudriavzevii* cells, including its fermentation performance. First, in order to improve the ability of *S. kudriavzeii* cells to consume fructose, we expressed genes encoding specific fructose transporters from the fructophilic and osmotolerant yeast *Zygosaccharomyces rouxii*. Second, one gene encoding a glycerol-uptake transporter from *Z. rouxii*, and three genes for alkali-metal-cation exporters from *S. cerevisiae* and *Yarrowia lipolytica*, respectively, were expressed in *S. kudriavzevii* to increase its osmotolerance.

2. Materials and methods

2.1. Yeast strains, cultivation media and plasmids

The yeast strains used in this work are listed in Table 1. Cells were grown either in complete YPD medium (1% yeast extract, 2% bacto-peptone, 2% glucose) or in minimal synthetic YNB medium (0.67% YNB without amino acids, 2% glucose, with auxotrophic supplements when necessary). YNB media for growth assays were supplemented with salts, sorbitol, glycerol, glucose and fructose at the indicated concentrations. Media with pH 5.5 and 7.0 were buffered with either 20 mM MES or 20 mM HEPES. pH was adjusted to the required value with NH_4OH to avoid an increase in sodium cations in the media. All strains were cultivated at 25 °C. The plasmids used in this study are listed in Table 2.

2.2. Fluorescence microscopy

Cells expressing transporters tagged with the GFP at their C-termini were viewed with an Olympus AX70 fluorescence microscope, using a U-MWB cube with a 450–480 nm excitation filter and a 515 nm barrier filter.

2.3. Growth assays

The growth of yeast cells was monitored in classical drop test assays. Stationary cells (pregrown on plates for 2 days and stored at 4 °C for 3 days) were suspended in sterile water to OD_{600} = 2.0 and 3 to 4 serial 10-fold dilutions of cell suspensions were spotted in a row on a series of YNB plates without or supplemented with increasing concentrations of various compounds as indicated, and cultivated at 25 °C for 5 days (media with salts) or 3 days (others). For osmotolerance and salt tolerance, the concentrations of solutes used were 15 mM–1.6 M KCl, 0–1.5 M NaCl, 0–100 mM LiCl, sorbitol 1.0–1.8 M, and 20% glucose. All experiments were repeated three times and representative results are shown.

Table 2
Plasmids used in this study.

Plasmid	Features (original names)	Source/reference
pGRU1	*ori, AmpR, 2 μ, ScURA3, GFP* (pGRU1)	Dr. B. Daignan-Fornier, NCBI Acc. No. AJ249649,
YEp352	*ori, AmpR, 2 μ, ScURA3* (YEp352)	Hill et al. (1986)
pScNHA1	derived from pGRU1, *ScNHA1^P-ScNHA1-GFP* (pNHA1-985-GFP)	Kinclova et al. (2001)
pScENA1	derived from pGRU1, *ScNHA1^P-ScENA1-GFP* (pENA1-GFP)	O. Zimmermannova (unpublished results)
pYlNHA2	derived from pGRU1, *ScNHA1^P-YlNHA2-GFP* (pYlNHA2-GFP)	Papouskova and Sychrova (2006)
pZrSTL1	derived from pGRU1, *ScNHA1^P-ZrSTL1-GFP* (pGRU1-ZrSTL1)	M. Duskova (unpublished results)
pZrFFZ1	derived from pGRU1, *ScNHA1^P-ZrFFZ1-GFP* (pZRF1-GFP)	Leandro et al. (2011)
pZr-ZrFFZ1	derived from YEp352, *ZrFFZ1^P-ZrFFZ1* (pZRF1)	Leandro et al. (2011)
pZrFSY1	derived from pGRU1, *ScNHA1^P-ZrFSY1-GFP* (pZRS1-N)	Leandro et al. (2013)
pZr-ZrFSY1	derived from pGRU1, *ZrFSY1^P-ZrFSY1* (pZRS1-S)	Leandro et al. (2013)

^{P} - promoter.

2.4. Synthetic must fermentations

Synthetic must was prepared according to Beltran et al. (2004) with the modifications of Stribny et al. (2016b). Inoculum cultures were prepared by cultivating cells aerobically in YNB medium to the stationary phase for 24 h, washing them with water and resuspending to OD_{600} = 25.0 in 1 ml of water. These suspensions were used as the inoculum of the 250 ml synthetic must, thus reaching an initial OD_{600} = 0.1. Fermentations (in 250-ml glass bottles) were performed in triplicate at 25 °C with gentle continuous orbital shaking (90 rpm) for the indicated time (15 days for cells expressing *Z. rouxii* fructose transporters and 10 days for cells with *Sc*Nha1). Glass bottles were closed with Müller valves and the progress of fermentation (release of CO_2) was measured each day by weight-loss monitoring on laboratory scales.

Data from weight-loss monitoring together with data on final sugar content obtained from HPLC analysis were used for estimating the fermentation parameters (m - maximum sugar consumption rate, l – lag phase period, t_{90} – time taken to consume 90% of sugars). Fermentation parameters were calculated as described in (Alonso-del-Real et al., 2017; Perez-Traves et al., 2014). *P*-values were calculated using the two-tailed Student's *t*-test.

2.5. Analytical determinations

For determining the final content of glucose, fructose, ethanol and glycerol in fermented must, samples were collected on the 10th day of fermentation. Yeast cells were removed by centrifugation, supernatants diluted 3-fold, filtered through a 0.22-μm nylon filter and analysed by HPLC using a refraction index detector and a HyperREZTM XP Carbohydrate H+ 8 μm column (Thermo Fisher Scientific) equipped with a HyperREZTM XP Carbohydrate Guard (Thermo Fisher Scientific). The analysis conditions were: eluent, 1.5 mM of H_2SO_4; 0.6 ml min-1 flux and a 50 °C oven temperature as described in Alonso-del-Real et al. (2017).

For analysis of the differences in glucose/fructose content in must