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Diversity of salt response among yeasts

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Abstract - Forty-two yeast strains from 27 species belonging to seven genera, selected for their ability to grow in 10% NaCl, have been analysed for their resistance to salt concentrations up to 5 M, by calculating the Minimum Inhibitory Concentrations (MIC). Using eight different NaCl concentrations from 0 to 5 M, results show that halotolerance (MIC) ranges from 1.7 to 3.8 M NaCl, with an average around 2.5 M and confirm that the most halotolerant strains belong to the species *Debaryomyces hansenii*. Since a real halophily could not be found in these isolates, and is generally questioned to be present among the yeast, the effects of NaCl has been measured as salt enhancement effect on growth (MSE), which is defined as the rate between the growth at a given NaCl concentration and the growth in the medium without addition of salt. The implications of these findings in food microbial ecology and technology are discussed.

Key words: salt resistance, halophily, yeast, biodiversity, biotechnology, environment.

INTRODUCTION

The various responses of microbes to salt presence have drawn the attention of several biologists attracted by the inherent interest of the phenomenon and by its applicability in different branches of biotechnologies (Vreeland, 1987), such as the production of salt resistant plants (Hasegawa *et al.,* 2000; Forment *et al.,* 2002; Borsani *et al.,* 2003; Gao *et al.,* 2003) the selection of halotolerant microbes to use in industrial processes (van der Sluis *et al.,* 2001) or in the bioremediation of polluted substrates with high salt concentrations (Feng *et al.*, 1997; Bastos *et al.*, 2000; Abadias *et al.*, 2001; Lahav *et al.*, 2002; Dan *et al.*, 2002, 2003) and the construction of genetically modified organisms for different industrial uses (Loray *et al.*, 1995; Ellul *et al*., 2003).

The convenience of yeast as model of eukaryote cell for salt stress studies and as biotechnological microorganisms (Serrano *et al.,* 1996; Prista *et al.,* 1997; Huh *et al.,* 2002; Lahav *et al.,* 2002) has encouraged the search for biodiversity in salt tolerance and investigations on the physiological and genetic mechanisms underlying halotolerance (Ferrando *et al.,* 1995; Blomberg, 1997; Mendizabal *et al.* , 1998; Lages *et al.,* 1999; Almagro *et al.*, 2000; Calderon-Torres and Thome, 2001; Cosentino *et al.,* 2001; Hansen *et al.,* 2001; Prista *et al.*, 2002; Ekendahl *et al.,* 2003; Jansen *et al.*, 2003; Schoondermark-Stolk *et al.*, 2003; Silva-Graca and Lucas, 2003; Turk *et al.*, 2004).

In spite of the large interest in salt tolerance, the positive effects of salt on yeast growth have been poorly considered and the possibility that real halophily can be found in yeast has been questioned (Silva-Graca and Lucas, 2003), probably because stringent and obligate halophily has never been found in yeast as in prokaryotes (Rothschild and Mancinelli, 2001; Donachie *et al.*, 2005). However, the positive effect of salt at relatively low concentrations on the yeast growth has been documented for some strains of the species *Debaryomyces hansenii* in particular conditions (Almagro *et al.*, 2000) and represent an intriguing topic in environmental biology. In fact, the potential presence of beneficial effects of salt in the yeast growth might enlarge our view on the adaptive mechanisms of yeast to environments with moderate concentrations of salt and contribute to the efforts aiming to select or construct better biotechnological strains for applications in salty substrates.

In this work we have studied halotolerance and effects of salt in 42 strains belonging to 27 species of seven yeast genera. These strains were chosen for their ability to grow at NaCl concentrations over 10%. The isolates from the *sensu stricto* group of the genus *Saccharomyces* were included for their undisguised biotechnological interest and as representatives of yeast species with little salt tolerance.

Scope of this paper is to carry out a preliminary screening on the extent and diffusion of the salt response among the most salt resistant yeast species and to develop parameters able to synthetically describe the behaviour, in presence of NaCl, of the yeast involved in food environment as beneficial starters or as spoilers.

MATERIALS AND METHODS

Yeast strains and media. The yeast strains employed in this study (Table 1) were partly obtained from the Industri-

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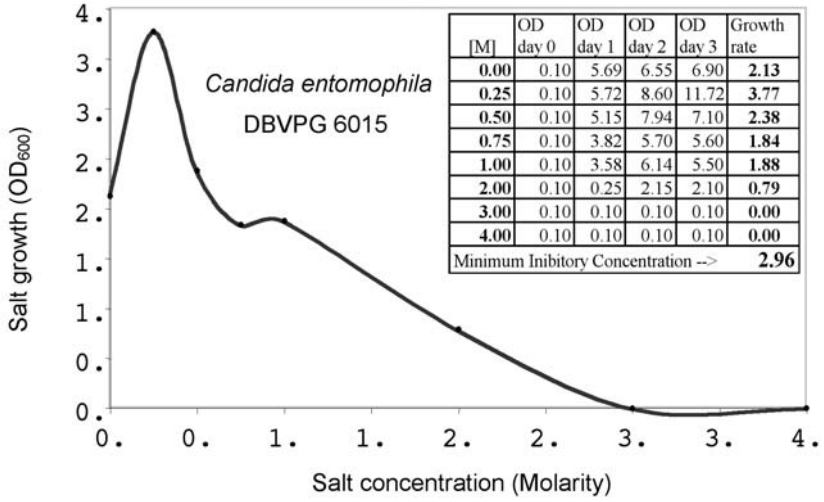
TABLE 1 – Minimum Inhibitory Concentration (MIC) and Maximum Salt Effect on growth (MSE) expressed along with the salt molar concentration and time at which the MSE was observed

Species	Strain*	MIC (M)	MSE**	(M)	Days	Typical environment of the species***
Candida bombicola	6870	2.0	1.10	0.25	3	Honey / juice
Candida diddensiae	6138	1.9	**1.34**	0.25	1	Shrimp / sugar
Candida friedrichii	6139	2.7	1.04	0.25	2	Cheese
Candida melibiosica	6144	3.0	1.10	0.25	3	Wine / soil
Candida parapsilosis	6150	2.7	**1.41**	0.25	2	Brines / skin
Candida salmaticensis	6026	2.9	**1.19**	0.75	3	Alpechin
Debaryomyces hansenii	6050	3.4	**2.08**	0.50	2	Sheep cheese
Debaryomyces hansenii	F1MC2	3.5	**1.63**	0.25	1	Sheep cheese
Debaryomyces hansenii	ORP5	3.5	**1.30**	0.50	2	Sheep cheese
Debaryomyces hansenii	P2P1	3.5	**1.44**	0.75	3	Sheep cheese
Debaryomyces hansenii	P2P6	3.4	**1.65**	0.25	3	Sheep cheese
Debaryomyces hansenii	T1C1	2.6	**2.33**	0.75	3	Sheep cheese
Debaryomyces hansenii	T3C2	3.8	**1.47**	0.50	3	Sheep cheese
Debaryomyces hansenii	TEP4	3.6	**1.67**	0.25	1	Sheep cheese
Metchnikowia pulcherrima	6184	3.0	**1.17**	0.25	3	Grapes / spoiled fruit / flowers
Pichia angusta	7139	1.9	**1.16**	0.25	2	Orange juice / alpechin
Pichia angusta	7140	2.0	**1.80**	0.25	1	Orange juice / alpechin
Pichia guilliermondii	6140	3.1	1.00	0.00	1	Alcohol / atmosphere
Pichia kluyveri	3634	1.8	**1.14**	0.25	3	Tropical products / cacao
Pichia membranaefaciens	6720	2.0	1.01	0.25	3	Grape must / wine
Pichia mexicana	6028	2.9	**1.83**	0.50	3	Grape must / cactus
Pichia ohmeri	6502	2.5	**1.23**	0.50	3	Cucumber brine / gooseberry
Saccharomyces bayanus	6299	1.8	1.10	0.50	3	Beer / grape must / pear juice
Saccharomyces cerevisiae	6173	1.7	1.00	0.00	1	Beer / brewery / grape must
Saccharomyces cerevisiae	6295	2.3	1.01	0.25	3	Beer / brewery / grape must
Saccharomyces cerevisiae	6820	1.8	**1.75**	0.25	1	Beer / brewery / grape must
Saccharomyces cerevisiae	6859	1.8	1.00	0.00	1	Beer / brewery / grape must
Saccharomyces paradoxus	6495	1.9	1.00	0.00	1	Winery / exudates / soil
Zygosaccharomyces bailii	6915	2.6	**1.26**	0.25	3	Food / apple juice
Zygosaccharomyces bisporus	6485	2.6	**1.43**	0.25	1	Food
Zygosaccharomyces mrakii	6289	1.9	**1.11**	0.25	2	Silage
Zygosaccharomyces rouxii	6187	2.8	**1.24**	0.25	1	Grape must / honey
Zygosaccharomyces rouxii	6440	2.5	1.00	0.00	1	Grape must / honey
Zygosaccharomyces rouxii	6459	2.2	**1.16**	0.25	1	Grape must / honey
Zygosaccharomyces rouxii	6462	2.3	1.09	0.25	3	Grape must / honey
Zygosaccharomyces rouxii	6463	2.8	**1.29**	0.25	1	Grape must / honey
Candida tropicalis	3224	2.8	1.05	0.50	1	Human patogen
Candida blankii	6204	2.0	**1.73**	0.25	3	Blood of *Putorius vison*
Candida entomophila	6015	3.0	**1.70**	0.25	3	*Crossotarsus externedentatus*
Candida membranifaciens	6145	2.7	**1.53**	0.25	3	Insects / wounds
Metchnikowia reukaufii	6185	1.7	1.03	0.25	3	Flower of *Epilobium angustifolium*
Rhodotorula aurantiaca	4372	2.1	1.04	0.25	2	Sea water

* Four digit numbers are accession of the DBVPG collection; ** MSE values over 1,1 M are in boldface; *** according to Kurtzman and Fell, 1998.

al Yeasts Collection, DBVPG at the University of Perugia (http://www.agr.unipg.it/dbvpg/home.html). Some strains were freshly isolated from a recent survey of local dairy products and identified as members of the species *Debaryomyces hansenii* by 26S rDNA partial sequencing (Kurtzman and Robnett, 1998). The strains from the DBVPG collection are labelled with four digits, whereas the fresh isolates are indicated with alphanumeric labels. Strains were chosen for their ability to grow on media with more than 10% (1.72 M) NaCl. The strains belonging to the *sensu stricto* group of the genus *Saccharomyces* were chosen as reference, representing scarcely halotolerant yeast species.

Yeast strains were maintained onto YEPD-A plates (1% yeast extract, 1% peptone, 2% glucose, 1.7% agar). Growth tests were carried out in 500 ml bottles containing 50 ml of Yeast Nitrogen Base (Difco Laboratories) added with glucose 2% (YNBD medium) and maintained at 25 °C under orbital shaking at 250 rpm. YNBD medium was added with NaCl in order to obtain media with the eight concentrations employed in the growth tests: 0.00, 0.25, 0.50, 0.75, 1.00, 2.00,



[M]	OD day 0	OD day 1	OD day 2	OD day 3	Growth rate
0.00	0.10	5.69	6.55	6.90	**2.13**
0.25	0.10	5.72	8.60	11.72	**3.77**
0.50	0.10	5.15	7.94	7.10	**2.38**
0.75	0.10	3.82	5.70	5.60	**1.84**
1.00	0.10	3.58	6.14	5.50	**1.88**
2.00	0.10	0.25	2.15	2.10	**0.79**
3.00	0.10	0.10	0.10	0.10	**0.00**
4.00	0.10	0.10	0.10	0.10	**0.00**
Minimum Inibitory Concentration -->					**2.96**

FIG. 1 – Example of response to different salt concentrations and calculation of the MIC. Growth rate was calculated as the angular coefficient of the linear regression-curve constructed with the growth data.

3.00, 4.00 M. The media were inoculated with 24-h pre-culture cells in order to obtain an initial cell density 0.1 (OD_{600} = 0.1). Growth was determined by reading the optical density of 1 ml culture with a Genova spectrophotometer (Jenway, UK). In order to ensure measure reproducibility and reciprocity between cell density and OD, samples were diluted, before reading, in the range between OD_{600} = 0.20 and OD_{600} = 0.25 samplings and analyses were carried out after 24, 48 and 72 h. Two independent measurement series were carried out with an average difference of +/- 10%; data presented are the average between the two series.

Calculation of the MIC. The sodium chloride Minimum Inhibitory Concentration (from here on referred to as MIC) was calculated by regression analysis of the specific growth rates relative to each salt concentration, in order to define the minimum NaCl concentration at which no growth could be detected. Specific growth rates were calculated for each strain at a specific salt concentration, as the slope of a regression equation, in which the independent variable was represented by the time (expressed in days) and the dependent variable by the growth (expressed as OD_{600}). The specific growth rates were plotted against the corresponding salt concentrations, obtaining a curve as that shown in Fig. 1. The linear regression equation, describing the points of the monotonic descending part of the curve, was employed to calculate the MIC as the point at which the line intercepts the axis of the NaCl concentration. The quality of the MIC calculation was evaluated as the rate between the standard error of the regression curve and the MIC, obtaining values ranging from 0.05 to 0.20. The MIC values reported are the average between two independent measurements.

Salt enhancing effect. An indicator of the response of yeast cultures to salt is the Maximum Salt Effect (MSE), defined as the maximum rate observed in the single cultural conditions (time and NaCl concentration), during the three days of growth, between the growth of the cultures in the salt added medium and that in the salt-free medium. MSE values are completed with time and concentration data, as displayed in Table 1 and are the mean of two independent measurements typically diverging by ±7%.

Statistical analyses. Data storage and statistical calculations were carried out with the Microsoft Excel, KaleidaGraph (http://www.creactive.net/Pages/091.asp) and Le Progeciel applications (http://www.bio.umontreal.ca/Casgrain/en/labo/R/v4/telecharger.html). Correlations were calculated according to the Pearson product moment correlation coefficient.

RESULTS AND DISCUSSION

Halotolerance

Halotolerance was evaluated as the minimum salt concentration able to inhibit yeast growth (MIC). Results (Tables 1 and 2) show that the MIC value range from 1.7 M (*Saccharomyces cerevisiae* DBVPG 6173) to 3.8 M (*D. hansenii* T3C2), with average 2.5 M, median 2.6 M, and a standard deviation of 0.6 M, corresponding to a variation coefficient of 0.2. After classification of MIC data in 0.2 M wide classes, the most frequent class were 2.0 M (10 occurrences), 2.8 M (8 occurrences) and 3.0 M (6 occurrences), the other classes containing from 0 to 3 species.

As expected, the highest MICs were found among strains of the species *D. hansenii*, which exhibit an interesting variability as deduced by a 1.1 M difference between the T1C1 and the P2P6 isolates. Considering that the observed values show a difference of 2.1 M (from 1.7 M to 3.8 M), the variability found in *D. hansenii* covers more than half of the whole range of values, indicating that this species includes strains with very different levels of halotolerance.

The osmotolerant species of the genus *Zygosaccharomyces* are spread in the region between 1.9 M and 2.8 M, most of this range is occupied by the five strains of the species *Z. rouxii* with MICs spanning from 2.2 M to 2.8 M.

The six strains belonging to three species included in the *sensu stricto* group of the genus *Saccharomyces* span from 1.7 and 2.3 M. Excluding the most resistant strain (DBVPG 6295), the other five cultures are very similar, ranging from 1.7 M to 1.9 M. These MIC values are in excellent agreement with the viability of *S. cerevisiae* colonies in NaCl containing plates (Blomberg, 1997).

Altogether, the three species studied in more detail, *D. hansenii* with eight strains, *Z. rouxii* with five and *S. cere-*

TABLE 2 – Average, median and standard deviation of different genera and of the complete dataset examined

Genus	Average	Median	Standard deviation
Candida			
MIC (M)	2.57	2.70	0.43
MSE	1.32	1.27	0.26
(M)	0.33	0.25	0.17
Debaryomyces			
MIC (M)	3.41	3.50	0.35
MSE	1.70	1.64	0.34
(M)	0.47	0.50	0.21
Metchnikowia			
MIC (M)	2.35	2.35	0.92
MSE	1.10	1.10	0.10
(M)	0.25	0.25	0.00
Pichia			
MIC (M)	2.31	2.00	0.52
MSE	1.31	1.16	0.35
(M)	0.29	0.25	0.17
Saccharomyces			
MIC (M)	1.88	1.80	0.21
MSE	1.14	1.01	0.14
MSE (M)	0.17	0.13	0.09
Zygosaccharomyces			
MIC (M)	2.46	2.55	0.61
MSE	1.20	1.20	0.33
(M)	0.22	0.25	0.19
Rhodotorula aurantiaca	2.30	1.01	0.25
All strains			
MIC (M)	2.55	2.60	0.61
MSE	1.32	1.21	0.33
(M)	0.30	0.25	0.19

TABLE 3 – Correlations among MIC and MSE in different subsets of strains

	MIC vs. MSE
All species	0.37
Debaryomyces hansenii	-0.78
Zygosaccharomyces	0.55
Saccharomyces	-0.28

visiae with four isolates, displayed average MIC values of 3.4, 2.5 and 1.9 respectively with variation coefficients around 0.1. This comparison indicates that the variability within the species is rather low, although isolates with MIC quite different from the average were found in *S. cerevisiae* (DBVPG 6295), and in *D. hansenii* (T1C1).

These findings suggest that wider biodiversity investigations at the species level are necessary to elucidate the distribution of this character and to find isolates with extreme characteristics, which could be useful for speculative studies as well as for biotechnological applications.

Effect of salt on growth

The maximum effect of salt on the yeast growth (MSE) is a rate indicating whether and how much the salt can increase the growth of the cells. MSE values equal to 1.00 indicate that salt did not improve the growth any time and concentration. In such cases, the NaCl concentration maximizing the growth was, by definition, 0 M.

The MSE values range from 1 to 2.33, and the average is 1.32. The most common NaCl concentration with a positive effect on growth resulted 0.25M (27 strains), followed by 0.5 M (7 strains) and 0.75 (3 strains). Only five strains did not show any salt enhancing effect. Half of the strains showed a positive salt effect on the growth after 3 days, seven after 2 days and 9 after one day.

In spite of their elevate osmotolerance and halotolerance, the strains of the genus *Zygosaccharomyces* display low MSE values ranging from 1.00 to 1.43 (average 1.20). The strains of the species *D. hansenii* exhibit the highest MSEs (2.3 the T1C1 strain and 2.08 the type strain DBVPG 6050), although the strain ORP5 gained very little (1.30) by the presence of salt.

The members of the genus *Saccharomyces* show very low MSE values (1.00 to 1.10) with the exception of the haploid *S. cerevisiae* DBVPG 6820. Whether the relatively high MSE could be attributed to its haploid state or to other features is matter for further investigations.

Correlation between MIC and MSE

The relative correlations of the two parameters describing the salt response (MSE and MIC) were analysed by considering all strains together and three subsets including the cultures of *Debaryomyces, Zygosaccharomyces* and *Saccharomyces*, respectively (Table 3). When all cultures are considered together, the correlation between MIC and MSE is weak (0.37), whereas it increases in the *Zygosaccharomyces* subset (0.55). Surprisingly, this correlation is very high and negative (-0.78) when the comparison is restricted to *Debaryomyces* strains. This strange result could be explained by the fact that the least resistant *Debaryomyces* strain (T1C1) exhibits the highest MSE (2.33), whereas the most halotolerant (T3C2) has one of the lowest MSE values (1.47). All together*, Debaryomyces* strains display the highest values of MIC and MSE, and exhibit a strong negative correlation between ability to take advantage of salt and to resist at the highest concentration, suggesting that the mechanisms governing the halotolerance should be quite different from those responsible of the faster growth in presence of salt.

Conversely, in *Zygosaccharomyces,* the halotolerance is positively correlated to the MSE suggesting that in this genus the halotolerance and the enhancing effects of salt could be directed by the same mechanisms.

Concluding remarks

In this paper we have shown that a relatively high variability exists among yeast in terms of salt resistance and ability to grow faster in presence of moderate salt concentrations. Although it is not possible to consider yeast real halophilic organisms (Silva-Graca *et al.*, 2003), namely because they can grow in absence of NaCl, salt can exert a positive role in their growth at concentrations ranging from 0.25 and 0.75 M.

From the biotechnological point of view, these data show that yeast represent an interesting reservoir of genes for the

production of plants able to grow and produce in salty environments (Hasegawa *et al.,* 2000; Forment *et al.,* 2002; Borsani *et al.,* 2003; Gao *et al.,* 2003). The ability of some strains to grow better with moderate salt concentrations is an important feature in industrial fermentations in which the addition of salt would both improve the growth rate of the fermenting yeast and decrease the risk of contamination by organisms with low halotolerance and reduced growth in presence of salt.

These results show a relatively large variation in the salt response of different food borne yeast. Particularly interesting is the large inter-specific variability shown by the *D. hansenii* strains, which could be positively used to select starter strains to be used in conserved olives and in cheese industry, considering that the usage of largely halotolerant strains allows to use more stringent technological conditions, reducing the probability of undesired contaminations.

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