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Effects of salts on *Debaryomyces hansenii* and *Saccharomyces cerevisiae* under stress conditions

Anabel Almagro[a], Catarina Prista[b], Santiago Castro[a], Célia Quintas[c], Amândio Madeira-Lopes[b], José Ramos[a], Maria C. Loureiro-Dias[b,c,*]

[a]*Department Microbiología, Escuela Técnica Superior de Ingenieros Agrónomos y de Montes, E-14071 Córdoba, Spain*
[b]*Department Botânica e Engenharia Biológica, Instituto Superior de Agronomia, 1349-017 Lisboa Codex, Portugal*
[c]*UCTA, Universidade do Algarve, 8000 Faro Cedex, Portugal*

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Abstract

The effect of Na^+ and K^+ on growth and thermal death of *Debaryomyces hansenii* and *Saccharomyces cerevisiae* were compared under stress conditions as those commonly found in food environments. At the supraoptimal temperature of 34°C both cations at concentrations of 0.5 M stimulated growth of *D. hansenii*, while K^+ had no effect and Na^+ inhibited growth of *S. cerevisiae*. At 8°C, close to the minimum temperature for growth in both species, both cations inhibited both yeasts, this effect being more pronounced with Na^+ in *S. cerevisiae*. At extreme pH values (7.8 and 3.5) both cations at concentrations of 0.25 M stimulated *D. hansenii* while Na^+ inhibited *S. cerevisiae*. K^+ inhibited this yeast at pH 3.5. Thermal inactivation rates, measured at 38°C in *D. hansenii* and at 48°C in *S. cerevisiae*, decreased in the presence of both cations. This protective effect could be observed in a wider range of concentrations in *D. hansenii*. These results call the attention to the fact that not all yeasts have the same behaviour on what concerns synergy or antagonism of salt together with other stress factors and should be taken into consideration in the establishment of food preservation procedures.

Keywords: *Debaryomyces hansenii*; *Saccharomyces cerevisiae*; Salt; Growth; Thermal death

1. Introduction

Debaryomyces hansenii is a common contaminant in several types of food and beverages being responsible for spoilage of dairy products (cheese, milk, yogurt, ice-creams), jam, confectionery, sausages and ham, brines (olives), salad dressing (Kreger van Rij, 1984; Tokuoka, 1993; Deak and Beuchat, 1996). This yeast is characterised by a high resistance to salt, some strains having been isolated from marine water (Norkrans, 1966) and solar saltworks. Underlying this behaviour, some physiological features have been identified: the production of glycerol and arabitol as compatible solutes (Nobre and da Costa,

*Corresponding author. Tel.: +351-21-3638161; fax: +351-21-3635031.
E-mail address: mcdias@isa.utl.pt (M.C. Loureiro-Dias)

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1985; André et al., 1988), transport systems to assure the homeostasis of glycerol (Lucas et al., 1990) and diversions in carbon metabolism associated with its production (Blomberg and Adler, 1993; Neves et al., 1997). It was previously shown that Na^+ is not toxic to *D. hansenii* since this yeast exhibits a good performance in the presence of high concentrations of this cation, even when the concentration of K^+ is very low (Prista et al., 1997; Thomé-Ortiz et al., 1998), reaching high levels of intracellular Na^+. Another special feature that we identified is that Na^+ does not inhibit K^+ uptake as it does in *Saccharomyces cerevisiae*. In the case of *D. hansenii* Na^+ even stimulated the transport of K^+ at pH 4.5 (Prista et al., 1997).

In the food environment, other forms of stress are frequently present besides high salt concentration, such as high and low temperatures, low pH and the presence of preservatives. The ability of stress agents to act synergistically or antagonistically has frequently been taken into consideration in the design of food preservation procedures (Silliker et al., 1990; Mossel et al., 1995; Deak and Beuchat, 1996). The aim of these procedures is to inactivate microorganisms either by inducing death or preventing growth. Many studies are being carried out on the mechanisms involved in these processes, since up to now they are not well understood (Guérin-Faubleu et al., 1995; Praphailong and Fleet, 1997; Sorensen, 1997).

The purpose of this work was to study the effect of NaCl on *D. hansenii* under stress conditions commonly found in the food environment, in particular high and low temperature and acidic pH, also in the presence of a weak acid preservative. High pH as in marine water (Sverdrup et al., 1946) was also included. To evaluate the specificity of the effect of Na^+ on *D. hansenii*, parallel experiments were performed with K^+ and sorbitol and with *S. cerevisiae*, as a reference yeast.

2. Materials and methods

2.1. Organisms

Debaryomyces hansenii IGC 2968 (CBS 767) and *Saccharomyces cerevisiae* IGC 4455 (CBS 1171) were routinely kept on solid YPD medium (10 g/l yeast extract (Difco Laboratories, Detroit, USA), 20 g/l peptone (Difco Laboratories, Detroit, USA), 20 g/l glucose (The British Drug Houses Ltd., Poole, UK), 20 g/l agar (BDH). Unless otherwise stated, the yeasts were grown at 28°C in liquid or solid YPD containing the desired concentration of NaCl, KCl, LiCl or sorbitol (all from Merck, Darmstadt, Germany).

2.2. Growth assays

The determination of the maximum temperature for growth was performed in a Temperature Gradient Incubator, Model TN-12 Advantec, Toyo, Roshi International Inc. (Tokyo, Japan). Exponential cells in YPD medium (pH 5.8, 28°C) were inoculated in the same medium, supplied with NaCl, KCl or sorbitol, the initial optical density (O.D.) being 0.05. Twelve tubes containing the cultures were incubated in the experimental temperature gradient of 32–40°C. Growth was considered positive when O.D. $>$ 0.5 after 14 days.

For determination of specific growth rates, assays were performed in liquid YPD at the desired temperature and pH, supplemented with NaCl, KCl, LiCl, sorbitol or benzoic acid, as indicated in Results. Optical density at 550 nm (OD_{550}) was measured up to the end of the exponential phase in a DU640 spectrophotometer (Beckman Instruments, Fullerton, USA). To study the effect of salts on growth at different pH values, the media were prepared as follows: (i) For high pH, the YPD medium, containing 30 mM *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (Sigma Chemical Co., St. Louis, USA), was brought to the desired pH with NH_4OH (Sigma). (ii) For low pH, cells were grown in mineral medium (van Uden, 1967) adjusted at the desired pH with HCl (BDH).

2.3. Thermal death assays

In order to measure specific thermal death rates cells were pre-grown in liquid YPD medium (pH 5.8, 25°C). Exponentially growing cultures were used to inoculate 100 ml of liquid YPD medium with the desired NaCl, KCl or sorbitol concentrations, in the range 0–2.5 M, contained in 500 ml flasks and magnetically stirred in water baths at 38 and 48°C for *D. hansenii* and *S. cerevisiae*, respectively. During the assays, 0.1 ml samples were taken from

the flasks and inoculated on YPD plates at room temperature (samples were taken in triplicate).

3. Results

3.1. Effects of Na^+ and K^+ on growth at high and low temperatures

The values measured for maximum temperature for growth for *Debaryomyces hansenii* and *Saccharomyces cerevisiae* in the temperature gradient incubator are summarised in Table 1. The presence of NaCl or KCl (0.5 M) raised the maximum temperature for growth of *D. hansenii* by 1.6°C, indicating a protective effect of the cations. In *S. cerevisiae*, there was a slight increase in the presence of KCl, while in the presence of NaCl there was a drop of 2°C. Sorbitol (0.855 M, for which a_w is identical to that obtained with 0.5 M NaCl or KCl) had in both yeasts an effect similar to that of KCl.

For a more detailed study on the effect of the salts on the specific growth rates at temperatures close to the maximum temperature for growth, experiments were performed at 33 and 34°C for *D. hansenii* and at 34, 35 and 36°C for *S. cerevisiae*. Fig. 1 shows the results obtained in a typical experiment with both yeasts in the presence of NaCl at 34°C. Clearly NaCl strongly stimulated the growth of *D. hansenii* at concentrations up to 1 M. On the contrary NaCl inhibited *S. cerevisiae* at all concentrations tested. When similar experiments were performed with KCl, the results were similar to those obtained with NaCl for *D. hansenii*, while no significant effect emerged for *S. cerevisiae* (results not shown). In parallel control experiments performed with both yeasts, sorbitol (0.855 M) had no significant effect on the specific growth rate.

Table 1
Maximum temperature for growth of *D. hansenii* and *S. cerevisiae* on YPD with 0.5 M NaCl, 0.5 M KCl or 0.855 M sorbitol[a]

Osmolite	Maximum temperature for growth (°C)	
	D. hansenii	*S. cerevisiae*
None	33.6	35.6
NaCl	35.2	33.8
KCl	35.2	36.1
Sorbitol	34.8	36.5

[a] Growth was monitored by optical density.

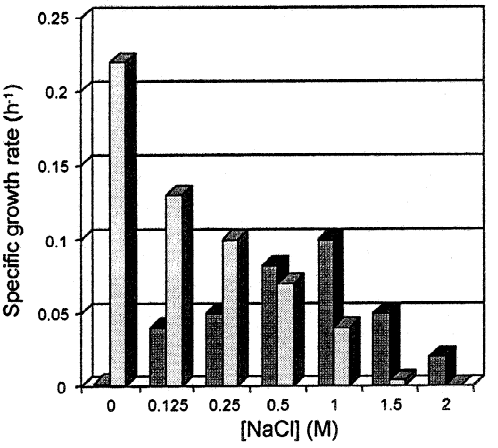

Fig. 1. Effect of Na^+ on the specific growth rate of *D. hansenii* (■) and of *S. cerevisiae* (▒) at 34°C, in complex medium (YPD), pH 5.8 containing the required concentration of NaCl.

As for low temperatures, experiments were performed at 8°C. This temperature is close to the minimum temperature for growth in both yeasts (Sá-Correia and van Uden, 1983; Prista and Madeira-Lopes, 1995). As Fig. 2 documents, both yeasts were inhibited by Na^+ and K^+, *S. cerevisiae* being more severely affected by Na^+ which, at a concentration of 0.5 M, inhibited growth completely.

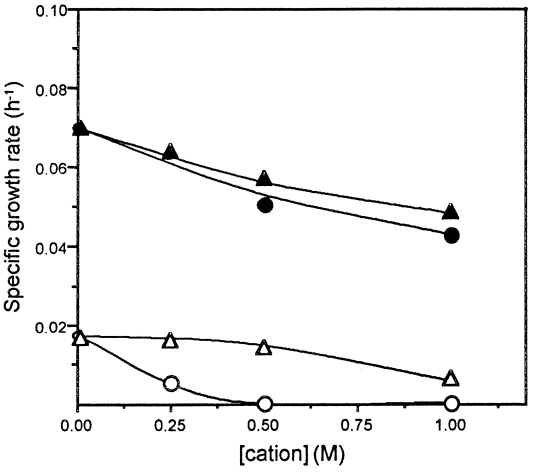

Fig. 2. Effect of Na^+ (○, ●) and K^+ (△, ▲) on the specific growth rate of *D. hansenii* (closed symbols) and *S. cerevisiae* (open symbols) at 8°C in complex medium (YPD), pH 5.8 containing the required concentration of NaCl or KCl.

3.2. Effects of Na^+ and K^+ on growth at high and low pH

When trying to establish conditions of high pH to perform the experiments, the first observation was that *D. hansenii* kept the ability to grow at a pH higher than *S. cerevisiae*. For example in plates at pH 8.2, no growth was observed in *S. cerevisiae* while *D. hansenii* formed normal colonies after 7 days at 28°C. In order to study the effect of the salts on specific growth rates, we chose liquid YPD at pH 7.8. In Fig. 3A the results obtained for the specific growth rates are summarised. Both cations stimulated *D. hansenii* and inhibited *S. cerevisiae*. The effect was in both cases more pronounced with Na^+ than with K^+. This tendency was confirmed in *D. hansenii* with plates at pH 8.5: in the absence of NaCl no growth was observed, while small colonies appeared when the content of Na^+ was 0.5 and 0.75 M. The effect of LiCl on *D. hansenii* was checked in plates at pH 8.2, but total inhibition was observed already at a concentration of 10 mM, confirming that Li^+ is toxic to *D. hansenii* (Prista et al., 1997).

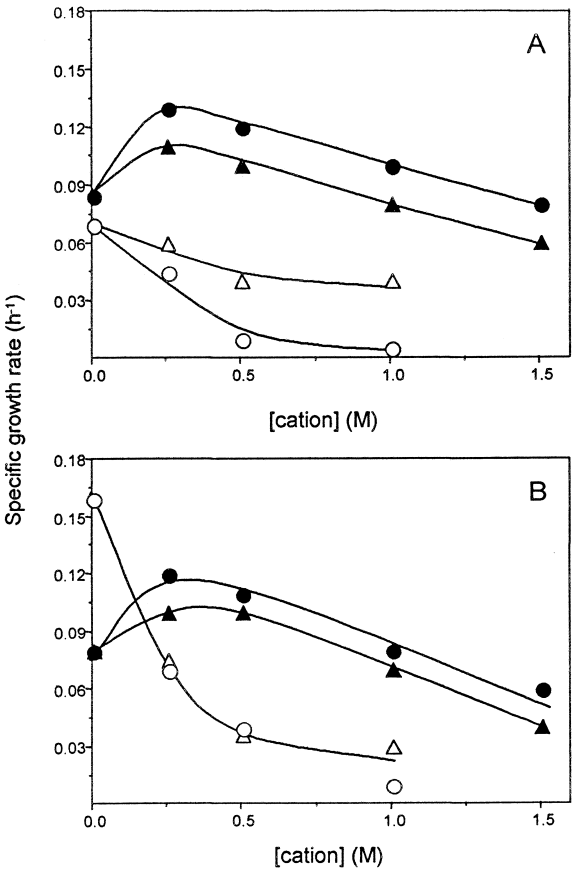

Fig. 3. Effect of Na^+ (○, ●) and K^+ (△, ▲) on the specific growth rate of *D. hansenii* (closed symbols) and *S. cerevisiae* (open symbols) at pH 7.8 (A) and pH 3.5 (B). Media were supplemented with the required amounts of NaCl or KCl.

We observed that the performance of *D. hansenii* at low pH was better in mineral medium than in YPD. For this reason the effects of the cations were evaluated in mineral medium at pH 3.5. Fig. 3B shows the results. In the absence of added salts, *S. cerevisiae* grew much better than *D. hansenii*. It is worth noting that at such a low pH, K^+ had an inhibitory effect on *S. cerevisiae* similar to that of Na^+. On the other hand, the growth of *D. hansenii* was stimulated by low concentrations of the cations, while higher concentrations had a reduced inhibitory effect. In parallel control experiments performed with both yeasts, either at low and high pH, sorbitol (0.855 M) had no significant effect on growth.

Since weak acids are commonly used as food preservatives in acidic food and beverages, the inhibitory effect of benzoic acid on growth was investigated at low pH in the presence of the salts. Benzoic acid (0.5 mM) at pH 4.0 reduced the growth rate of *D. hansenii* by 43% and that of *S. cerevisiae* by 22% in the absence of salts. Both cations potentiated the inhibitory effect of benzoic acid on the growth of *S. cerevisiae*. As for *D. hansenii* no additional inhibition of growth was observed for concentrations of the salts up to 1 M (results not shown).

Under all conditions tested the presence of the salts in the range of concentrations indicated above delayed growth much more severely in *S. cerevisiae* than in *D. hansenii*. For example, at pH 7.5 the duration of the lag phase was 5 h with or without 0.5 M NaCl in *D. hansenii* while in *S. cerevisiae* it increased from 4 to 9 h under the same conditions.

3.3. Effects of Na^+ and K^+ on thermal death

The experimental conditions to study the effects of salts on thermal inactivation were based on previous works (van Uden, 1984; Prista and Madeira-Lopes, 1995) that had shown that *D. hansenii* is more sensitive to high temperature than *S. cerevisiae*. We conducted experiments to determine thermal death

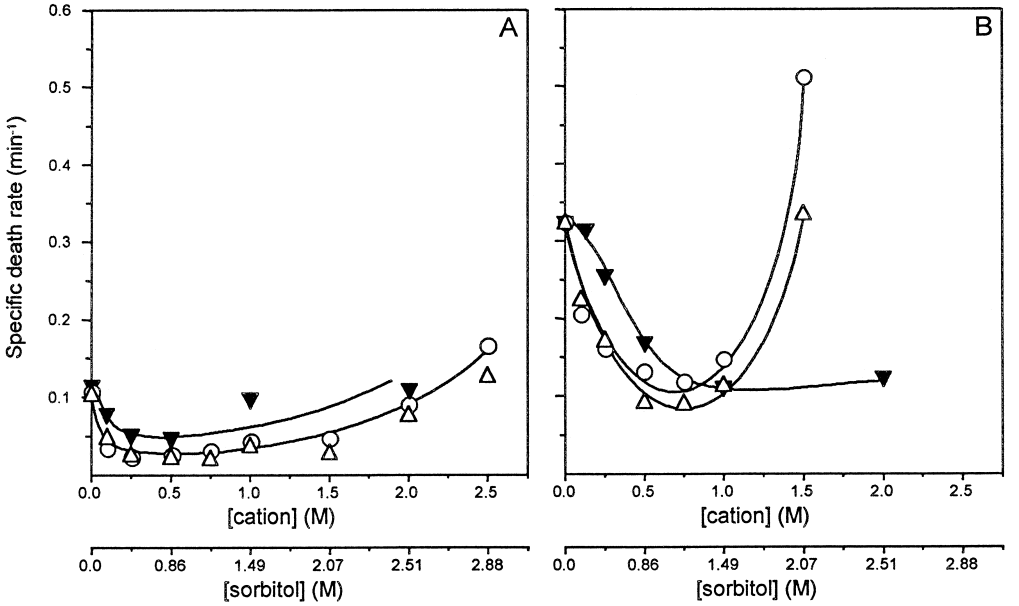

Fig. 4. Effect of Na^+ (○), K^+ (△) and sorbitol (▼) on the specific death rate of *D. hansenii* (A) at 38°C and *S. cerevisiae* (B) at 48°C. Cells were grown on liquid YPD (pH 5.8) at 25°C up to mid-exponential phase and inoculated in flasks containing YPD medium, at 38°C or 48°C, with the desired NaCl, KCl or sorbitol concentrations. Concentrations of sorbitol were chosen in order to obtain a_w values similar to those obtained with NaCl and KCl.

rates in the range 38–42°C for *D. hansenii* and 47–49°C for *S. cerevisiae* at different concentrations of NaCl and KCl. Fig. 4 shows the results of typical experiments performed at 38°C for *D. hansenii* (Fig.4A) and 48°C for *S. cerevisiae* (Fig. 4B). In both cases, the presence of salts even at a low concentration (0.1 M) resulted in a strong reduction of the death rate. The maximum reduction observed was about 75% for *D. hansenii* in presence of both cations and for *S. cerevisiae* it was 60% and 70% in presence of NaCl and KCl, respectively. The range of concentrations in which protection against thermal death was observed was wider in *D. hansenii* (up to 2 M), while in *S. cerevisiae*, 2 M induced a very fast death (in 5 min all the cells were inactivated). A similar behaviour was observed previously in another strain of *D. hansenii* isolated from solar saltworks (results not shown).

Parallel experiments were performed with sorbitol (closed symbols in Fig. 4) using a concentration range of 0–2.5 M for which a_w is similar to that obtained in the concentration range of 0–2 M NaCl or KCl. Sorbitol was slightly less effective than NaCl or KCl as protective agent. The important difference was observed for *S. cerevisiae* at higher concentrations: in the presence of 1.5 M NaCl or KCl the protective effect was eliminated while it was still present with 2.5 M sorbitol.

4. Discussion

A characteristic feature of the physiology of *D. hansenii* is its resistance to NaCl. Since this yeast has been described as involved in spoilage, it is particularly important to understand the behaviour of the yeast when a high salt concentration is present together with other stress agents in the food environment. A global analysis of the results obtained with *D. hansenii* and *S. cerevisiae* fits with previous observations on the relations of both yeasts with alkaline cations. In general Na^+ was toxic to *S. cerevisiae* but improved the growth of *D. hansenii*. This was evident for the growth at supraoptimal temperature. On the contrary, K^+, a non toxic cation did not affect *S. cerevisiae*, but still improved growth of *D. hansenii*. The same pattern was found at high and low pH.

Interestingly, growth at low temperature was in all cases inhibited by the presence of the cations, although a more pronounced effect was exerted by Na^+ on *S. cerevisiae*. Presumably low temperature and the presence of the salts are antagonistic as for their effects on the synthesis of appropriate lipids for adaptation to each of these situations. For example, it has been shown that lipid saturation is important for salt tolerance in *Zygosaccharomyces rouxii* (Yoshikawa et al., 1995) while adaptation to low temperature results in an increase of the degree of lipid unsaturation in several yeast species (Arthur and Watson, 1976).

The reduction in cell death provoked by the salts was originated by some physiological event occurring very fast (within less than 5 min) and did not imply a previous adaptation of the cells, since in the experiments of thermal death cells were growing in YPD without added salts, at 25°C and at time 0, were transferred to batches of the same medium at high temperature with different concentrations of the salts. These facts, together with the effect observed with sorbitol are in accordance with the previous knowledge in the food industry that conditions of low water activity protect against thermal death. Our observations lead to the understanding that the reduction of water activity, even for concentrations of solutes as low as 0.1 M, are effective. It is interesting to notice that in the case of *S. cerevisiae* both NaCl and KCl had a toxic effect at 1.5 M which prevailed over the protective osmotic effect. This toxic effect was not observed in *D. hansenii*.

The food industry is aware of the importance of crossed effects of stress agents in food preservation procedures. Our work is a contribution to this field, calling the attention to the fact that not all yeasts have the same behaviour on what concerns synergy or antagonism of salt together with other stress factors. It would be important to perform the same type of experiments with dangerous spoilage yeasts belonging to the genus *Zygosaccharomyces*, since the preservation procedures are often established taking into account the resistance parameters of these yeasts.

We have previously reported that the presence of salt improves the performance of *D. hansenii* under normal conditions. From the results of this work we conclude that this improvement is more significant under stress conditions, contributing to protect the cells against those factors.

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