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Osmotic adjustment in marine yeast

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Abstract. The effect of environmental salinity on cell growth, and on the composition and accumulation of compatible solutes, or osmotica, of five yeast strains (*Aureobasidium pullulans*, *Candida* sp., *Cryptococcus albidus* var. *albidus*, *Debaryomyces hansenii* and *Rhodotorula rubra*) was compared. All these yeast were isolated from marine environments, but were able to grow in the absence of salt and should therefore be considered as halotolerant strains. According to their specific cell growth rates at different salt concentrations, these strains vary in their capacity to osmotically adjust to modifications in external salinity. *Candida* sp. appears to be the most sensitive since the maximum salt concentration at which it can grow is 1.54 mol l^{-1} NaCl; however, it showed the highest specific cell growth in the range of 0 to 1.54 mol l^{-1} NaCl. *Aureobasidium pullulans*, on the other hand, showed the lowest specific growth rate, but the highest halotolerance range from 0 to 5.13 mol l^{-1} NaCl. *Debaryomyces hansenii*, in contrast, showed higher specific growth at this salinity range *Cryptococcus albidus* var. *albidus* and *Rhodotorula rubra* showed similar specific cell growth rate values and halotolerance between 0 and 2.45 mol l^{-1} NaCl. The protein and carbohydrate content of the biomass of the different yeast cells, as a result of external salinity variation, remained practically constant. The most important effects of the increase in salt concentration in the culture medium were the reduction of cell volume and the accumulation of low-molecular-weight metabolites (LMWM), which appear to act as osmoregulators. Glycerol was found as the major compatible solute in the different marine yeasts studied herein with a total contribution of 64–96% of the internal cell osmolarity. Other LMWM, like carbohydrates and amino acids, contributed to a lesser extent to compensate for the rise in osmotic pressure promoted by the salinity of the external environment.

Introduction

Fungi are a heterogeneous group of organisms with many kinds of vegetative, as well as sexual, life cycles. Less than 1% of the described fungal species are obtained from marine environments and only 0.36% are marine-occurring yeasts (Kohlmeyer and Kohlmeyer, 1979). The biology of marine fungi has been reviewed (Moss, 1986), including the physiology of their osmotolerance (Blomberg and Adler, 1992), based on a few cases: *Debaryomyces hansenii* (Gustafsson and Norkrans, 1976; Adler *et al.*, 1985; Larsson *et al.*, 1990), *Zygosaccharomyces rouxii* (Van Zyl and Prior, 1990), *Hansenlua anomala* (Van Eck *et al.*, 1989), *Sporobolomyces salmonicolor* (Gervais, 1991) and *Candida tropicalis* (Saubenova *et al.*, 1989). Thus, it is necessary to increase our knowledge about the mechanisms of haloadaptation, halophilism or halotolerance of a larger number of examples since there may be different strategies that may be ecologically important.

The terminology currently employed in studies concerned with the osmotic responses of the cell has been reviewed (Reed, 1984). The physiological adaptation involving an increase in the concentration of compatible solutes in the cell is called osmotic adjustment and the compatible solutes are called osmotica.

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A common mechanism of osmotic adjustment in microorganisms is the intracellular accumulation of compatible solutes or osmotica (Yancey *et al.*, 1982; Mackay *et al.*, 1984; Wegmann, 1986; Blomberg and Adler, 1992). It is presumed that this accumulation compensates for the change in internal water potential promoted by an increase in salinity in the external environment. In yeasts, sugars (Saubenova *et al.*, 1989) and polyols (Gustaffson and Norkrans, 1976; Adler and Gustafsson, 1980; Brown and Edgley, 1980; Adler *et al.*, 1981, 1985; André *et al.*, 1988; Van Zyl and Prior, 1990) have been reported as the major compounds involved in osmoregulation. An important part of the studies on osmoregulation in yeast has been done using sugars and other organic solutes as osmolites, and in some cases the cell response was found to be solute dependent (Blomberg and Adler, 1992).

Other adaptation mechanisms to high-salinity environments include modifications of cell wall and membrane components (Clipson and Jennings, 1990), salt vacuolization, internal Na^+/K^+ pools (Norkrans and Kylin, 1969), as well as molecular changes in their enzymes (Comerford *et al.*, 1985), metabolic and transport systems (Clipson and Jennings, 1990). Burke and Jennings (1990) have also emphasized that the yeasts not only produce compatible solutes to maintain turgidity when challenged by high salinity, but also as a means for energy dissipation through substrate recycling and as a physiological response of buffering capacity. All these modifications often preclude, and/or complicate, the correct identification and classification of marine yeasts. Thus, further work is needed to clarify the different mechanisms by which marine yeasts may survive in the sea. In this report, we describe the effects observed on the morphology as well as on the chemical composition of intracellular metabolites of five different marine yeasts exposed to increasing salinity. The results support our hypothesis that different strategies and mechanisms of adaptation in every marine yeast species may operate to allow their survival and propagation in the marine environment.

Method

Yeast strains

Three yeast strains were isolated from solar salt ponds in La Paz, Baja California Sur (Mexico), during the winter of 1990. The waters in the salt ponds, located between 24°6′5″N and 110°20′10″W, had a salinity and pH between 1.025 and 1.537 mol l^{-1}, and 7.3 and 7.9, respectively. The yeast strains were isolated using a liquid medium containing 10% glucose, 5% yeast extract, and 0, 0.86, 1.71, 2.45 or 5.13 mol l^{-1} NaCl. The pH was adjusted to 4.5 with 0.1 N HCl, in order to reduce bacterial growth. Purification of each strain was carried out in a solid medium containing 2% glucose, 1% peptone, 5% yeast extract and 2% agar in filtered seawater. The pH was adjusted to 4.5 with 0.1 N HCl.

The three strains were characterized according to the procedure of Van de Walt and Yarrow (1983). The morphological and physiological characteristics of the strains showed that they are *Aureobasidium pullulans*, *Candida* sp. and *Cryptococcus albidus* var. *albidus*. Two strains from the marine yeast collection

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of the Biological Research Center at La Paz were also studied—*D.hansenii* (C-11) and *Rhodotorula rubra* (A-18)—which were isolated from the Pacific Ocean (Hernández-Saavedra, 1990).

Yeast culture and growth determination

A 36 h inoculum was prepared in MB medium (Lodder and Kreger van Rij, 1983) without NaCl. The inoculum culture was then centrifuged at 10 000 *g* for 10 min and the cell suspension was adjusted to 1×10^8 cells ml^{-1} with fresh sterile MB medium.

For the determination of specific growth rate, 0.25 ml of the inoculum cell suspension was added to a 125 ml conical flask containing 25 ml of MB medium with different NaCl concentrations. The initial cell concentration was 1×10^6 cells ml^{-1}. The liquid cultures were incubated on a rotary shaker at 100 r.p.m. at 30°C. The cell concentration was determined every 2 h until the stationary phase, and the specific growth rate (μ) was determined as follows:

$$\mu = \ln(N_t/N_0)t^{-1}$$

where N is the cell concentration and t is the time of culture. All the determinations were carried out on three independent replicate experiments.

Determination of biomass composition and low-molecular-weight metabolites

The dry biomass was determined by the Bauer and Shiloach (1974) method, which consists of determining (by independent triplicates) the constant dry weight of 500 ml of cell suspension with a known number of cells. The cellular concentration was determined microscopically by the hemocytometer technique. Lipid extraction and biomass analysis were performed as previously reported (Vazquez-Duhalt and Arredondo-Vega, 1991; Band *et al.*, 1992) using a mixture of CH_3OH:$CHCl_3$ (2:1) and obtaining two late phases. The lower $CHCl_3$ phase was evaporated under vacuum at 45°C and dried under N_2, and the total lipids weighed. The protein and polysaccharide contents of the biomass, and the amino acid, carbohydrate and glycerol contents in the aqueous phase, containing the low-molecular-weight metabolites (LMWM), were estimated according to Band *et al.* (1992) by standard spectrophotometric techniques. The cellular volume was estimated measuring the cellular size, under different saline conditions, by optic microscopy with a micrometer. The data obtained were processed by means of the formula:

$$V = 4/3\ h\ w\ l$$

where V = volume, h = height, w = width and l = length.

Intracellular osmotic pressure estimation

The concentrations of amino acids, glycerol and carbohydrates in the LMWM

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fraction on a dry biomass basis were multiplied by the cell dry weight and divided by the cell volume to obtain the intracellular LMWM concentration in molar units. The osmotic pressure was estimated using the equation describing the relationship between solute concentration in kg m^{-3} (C) and osmotic pressure in Os/kg for each solute (Weast, 1986):

$$\text{glucose Os/kg} = 2.991 \times 10^{-3}\ X^{0.985}$$

$$\text{glucose Os/kg} = 6.181 \times 10^{-3}\ X^{0.160}$$

$$\text{NaCl Os/kg} = 0.025\ X^{1.055}$$

The osmotic pressure of amino acids was estimated using Bonner's (1982) factor for lysine.

Results and discussion

Three different yeast strains were isolated from salt ponds near La Paz, Baja California Sur, Mexico, using the procedure described in the Method section. As discussed by Phaff (1986), the kind of medium employed by us is a non-specific isolation medium since any yeast that can utilize the different carbon sources available in the mixture may readily grow; however, in our case, selection is also provided by the amount of salt present in the medium. Since the salinity in the ponds was between 1.025 and 1.537 mol l^{-1} NaCl, and the pH was between 7.3 and 7.9, at the time of isolation, we may expect some natural selection and, therefore, a limited distribution of yeast species in the ponds. The three isolates corresponded, according to Van der Walt and Yarrow (1983), to *A.pullulans*, *Candida* sp. and *C.albidus* var. *albidus*. These yeast species are frequently encountered in marine environments (Roth *et al.*, 1962; Fell and van Uden, 1963; Meyers *et al.*, 1967; Ahearn *et al.*, 1968). As has been reported, the presence of NaCl in seawater is not an exacting factor for most of the yeasts isolated from marine niches (Roth *et al.*, 1962; Norkrans, 1966). All the strains studied here were able to grow in the presence of NaCl at similar levels to that in seawater. Ranges of growth have been reported in four of the studied species in media with different NaCl concentrations from 0 to: 4.8 mol l^{-1} for *D.hansenii*, 2.57 mol l^{-1} for *C.albidus*, 2.47 mol l^{-1} for *R.rubra* and a wide range of salinities for species belonging to the *Candida* genus (Yamasato *et al.*, 1974); this report is very close to our findings. Also a wide capacity for growth at different temperatures and pH values has been reported for our studied species: from 0 to 37°C and from pH 2.2 to 8.6 (Norkrans, 1966, 1968; Yamasato *et al.*, 1974). Javor (1989) has reported the studied species like ones of the most halotolerant species; however, there are few reports of yeast from extremely hypersaline environments. Based on temperatures, hypersaline brines which experience extreme solar heating would be expected to have few or no yeast populations; in most cases, they only can be developed in the cooler sediments. In this work, the isolation of yeast from salt pond water was possible because of the sampling

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season. While the environmental temperature during summer is >35°C, in the sampling time (winter) it is generally <15°C; solar irradiation is consequently low in winter. In general, it has been accepted that yeast and fungi may be halotolerant, but not obligatory halophilic microorganisms.

The growth and biomass composition of cells grown in the presence of different NaCl concentrations are shown in Table I. *Cryptococcus albidus* var. *albidus* and *R.rubra* showed their maximum growth rate in a medium without NaCl, while *A.pullulans*, *Candida* sp. and *D.hansenii* showed their highest growth rate in media containing 2.05, 1.51 and 1.71 mol l^{-1}, respectively. The growth rate has been reported as the major parameter in studies on the effect of water activity of the medium on yeast cells (Anand and Brown, 1968). These authors measured growth rates of 16 yeast strains at 30°C in media adjusted to

Table I. Specific cell growth and biomass composition of five marine yeast isolates grown at different salinities

Marine strain	NaCl concentration (M)	Specific cell growth (day^{-1})	Protein (% dry wt)	Polysaccharides (% dry wt)
Aureobasidium pullulans				
	0.00	0.8 (±0.1)[a]	41.5 (±7.2)	35.4 (±2.2)
	1.02	1 2 (±0 1)	42.0 (±1.5)	31.1 (±2.0)
	2.05	1.7 (±0 1)	43.5 (±2.3)	35.5 (±2.3)
	4.11	0.5 (±0.1)	44.4 (±2.7)	33.1 (±1.2)
	5.13	No growth		
Candida sp.				
	0.00	10.3 (±0.2)	42.8 (±1.0)	25.5 (±3.6)
	0.51	11.3 (±0.4)	43 2 (±0 8)	24.7 (±1.5)
	1.54	5 0 (±0.8)	42.7 (±1.9)	25.4 (±2.8)
	2.57	No growth		
Cryptococcus albidus var. *albidus*				
	0.00	7.5 (±0.5)	37 4 (±1.0)	22.8 (±2.3)
	0.68	6.7 (±0.4)	35.8 (±1.5)	21.5 (±1 0)
	1.37	6.3 (±0.4)	36 7 (±3.0)	18.9 (±1.9)
	2.05	No growth		
Debaryomyces hansenii (C11)				
	0.00	7.6 (±0.9)	30.1 (±5.4)	44.3 (±5.5)
	0.86	7.2 (±0.8)	31.0 (±0.3)	39.7 (±3.7)
	1.71	8.8 (±0.9)	29.8 (±1.2)	39.3 (±2.7)
	2.40	ND[b]	29.9 (±0.9)	40.5 (±4.1)
	3.42	No growth		
Rhodotorula rubra (A18)				
	0.00	7.5 (±0.7)	43.8 (±3.4)	26.7 (±2.5)
	0.68	7.5 (±1.0)	40.3 (±1.0)	23.1 (±1.8)
	1.37	5.6 (±0.5)	39.3 (±5.8)	24.3 (±1.5)
	2.05	3.5 (±0.7)	38.3 (±1.5)	24.1 (±0.9)
	2.47	No growth		

[a]The SD (in parentheses) was calculated from three values obtained in independent replicate experiments.
[b]Not determined.

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different water potentials with poly(ethylene glycol). Osmotolerant and osmophilic organisms were defined on the basis of maximum, optimum and minimum growth rates in the different media. In our case, all the strains seemed to be slightly osmophilic, except for *C.albidus* var. *albidus*, which is only an osmotolerant species. In spite of the low growth rate obtained with *A.pullulans* cultures, this marine isolate was able to grow at the highest salt concentration (4.11 mol l^{-1}). *Cryptococcus albidus* var. *albidus* was the most sensitive to the NaCl concentration, and was unable to grow in a medium containing 2.02 mol l^{-1} NaCl. On the other hand, *D.hansenii* cultures showed the highest growth rate at high salinity (Table I).

The protein and polysaccharide contents in the cell biomass were not affected by the increase in salinity of the medium (Table I). These results are in agreement with those found by Hamada *et al.* (1984), suggesting that these cell fractions are not involved in the osmoadaptation process.

In fungi, the intracellular accumulation of compatible solutes or osmotica, such as polyols, amino acids and small carbohydrates, was the most important response to the increase in osmotic pressure of the culture medium (Blomberg and Adler, 1992). During the extraction procedure, these compatible solutes were extracted with the LMWM, as in the case of other eukaryotic microorganisms (Vazquez-Duhalt and Arredondo-Vega, 1991; Band *et al.*, 1992).

The analysis of the LMWM fraction showed that the chemical composition of this fraction changed significantly with the increase in NaCl concentration in the culture medium for all the marine isolates. The glycerol, carbohydrate and amino acid contents of the five marine yeast species are shown in Figure 1. In general, there was an increase in the cell concentration of LMWM, resulting in an increase in the internal osmotic pressure of cells. The carbohydrate content in the LMWM fraction decreased with the increase in salinity of the medium in the cases of *R.rubra*, *C.albidus* var. *albidus* and *D.hansenii*, while in *A.pullulans* and *Candida* sp. this content increased with the salinity. A decrease in the amino acid contents was also observed in all the species, except in the case of *A.pullulans*, in which a correlated increase was observed (Figure 1). As shown in Figure 1, the most important effect of the increase in salinity was the accumulation of glycerol in the cells of the five marine yeast species. It appears that glycerol is the major osmoticum in marine yeasts and in most of the cases its concentration was directly correlated with the salt concentration in the medium. The glycerol concentrations found in *D.hansenii* are not in agreement with those reported by Adler *et al.* (1985). In our strain, glycerol is apparently not the major compatible solute; however, its concentration in the five marine yeast strains was higher than those found in the non-marine yeast *Saccharomyces cerevisiae* (Blomberg and Adler, 1989).

The nature of the external solute may influence the type of osmoregulator which is internally accumulated. *Zygosaccharomyces rouxii* accumulates glycerol under salt stress (Brown, 1978), while both arabinol and glycerol were accumulated under osmotic stress promoted by glucose or poly(ethylene glycol) (Moran and Witter, 1979; Van Zyl and Prior, 1990). Glycerol, trehalose and γ-decalactone have been reported as osmotica in some yeasts in response to a

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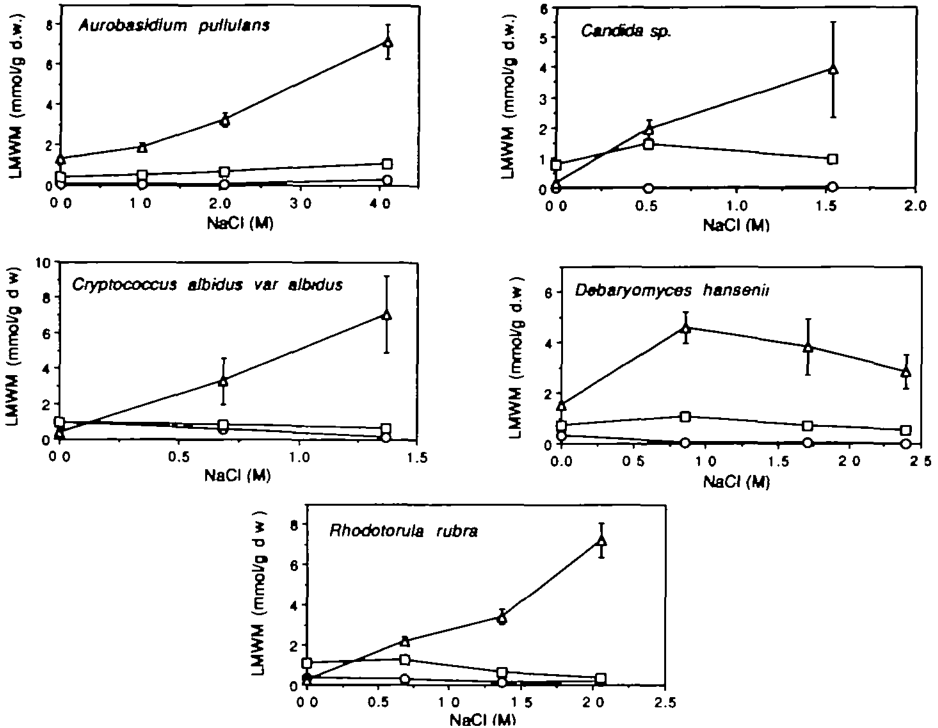

Fig. 1. LMWM concentrations in the dry cell biomass of five marine yeast strains grown at different salinities. Glycerol (△), carbohydrates (□) and amino acids (○).

salinity increase (Gustafsson and Norkrans, 1976; Brown, 1978; Adler *et al.*, 1982; Saubenova *et al.*, 1989; Gervais, 1991). Our results, obtained from four marine yeast species, in which glycerol accumulation appears to be the most important response to salt stress, are in agreement with those obtained with a few other marine strains (Saubenova *et al.*, 1989; Van Eck *et al.*, 1989; Larsson *et al.*, 1990; Van Zyl and Prior, 1990; Gervais, 1991). Thus, glycerol seems to be the preferred compatible solute in yeast grown under salt stress, while longer polyols accumulated when the culture occurs in a sugar-rich medium (Adler *et al.*, 1982).

The cell volume was reduced by the increase in the NaCl concentration of the medium (Figure 2). In all cases, the decrease in cell volume correlated with the NaCl concentration in the medium, except in the case of *R.rubra*, in which there was a reduction of 60% of cell volume in 0.68 mol l^{-1} NaCl and this volume remained unchanged at 2.05 mol l^{-1} NaCl. The reduction of cell volume as a response to osmotic stress has also been obtained with *S.cerevisiae* (Niedermeyer *et al.*, 1977) and *D.hansenii* (Lindman, 1981). Regulation of cell volume through alternation of cell growth rate in response to osmotic stress, with which the cell can increase its osmotic pressure by concentrating the existing solutes, seems to be a part of the osmotic adjustment process.

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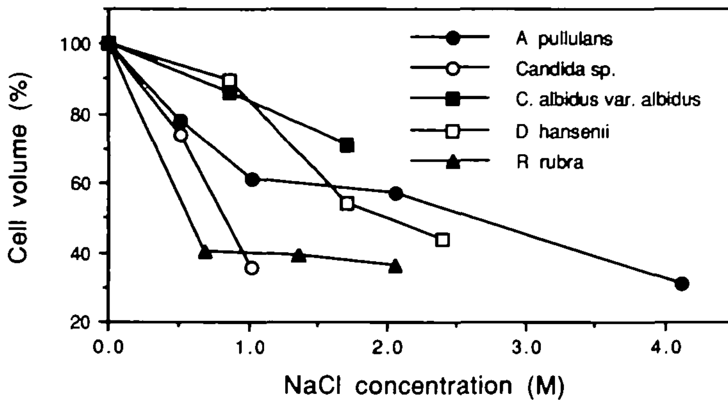

Fig. 2. Cell volume of five marine yeast strains grown at different salinities. The 100% cell volume is the cell volume found in the cultures without sodium chloride which are: *A.pullulans*. 33 μ^3; *Candida* sp. 17 μ^3. *C albidus* var *albidus*. 162 μ^3; *D.hansenii*. 23 μ^3; *R.rubra*. 53 μ^3

In order to determine the participation of each LMWM in the osmoregulation process, the intracellular osmotic pressure was estimated using the cellular concentration of each metabolite and cell volume at the different NaCl concentrations. The external osmotic pressure was completely compensated for by the concentration of LMWM in the cells (Table II). In a medium without NaCl, the participation of the carbohydrates from the LMWM fraction in the intracellular osmolarity represented only between 0.6% (in the case of *Candida* sp.) and 44.8% (*C.albidus* var. *albidus*). The amino acid cell concentration in cultures from no-saline media produced from 20.8 to 86.6% of the intracellular osmolarity, while the glycerol participated with 10.7–68.5% of the osmolarity. However, glycerol was found to be the major compatible solute in osmotica in the cultures from a medium with high osmotic pressure, and its participation represented from 8.4 to 95.6% of the total intracellular osmolarity (Table II). The participation of amino acids in osmoregulation was significant and decreased with salinity, while the participation of carbohydrate was the lowest in all the marine species.

In some cases (e.g. *A.pullulans* at 2.05 mol l^{-1} and *D.hansenii* at 2.40 mol l^{-1}), the internal osmolarities were substantially lower than the external ones (Table II). This suggests a possible participation of other solutes, such as polyols or ions, which have not been considered in this study.

In conclusion, we have here shed some light with regard to the different strategies that marine yeasts may follow to adapt themselves to the seawater environment. The consideration of various properties, such as specific cell growth rate, cell volume modification and accumulation of osmotica, among others, indicates that different yeasts employ distinct mechanisms to counteract the effect of the osmotic pressure of external salinity and survive in the marine environment. In conclusion, the accumulation of glycerol appears to be the most important osmotic-adjustment mechanism in marine yeasts. The concentration of LMWM in the cell compensated for the osmolarity of the extracellular

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Table II. Intracellular and culture media osmolarities, and participation of LMWM in the intracellular osmolarity of five marine isolates grown at different salinities

Marine strain	NaCl concentration (M)	Osmolarity of the medium (Os/kg)	Intracellular osmolarity (Os/kg)	% of intracellular osmolarity		
				Carbohydrates	Amino acids	Glycerol
Aureobasidium pullulans						
	0.00	0.2	0.35	3.7	20.8	68.5
	1.02	2.00	0.83	1.8	17.9	80.3
	2.05	4 40	1 56	0.9	14.3	84.8
	4 11	11.91	9.17	2.2	6.9	90.9
Candida sp.						
	0 00	0.22	0.16	0.6	86.6	12.8
	0.51	0.96	0.91	0.1	39.1	60.8
	1.54	3.12	3.33	0.7	14.9	80 4
Cryptococcus albidus var. *albidus*						
	0.00	0.22	0.43	44.8	40.1	15.1
	0.68	1.30	1 14	12 1	14.8	73.0
	1.37	2.73	2 66	1.3	6.4	92.3
Debaryomyces hansenii (C11)						
	0.00	0.22	0.50	12.3	26.3	61.4
	0.86	1.64	1.37	0.8	15.9	83.3
	1.71	3.53	2.15	0.6	12.5	86.9
	2.40	5.34	1.94	0.0	11.1	88.9
Rhodotorula rubra (A18)						
	0 00	0.22	0 29	22.6	66.7	10.7
	0.68	1.30	1.93	6.9	29.4	63.7
	1.37	2 73	2 57	2.4	11.8	85.8
	2.05	4.40	6.85	1.7	2.7	95.6

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medium when the salt concentration was increased. However, additional studies are necessary to increase our knowledge about these mechanisms since the presence of yeasts in the sea is ecologically relevant to maintain a healthy and equilibrated environment.

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