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ORIGINAL PAPER

Physiological characterization of polyextremotolerant yeasts from cold environments of Patagonia

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Abstract

Yeasts from cold environments have a wide range of strategies to prevent the negative effects of extreme conditions, including the production of metabolites of biotechnological interest. We investigated the growth profile and production of metabolites in yeast species isolated from cold environments. Thirty-eight strains were tested for their ability to grow at different temperatures (5–30 °C) and solute concentrations (3–12.5% NaCl and 50% glucose). All strains tested were able to grow at 5 °C, and 77% were able to grow with 5% NaCl at 18 °C. We were able to group strains based on different physicochemical/lifestyle profiles such as polyextremotolerant, osmotolerant, psychrotolerant, or psychrophilic. Five strains were selected to study biomass and metabolite production (glycerol, trehalose, ergosterol, and mycosporines). These analyses revealed that the accumulation pattern of trehalose and ergosterol was related to each lifestyle profile. Also, our findings would suggest that mycosporines does not have a role as an osmolyte. Non-conventional fermentative yeasts such as *Phaffia tasmanica* and *Saccharomyces eubayanus* may be of interest for trehalose production. This work contributes to the knowledge of non-conventional yeasts with biotechnological application from cold environments, including their growth profile, metabolites, and biomass production under different conditions.

Keywords Ergosterol · Mycosporines · Glycerol · Polyextremotolerant · Trehalose

Introduction

Cold environments comprise nearly 80% of the geosphere, with average temperatures below 5 °C (Cavicchioli et al. 2011). These environments have physicochemical factors that affect life development, such as high UV radiation, low water potential, low nutrient availability, high hydrostatic pressure, and oxidative stress (Margesin and Miteva 2011; Goordial et al. 2013; Buzzini et al. 2017). The above conditions make cold environments extreme for life and are generally inhabited by organisms with special adaptations belonging to Bacteria, Archaea, and Eukarya, including yeasts, filamentous fungi, protists, and lichens (Cantrell 2017; Buzzini et al. 2018). Yeasts exhibit various adaptations to living and survive under extreme conditions (Yusof et al. 2021), and some are considered polyextremotolerant because they can tolerate many different types of stress (Gostincăr et al. 2018). On the other hand, there are specialized extremophilic and extremotolerant species that cope efficiently with a given stressor and are limited in their ability to shift habitat (Gostincăr et al. 2010; Buzzini et al. 2018).

Yeasts adapted to extreme cold conditions are able to synthesize molecules that act as cryoprotectants and/or osmolytes, protecting cells from damage due to intracellular ice formation and desiccation during freeze–thaw cycles. Metabolites recognized as cryoprotectants include: Glycerol (Alcaíno et al. 2015), trehalose (Eleutherio et al. 2015; Feofilova et al. 2014; Ianutsevich et al. 2020), ergosterol (Lingwood and Simons 2010; Liu et al. 2015; Dupont et al. 2012; Ma et al. 2023), and mycosporines

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(Mycosporine-glutaminol-glucoside: MGG) (Moliné et al. 2014; Kogej et al. 2006). In yeast and other fungi, glycerol is the major solute regulating cell turgor under conditions of high extracellular osmolarity. Its production plays a role not only in protecting against osmotic stress, but also in maintaining redox balance and the pool of inorganic phosphate in the cytoplasm. In addition, glycerol can be used as a carbon and energy source by some yeast cells (Dakal et al. 2014; Semkiv et al. 2017). Trehalose has been described for its role in protecting against the effects of freezing, low temperatures, and desiccation. It is a unique cryoprotectant that protects not only cell macromolecules but also membranes under stress conditions and has antioxidant and regulatory functions (Li et al. 2008; Tsuji 2016; Ianutsevich et al. 2020). In yeast *S. cerevisiae*, stress or starvation can lead to fluctuations in trehalose content from less than 1% to more than 20% of cell dry weight (Eleutherio et al. 2015; Feofilova et al. 2014).

Ergosterol is a component found in the membrane of all fungi. Concentration levels of ergosterol can change due to desiccation and osmotic stress resistance (Dupont et al. 2012; Ma et al. 2023). It contributes to fluidity, permeability, microdomain formation, and membrane protection, along with other sterols (Lingwood and Simons 2010; Dupont et al. 2012). Yeast mycosporines protect against UV radiation and have antioxidant activity (Moliné et al. 2014; Pajarola 2015). Kogej et al. (2006) suggested that MGG could serve as a compatible osmolyte for osmoregulation since the concentration of mycosporine-like amino acids (MAAs) and MGG in some microorganisms is linked to the salinity of the medium. These metabolites are crucial for survival in extreme environments and have biotechnological potential as well.

We conducted a study on the growth profile of 38 yeasts isolated from Patagonia Argentina and Antarctica. We evaluated their production of secondary metabolites under different temperatures and osmotic concentrations to understand their response to cold and osmotic stress.

Materials and methods

Yeast strain

Thirty-eight yeast strains belonging to the classes Microbotryomycetes (*Glaciozyma* sp., *Cryolevonia* sp., *Leucosporidium* sp., *Sampaiozyma* sp.); Tremellomycetes (*Phaffia* sp., *Mrakia* sp., *Tausonia* sp., *Vishniacozyma* sp.); Dothideomycetes (*Aureobasidium* sp.); and Saccharomycetales (*Saccharomyces* sp., *Metschnikowia* sp., *Debaryomyces* sp.) were studied (Table 1).

All strains come from the yeast culture collection of the Centro de Referencia en Levaduras y Tecnología Cervecera (CRELTEC), IPATEC (CONICET-Universidad Nacional del Comahue). *Phaffia* sp. and *Saccharomyces* sp. strains were included as references with high biotechnological potential.

Cultivation of yeasts in solid medium

The effect of osmotic and thermal stress on the growth of the selected yeasts was assessed in plates containing MYP media (g L^{-1}: malt extract 7; yeast extract 0.5; soy peptone 2.5; agar 2) supplemented with different concentrations of NaCl: 0%, 3%, 5%, 7.5%, 10%, 12.5%; and glucose: 50%; and both 10% NaCl + 5% glucose. The plates were inoculated with 10 µL of a calibrated suspension (1×10^6 cells mL^1) of yeast cells and grown for 24–48 h. Each one of these plates was incubated at different temperatures: 5 °C, 12.5 °C, 18 °C, 25 °C, 30 °C and 35 °C. Colony development was recorded at 5, 10, and 20 days according to Kurtzman et al. (2011). Positive growth, was considered when observed growth after 5 days of inoculation (value 1 in cluster analysis); delayed growth, positive growth was observed after 10 days of inoculation (value 0.66 in cluster analysis); slow growth, growth develops slowly over a period exceeding 2 weeks (value 0.33 in cluster analysis); 0 negative growth.

Strain selection and liquid culture

Based on their growth profile, taxonomy, and biotechnological potential, five yeast strains were selected for the analysis of secondary metabolites accumulation in liquid culture: *Vishniacozyma victoriae* CRUB 2064, *Debaryomyces hansenii* CRUB 2079, *Phaffia tasmanica* PYCC 6858 ^{T}, *Saccharomyces eubayanus* CRUB 1939 and *Mrakia* sp. CRUB 1272. Selected strains were cultured in 250 mL Erlenmeyer flasks with 50 mL of modified MMS liquid medium (g L^{-1}: glucose 10, bacto-peptone 4.5, KH_2PO_4 2, $MgSO_4.7H_2O$ 0.5, yeast extract 1), and inoculated with the respective cultures in order to obtain 1×10^6 cells mL^1 and cultivated at 200 rpm under two different osmotic concentrations (without NaCl and with 5% NaCl) and two incubation temperatures (5 °C and 18 °C). The number of cells per mL was estimated for each sample using a Neubauer chamber. Samples from flasks incubated at 5 °C samples were taken every 48 h, and samples from flasks incubated at 18 °C were taken every 24 h until the stationary phase was reached. Growth parameters including maximum specific growth rate and lag phase duration, were calculated from each treatment by directly fitting cell count versus time to the reparameterized Gompertz equation proposed by Zwietering et al. (1990):

$y = A^* \exp(-\exp(((m * e)/A) - (1-t) + 1))$.

where $y = \ln (N_t / N_0)$, $N0$ is the initial cells/mL and N_t cells at time t; $A = \ln (N_\infty / N_0)$ is the maximum cells

Table 1 Yeast strains information about taxonomic affiliation, growth profile group, and environmental origin

Class	Species	Strain	GROUP	Substrate and isolation location	References
Saccharomycetes	*Metschnikowia australis*	CRUB 2081	G1	Bellingshausen Sea water (Antarctica)	This study
	Debaryomyces hansenii	CRUB 2079	G1	Bellingshausen Sea water (Antarctica)	
		CRUB 2080	G1	Weddell Sea water (Antarctica)	
Tremellomycetes	*Vishniacozyma victoriae*	CRUB 2064	G1	Bellingshausen Sea water (Antarctica)	This study
	V. psychrotorelans	CRUB 2065	G1	Scotia Sea water (Antarctica)	
Saccharomycetes	*Saccharomyces eubayanus*	CRUB 1568 ^{T}	G2A	*C. harioti* in *N. dombeyi*, Nahuel Huapi NP, Argentina	Libkind et al 2011a
		CRUB 1939	G2A	*N. antarctica* bark, Los Alerces NP, Argentina	
		CRUB 1974	G2A	Soil beneath *N. antarctica*, Nahuel Huapi NP, Argentina	
		CRUB 1977	G2A	Soil beneath *N. pumilio*, Lanin NP, Argentina	
		CRUB 1979	G2A	Soil beneath *N. antarctica*, Tierra del Fuego NP, Argentina	
	Saccharomyces uvarum	CBS 7001 ^{T}	G2A	*Mesophylax adopersus*, Avila, España	Almeida et al 2014
		CRUB 1595	G2A	*C. harioti* in *N. antarctica*, Nahuel Huapi NP, Argentina	
		CRUB 2082	G2A		
		CRUB 2083	G2A	*N. dombeyi* bark, Nahuel Huapi NP, Argentina	
		CRUB 2085	G2A	Soil beneath *N. betuloides*, Los Glaciares NP, Argentina	
Dothideomycetes	*Aureobasidium pullulans*	CRUB 2078	G2B	Bellingshausen Sea water (Antarctica)	This study
Microbotryomycetes	*Glaciozyma martinii*	CRUB 2066	G2B	Bellingshausen Sea ice (Antarctica)	
	Glaciozyma martinii	CRUB 2067	G2B	Bellingshausen Sea water (Antarctica)	
Tremellomycetes	*Leucosporidium fragarium*	CRUB 1200	G2B	Meltwater of Mount Tronador, Argentina	de Garcia et al 2007
	Sampaiozyma sp.	CRUB 2077	G2B	Scotia Sea water (Antarctica)	This study
	Tausonia pullulans	CRUB 2072	G2B	Sea water of Drake Passage	
	Phaffia sp. I	ZP 875	G2B	*C. gunni* in *N. cunninghamii*, Mount Field NP, Tasmania	
	Phaffia sp. II	ZP 938	G2B	*N. mooreii* leaves, Queensland, Australia	
	Phaffia rhodozyma	CBS 7918	G2B	Exudate of *Betula verrucosa*, Moscú, Rusia	
	Phaffia rhodozyma	ATCC 24229	G2B	Exudate of *Cornus brachypoda*, Hiroshima, Japón	
	Phaffia rhodozyma	ZP 874	G2B	*C. gunni* in *N. cunninghamii*, Tasmania	
	Phaffia rhodozyma	CRUB 1149	G2B	Water near *N. pumilio*, Ilón Lake, Argentina	
	Vishniacozyma victoriae	CRUB 2063	G2B	Bellingshausen Sea water (Antarctica)	This study
Tremellomycetes	*Mrakia* sp.	CRUB 1272	G2C	Meltwater of Mount Tronador, Argentina	de Garcia et al 2007
	Mrakia frígida	CRUB 2073	G2C	Wood with plasmodium, Ushuaia, Argentina	This study
	Mrakia robertii	CRUB 2074	G2C	Bellingshausen Sea water (Antarctica)	
	Mrakia robertii	CRUB 2075	G2C	Bellingshausen Sea water (Antarctica)	
	Mrakia robertii	CRUB 2076	G2C	Bellingshausen Sea water (Antarctica)	
Microbotryomycetes	*Cryolevonia giraudoae*	CRUB 1739	G3	Ice from Glacier Perito Moreno, Los Glaciares NP	de Garcia et al. 2020
	Cryolevonia giraudoae	CRUB 2068	G3	Bellingshausen Sea water (Antarctica)	
	Cryolevonia giraudoae	CRUB 2069	G3	Bellingshausen Sea water (Antarctica)	
	Cryolevonia giraudoae	CRUB 2070	G3	Bellingshausen Sea water (Antarctica)	
	Cryolevonia giraudoae	CRUB 2071	G3	Bellingshausen Sea water (Antarctica)	

production with N_∞ as the asymptotic maximum, m is the maximum growth rate ($h-1$), and l the period of time needed to reach stationary phase.

The growth data from each treatment and strain were fitted by a non-linear regression procedure, minimizing the sum of squares of the difference between experimental data and the fitted model, i.e., loss function (observed − predicted). This analysis was conducted using the non-linear module of Statistica 6.0 software package and its Quasi-Newton option (Arroyo Lopez et al. 2009).

Extraction and analysis of secondary metabolites

Trehalose, ergosterol, glycerol, and mycosporines, were measured in biomass when each strain reached stationary phase at 5 and 18 °C. Glycerol was also measured in cell-free supernatant. Cultures were incubated in the conditions mentioned above. For trehalose extraction, the method of Hodge (1962) with modifications was used; 1 g of dry cells was incubated for 40 min with 200 uL of 500 mM trichloroacetic acid (TCA) at room temperature. The extract was measured at 620 nm by the anthrone method, and trehalose standard 99% Sigma-Aldrich® was used for quantification. For ergosterol extraction, the method described by Shaw and Jefferies (1953) with modifications was used. Known aliquots of yeast biomass were suspended in 1.5 ml of methanol: KOH (25:10) and 1.5 ml of distilled water and incubated at 90 °C for 2 h. Petroleum ether (3 ml) was used as a solvent for ergosterol, and the complete extraction was attained after three subsequent extractions. The final fractions of ether were collected, washed with 10 mL of distilled water, and recorded. Quantitative analysis was carried out at 283 nm using ergosterol molar extinction coefficient, $\mathcal{E}=1.26\times10^4$ (Shaw & Jefferies 1953; Olson and Knizley 1962). For glycerol determination, Free Glycerol Reagent—Sigma-Aldrich® commercial kits were used in the cell-free supernatant. for this 2 mL of culture was centrifuged and washed, suspended in 1 mL of water, heated to 90 °C for 10 min, and centrifuged for 1 min at maximum speed. The supernatant was recovered, and the glycerol content was determined using the commercial kit mentioned before. A solution of glycerol 99% Sigma-Aldrich® 26 mg mL^{-1} was used as an internal standard.

MGG production was carried out in flasks incubated under two conditions, light and darkness. Darkness was achieved by covering the flask with aluminum foil, while flasks in light conditions were incubated in a Sedemix I500PF incubator chamber, under constant light provided by 6 white fluorescent lamps, General Electric T8, and 3 Toshiba FL40T8BRF lamps. Extraction and quantification were performed according to the method described by Moliné et al. (2011). Quantitative analysis was carried out at 310 nm using the MGG molar extinction coefficient, $\mathcal{E}=2.5\times10^4$. The concentration of all analyzed metabolites was expressed as mg per g of dry biomass. Dry cell biomass was measured in dried samples (85 °C until constant weight) obtained from 0.5 mL of culture in the stationary phase.

Statistical analysis

The growth profile of 38 studied strains (19 species) was analyzed using cluster analysis with Euclidean distance in R package gplots (Warnes et al. 2015). To investigate the effects of temperature and osmotic stress on metabolite production, a one-way analysis of variance (ANOVA) was performed, adjusting the significant differences by Bonferroni correction. When parametric analysis was not possible, an ANOVA on ranks was performed by the Kruskal–Wallis method ($p<0.05$). The statistical software SigmaPlot 11.0 and GraphPad Prism 5 were used for data analysis.

Results

Analysis of yeast growth in different culture conditions

We studied the growth patterns of 38 strains of yeast under different osmotic and temperature conditions and found high variability. Through cluster analysis and heatmap analysis, we identified three groups (Fig. 1).

The first group (G1) included polyextremotolerant yeast strains that can grow in all tested salinity conditions, including 10% NaCl and 5% glucose, and a wide range of temperatures. This group comprised five strains belonging to different taxonomic groups, including *Metschnikowia australis, Debaryomyces hansenii* (Order: Saccharomycetales), and *Vishniacozyma victoriae* (Order: Tremellales), all of which were isolated from Antarctic seawater (Table 1).

The second group (G2) was the most heterogeneous, with 28 strains separated into three subgroups (A, B, and C). Subgroup A included ten yeast strains from the *Saccharomyces* genus that were osmotolerant and able to grow in a range of 5–30 degrees with salt concentrations up to 7.5% and showed growth with glucose concentrations up to 50% at 25 °C. Subgroup B comprised 13 strains belonging to different genera (*Aureobasidium, Sampaiozyma, Phaffia, Tausonia, Vishniacozyma*, and *Glaciozyma*) isolated from various substrates, which can be considered psychrotolerant. Subgroup C included five yeast strains from the *Mrakia* genus that exhibited a psychrophilic profile.

Finally, the third cluster (G3) contained only yeasts of the recently described species *Cryolevonia giraudoae* isolated from glacier ice (Fig. 1) (de Garcia et al. 2020). These strains were able to grow only at 5 °C without NaCl and had an extreme profile with low capability to survive

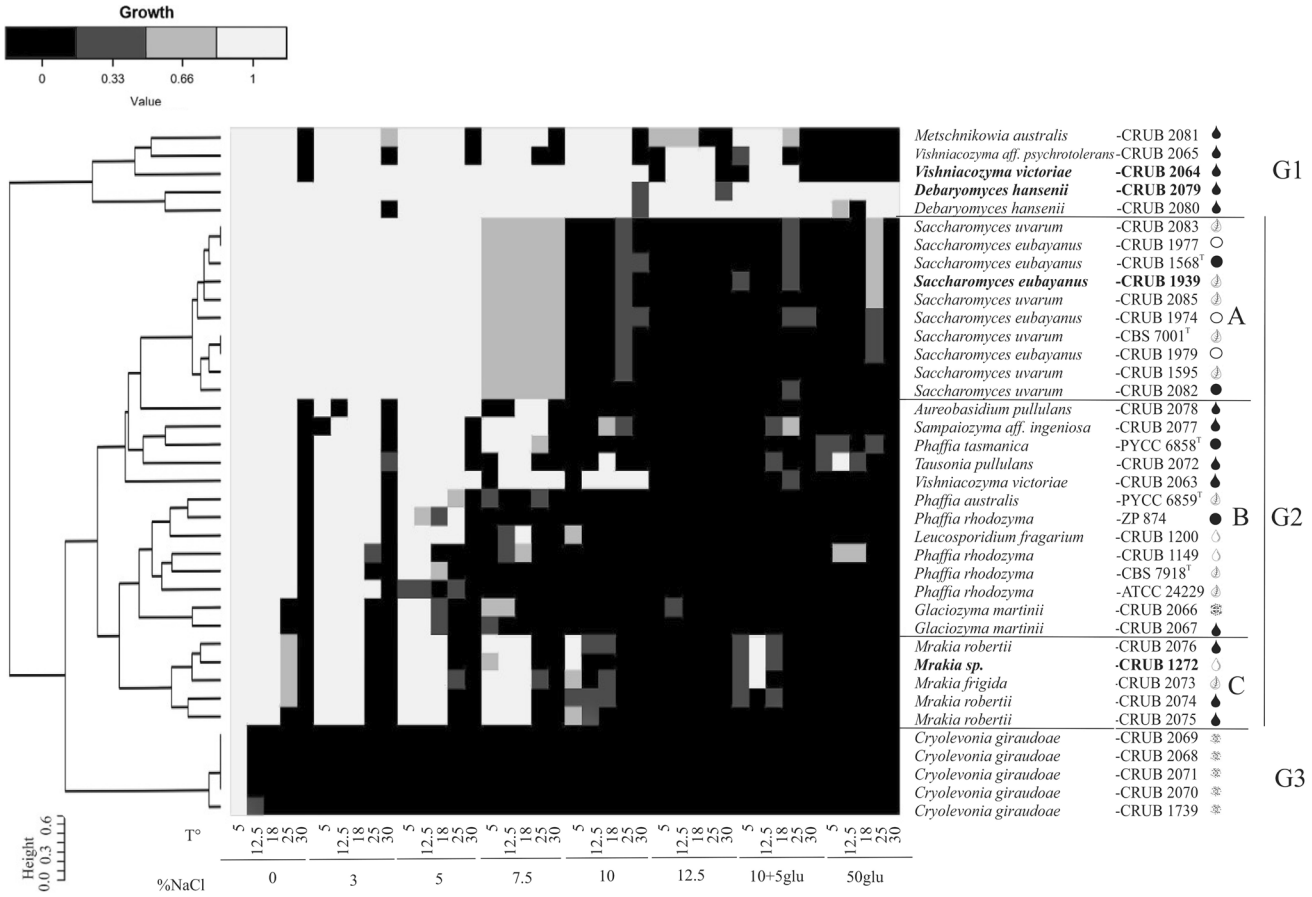


Fig. 1 Heatmap showing yeast strain's growth under different temperatures and osmotic conditions (indicated in the horizontal axe). The vertical axes of the cluster dendrogram show the fusion level. Clustering among samples was based on Euclidean distance. Different groups are indicated as G1, G2 (with 3 subgroups A, B, and C), and G3. T°: Temperature; % NaCl: percentage of NaCl included in the culture media; Glu: Glucose; leaf: tree ecosystem; black circle: soil or sediments; white circule: *Cyttaria* spp. stromata; white water drop: meltwater; black water drop: Antarctic sea water; snowflake: glacier ice

in a changing environment in terms of temperature and salinity.

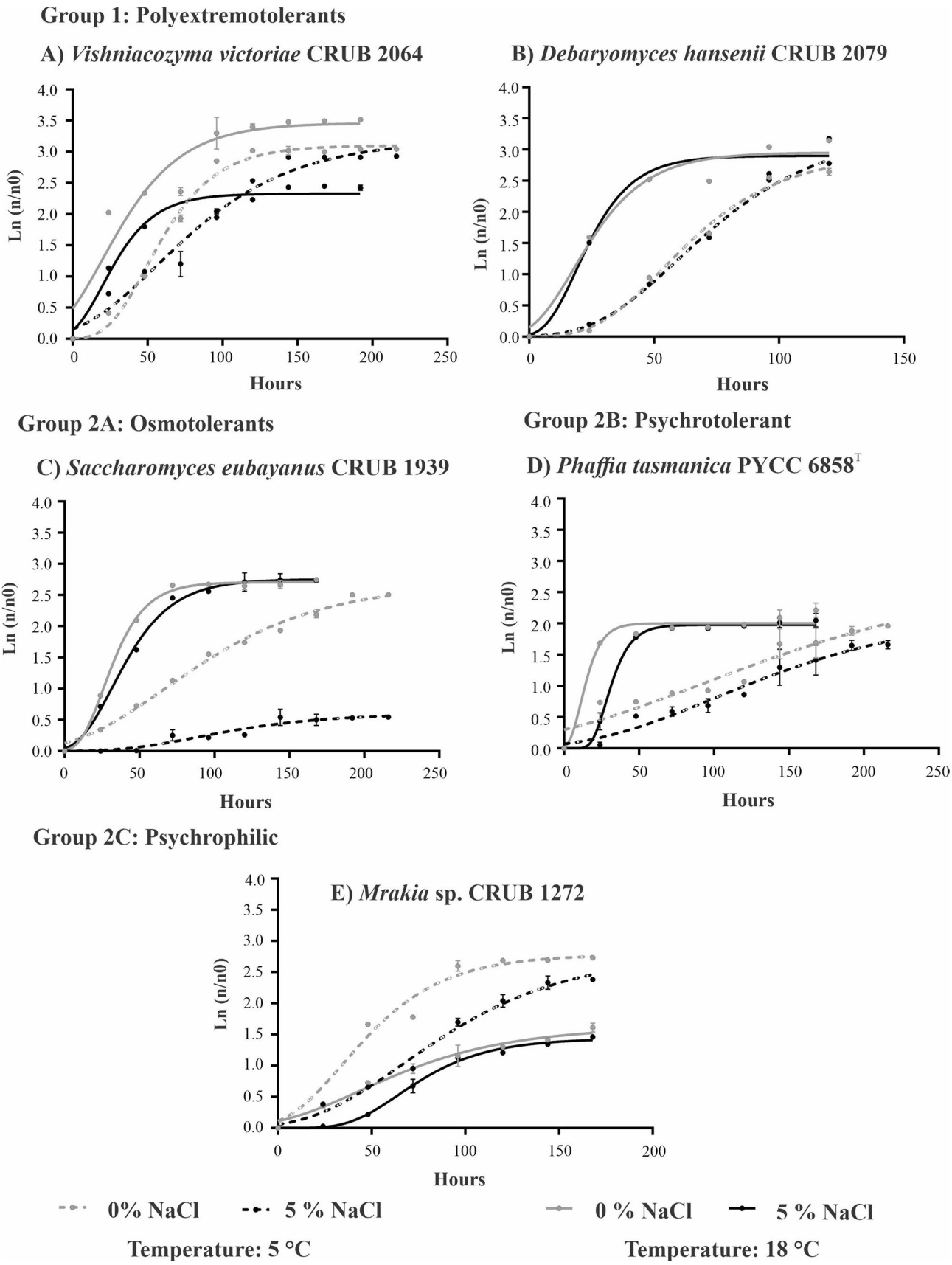
We selected two strains from G1 (*Debaryomyces hansenii* CRUB 2079, *Vishniacozyma victoriae* CRUB 2064), and representatives of the subgroups A, B, and C from G2 (*Saccharomyces eubayanus* CRUB 1939, *Phaffia tasmanica* PYCC 6858 ^{T} and *Mrakia* sp. CRUB 1272) to study their growth patterns. They were grown in liquid culture at two temperatures (5 and 18 °C) and two different osmotic conditions (0% NaCl and 5% NaCl), showing variable growth rate (μ) ranging from 0.157 to 0.003, and significant differences ($p < 0.05$) (Table 2; Fig. 2). Each strain exhibited a growth pattern consistent with its specific profile, which we describe in detail.

Vishniacozyma victoriae

Strain *V. victoriae* CRUB 2064 did not show significant differences in growth values, except for 5 °C with 5%

Table 2 Specific growth rate (μ) for 5 selected strains for different culture conditions. Letters indicate significant differences

Strains	5 °C		18 °C	
	Control	5% NaCl	Control	5% NaCl
Vishniacozyma victoriae CRUB 2064	0.04	0.02 a	0.04	0.04
Debaryomyces hansenii CRUB 2079	0.04	0.04	0.16 a	0.07 b
Phaffia tasmanica PYCC 6858 ^{T}	0.01	0.01	0.09 a	0.07 b
Saccharomyces eubayanus CRUB 1939	0.02 a	0.003 b	0.06 c	0.04 d
Mrakia sp. CRUB 1272	0.03 a	0.02 b	0.01c	0.02 b


Group 1: Polyextremotolerants
A) Vishniacozyma victoriae CRUB 2064
B) Debaryomyces hansenii CRUB 2079
Ln (n/n0)
Hours
Group 2A: Osmotolerants
Group 2B: Psychrotolerant
C) Saccharomyces eubayanus CRUB 1939
D) Phaffia tasmanica PYCC 6858T
Group 2C: Psychrophilic
E) Mrakia sp. CRUB 1272
0% NaCl
5 % NaCl
0 % NaCl
5 % NaCl
Temperature: 5 °C
Temperature: 18 °C

◂**Fig. 2** Growth kinetics of the selected yeast strains in MMS liquid culture at two temperatures (18 °C and 5 °C) and two osmotic concentrations (Control without NaCl and 5% NaCl). The value of each point corresponds to the mean, and the error bars represent the standard deviation from 3 replicates

NaCl, in these conditions the lowest μ value was observed (0.02) ($p < 0.05$) (Table 2; Fig. 2a). The final biomass production was similar in all the tested conditions (0.026 to 0.03 g mL^{-1}). Glycerol production was highest at 5 °C with 5% NaCl (562.7 ± 34.2 mg g^{-1}). Extracellular glycerol was also detected in this condition (19.7 ± 6.5 mg g^{-1}) (Fig. 3). Trehalose was produced in higher amounts at 5 °C without NaCl, 103.1 ± 12.1 mg g^{-1} (Fig. 4). Ergosterol values were significantly higher ($p < 0.05$) at 18 °C with NaCl (6.2 ± 0.15 mg g^{-1}) (Fig. 5).

V. victoriae CRUB 2064 produces mycosporines, the higher values were observed at 18 °C with light and without NaCl (3.5 ± 0.7 mg g^{-1}). NaCl had a negative impact on the accumulation of this metabolite, it was 78% lower (1.17 ± 0.35 mg g^{-1}) under this condition. A similar effect was observed when the strain was cultured in darkness (Fig. 6). No mycosporines accumulation was observed when the growth temperature was set at 5 °C in any of the mentioned conditions.

Debaryomyes hansenii

Debaryomyes hansenii CRUB 2079 showed significant differences in growth at different temperatures ($p < 0.05$), but no differences were observed between different NaCl concentrations at each temperature. The final biomass production varied from 0.02 to 0.05 g mL^{-1} (Table 2; Fig. 2b). Glycerol production showed the lowest concentrations at 18 °C without NaCl (60 ± 0.7 mg g^{-1}), but did not show significant differences among treatments ($p < 0.05$) (Fig. 3). For trehalose exhibited higher amounts at 5 °C without NaCl (49.72 ± 9.7 mg g^{-1}), and its accumulation was negatively affected at 5 °C and 18 °C with NaCl ($p < 0.05$) (Fig. 4). Ergosterol concentration was significantly higher at 18 °C with 5% NaCl (6.15 ± 0.9 mg g^{-1}), while no statistically significant differences were observed for the rest of the culture conditions ($p < 0.05$) (Fig. 5). No mycosporines were produced in any condition tested.

Saccharomyces eubayanus

Saccharomyces eubayanus CRUB 1939 showed significant differences for each treatment and had maximum growth rate at 18 °C without 5% NaCl (μ: 0.06) and minimum growth rate at 5 °C with 5% NaCl (μ: 0.003) (Fig. 2b, Table 2). Intracellular glycerol was higher under 5% NaCl regardless of the temperature (5°C: 175.8 ± 3.83; 18 °C: 166.2 ± 6.78 mg g^{-1}) (Fig. 3). Trehalose production only showed significantly lower values at 5 °C with NaCl, in the remaining condition the production ranging from 97 to 103 mg g^{-1} (Fig. 4). Regarding ergosterol production, no significant variation was observed among treatments (5.39 ± 0.84 to 4.46 ± 0.17 mg g^{-1}) ($p < 0.05$) (Fig. 5). No mycosporines were produced in any condition tested.

Phaffia tasmanica

Phaffia tasmanica PYCC 6858 ^{T} showed significant differences in growth at 5 °C with and without 5% NaCl (μ: 0.01), where at these conditions' biomass production was significantly lower (0.0059 g mL^{-1}) ($p < 0.05$) (Table 2; Fig. 2d). However, intracellular glycerol production was higher at 5 °C with 5% NaCl (804.9 ± 148.3 mg g^{-1}) (Fig. 3). Regarding trehalose, the maximum values were observed at 18 °C (110 ± 10.4 mg g^{-1}), and at 5 °C the production was significantly lower (48.3 ± 2.43 mg g^{-1}) ($p < 0.05$) (Fig. 4). Similarly, the ergosterol accumulation was significantly higher at 18 °C with NaCl (7.72 ± 0.8 mg g^{-1}), while no statistically significant differences were observed for the rest of the culture conditions ($p < 0.05$) (Fig. 5).

Phaffia tasmanica PYCC 6858 T can synthesize mycosporines, the higher production was registered at 18 °C with light and without NaCl (6.9 ± 1.3 mg g^{-1}), in this condition with NaCl, the accumulation was 75% lower (3.9 ± 0.06 mg g^{-1}). A similar effect was observed when the strains were cultured in darkness. No MGG accumulation was observed when the growth temperature was set at 5 °C (Fig. 6).

Mrakia sp.

Finally, *Mrakia* sp. CRUB 1272 showed significantly higher growth at 5 °C without NaCl (μ: 0.03) ($p < 0.05$), at 5 °C with 5% NaCl (μ: 0.02) had a longer lag phase, at 18 °C with 5% NaCl the lag phase was longer and half of the biomass obtained at 5 °C with 5% NaCl was achieved (0.009 and 0.0144 g mL^{-1} respectively) (Fig. 2e).

Intracellular glycerol production was at its peak at 18 °C with 5% NaCl (422.6 ± 35.7 mg g^{-1}). However, during this condition, the strain had the slowest rate of growth and biomass production. We also noticed extracellular glycerol production with and without NaCl at 18 °C (26.61 ± 0.18 mg g^{-1} and 18.8 ± 0.14 mg g^{-1} respectively) (Fig. 3). The highest production of trehalose was observed at 5 °C without NaCl (55.4 ± 10.9 mg g^{-1}), but in other conditions, we observed significantly lower production of this metabolite ($p < 0.05$) (Fig. 4). Ergosterol was notably higher at 18 °C with NaCl (9.5 ± 0.7 mg g^{-1}), but there were no statistical differences ($p < 0.05$) observed for the remaining culture conditions (Fig. 5). No mycosporines were produced in any condition tested.

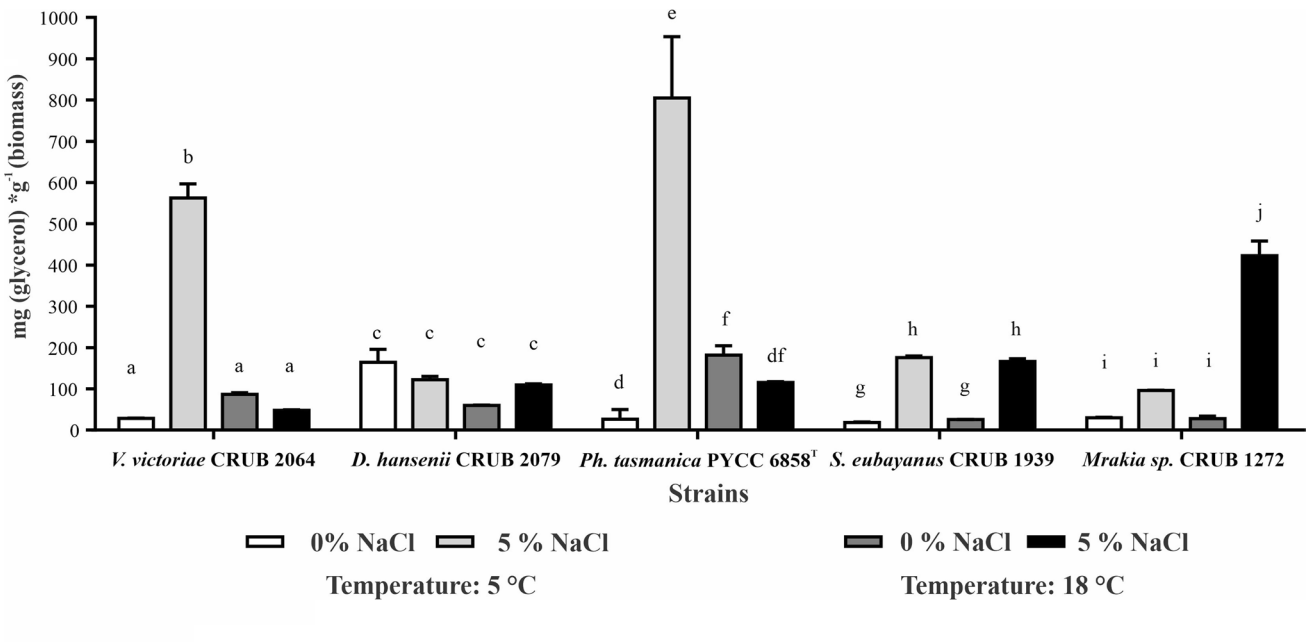


Fig. 3 Accumulation of glycerol in biomass of the strains *Vishniacozyma victoriae* CRUB 2064; *Debaryomyces hansenii* CRUB 2079; *Phaffia tasmanica* PYCC 6858.^{T}; *Saccharomyces eubayanus* CRUB 1939; and *Mrakia* sp. CRUB 1272. In MMS cultures as the control and MMS cultures + 5% NaCl. Significant differences were evaluated for each strain, indicated by different letters ($p < 0.05$)

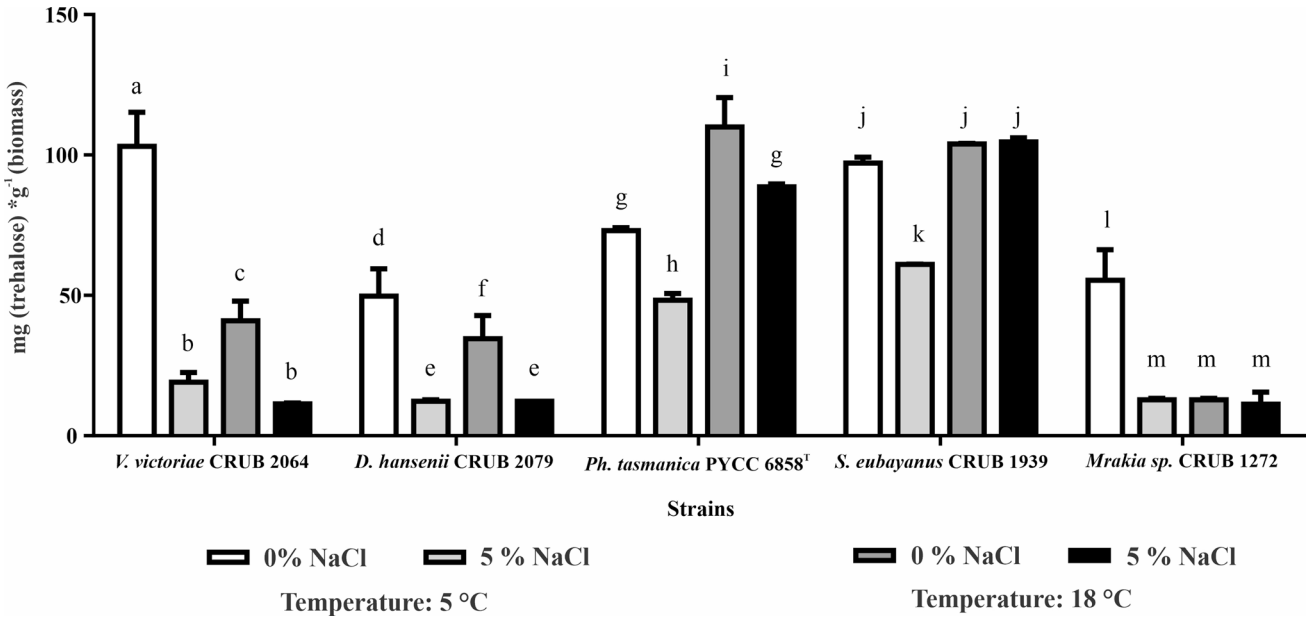


Fig. 4 Accumulation of trehalose in biomass of the strains *Vishniacozyma victoriae* CRUB 2064; *Debaryomyces hansenii* CRUB 2079; *Phaffia tasmanica* PYCC 6858.^{T}; *Saccharomyces eubayanus* CRUB 1939; and *Mrakia* sp. CRUB 1272. In MMS cultures as the control and MMS cultures + 5% NaCl. Significant differences were evaluated for each strain, indicated by different letters ($p < 0.05$)

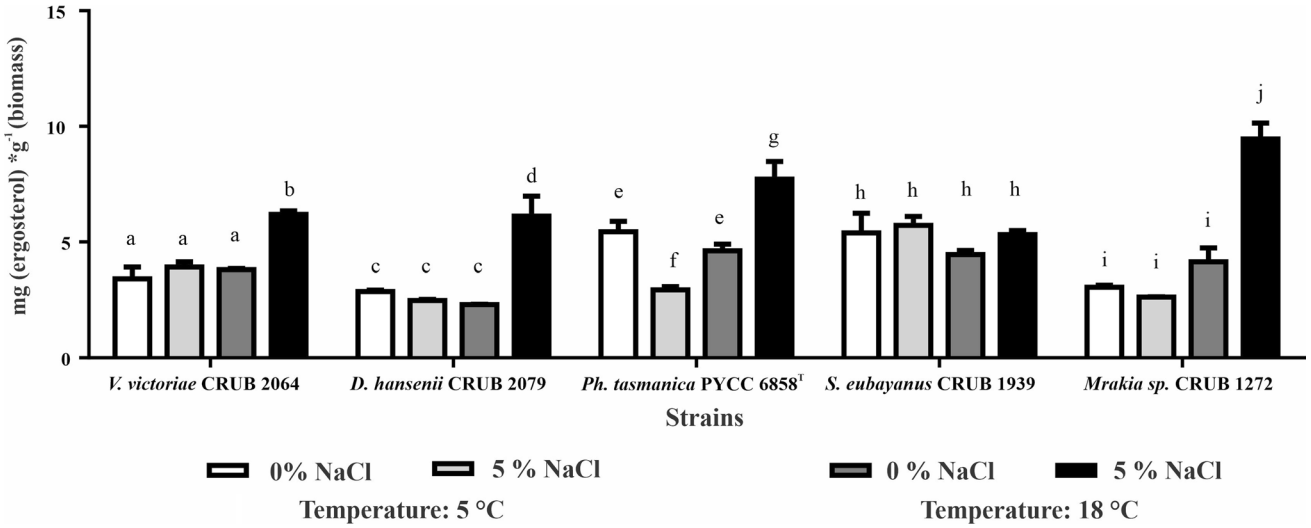


Fig. 5 Accumulation of ergosterol in biomass of the strains *Vishniacozyma victoriae* CRUB 2064; *Debaryomyces hansenii* CRUB 2079; *Phaffia tasmanica* PYCC 6858.[T]; *Saccharomyces eubayanus* CRUB 1939; and *Mrakia* sp. CRUB 1272. In MMS cultures as the control and MMS cultures + 5% NaCl. Significant differences were evaluated for each strain, indicated by different letters ($p < 0.05$)

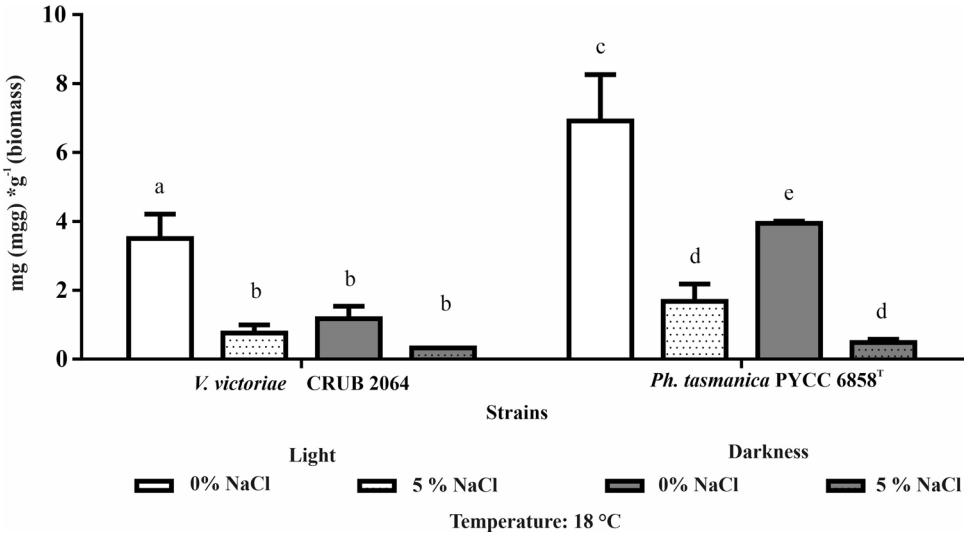


Fig. 6 Accumulation of mycosporines (MGG) in biomass of the strains *Vishniacozyma victoriae* CRUB 2064 and *Phaffia tasmanica* PYCC 6858.[T]. In MMS cultures as the control and MMS cultures + 5% NaCl. Significant differences were evaluated for each strain, indicated by different letters ($p < 0.05$)

Discussion

In this study, we categorized 38 yeast strains found in extreme environments into five groups: osmotolerant, polyextremophilic, psychrotolerant, psychrophilic, and extreme psychrophilic. The three strains that were classified into the polyextremotolerants group (G1) were isolated from seawater in Antarctica and belong to different taxonomic groups *Metschnikowia australis*, *Debaryomyces hansenii* (Order: Saccharomycetales) and *Vishniacozyma victoriae* (Order: Tremellales). Generally, marine yeasts have developed a series of adaptive mechanisms that include the production of osmolyte compounds that allow them to survive in saline environments (Musa et al. 2018). The species in the G1 group have been previously described as ubiquitous (de Garcia et al. 2014), and it has been mentioned that their metabolic plasticity allows them to play an ecological central role in these extreme environments (Gostinčar et al. 2014; Buzzini et al. 2018; Touchette et al. 2023).

The 28 strains of group G2, presented a wide range of responses either to temperature or to osmotic conditions. The subgroup A was represented by yeasts of the *Saccharomyces* genus. Our results related to temperature profile coincide with the data obtained from different psychrotolerant species of the genus, which are capable of growing at low temperatures (8–15 °C) and have a maximum growth

temperature in the range of 33–35 °C (Sampaio 2018). *S. eubayanus* and *S. uvarun* not only have diversity hotspots in common but also share an ecological association with *Nothofagus* spp. (Almeida et al. 2014; Eizaguirre et al. 2018). While *S. eubayanus* has been found in other regions (Peris et al. 2016; Sampaio 2022), the limited number of isolated strains and their lower genetic diversity indicate that the cold environment of South America serves as the leading center for their evolutionary radiation (Eizaguirre et al. 2018; Nespolo et al. 2020). In our results, native strains of *Saccharomyces* genus showed in its growing profile the adaptation to high sugar concentration and low temperatures, both characteristics of *Nothofagus* spp. forests and *Cyttaria* stromata, a fungus with a fructified body that can present up to 10% of w/v of simple sugars (Ulloa et al. 2009; Cadez et al. 2019). *Saccharomyces cerevisiae* response to osmotic stress and low temperatures shows a marked effect on the plasma membrane, the rigidity hinders the incorporation of soluble molecules, preventing the degradation and subsequent utilization of nutrients by the cell (Teixeira et al. 2011; Shokoohi et al. 2016). This could explain the low growth of *Saccharomyces* sp. strain under conditions that combine the effect of cold and osmotic stress.

On the other hand, all the genera included in subgroups B and C, *Aureobasidium*, *Sampaiozyma*, *Tausonia*, *Vishniacozyma*, *Phaffia, Leucosporidium, Mrakia*, and *Glaciozyma*, have been previously described as cold-adapted (Fell et al. 2011; Gostinčar et al. 2014; de Garcia et al. 2015; Wang et al. 2015). The environments from which they were isolated include cold environments such as substrates associated with the *Nothofagus* spp. tree system (Sampaio and Goncalves, 2017), glacial meltwater (de Garcia et al. 2007, 2012), and Antarctic marine ice (de Garcia, data not shown). These species exhibit broad profiles and employ multiple strategies (cold-adapted enzymes, pigments production, polysaccharides production, etc.) to deal with high salinity and low-temperature conditions, they also constitute the dominant species in different cold environments around the world (Buzzini et al. 2018).

Finally, the extreme psychrophilic group (G3), consists entirely of a recently described species *Cryolevonia giraudoae* (de Garcia et al. 2020), a yeast species that shows an extreme profile and low capability to survive in a changing environment in terms of temperature and salinity. Highly specialized organisms with low phenotypic plasticity are highly adapted to very specific environmental conditions (Touchette et al. 2023). This means that they are unable to survive in other environments that differ significantly from their natural habitat. These organisms are among the most vulnerable in the context of climate change, so understanding them is important for the conservation of biodiversity, these point to the importance of studying the biodiversity of glacial ice and the preserved microbiota present on it (Rosa et al. 2019). However due to their very low phenotypic plasticity and their low growth rate we did not include this group in the typification of their biotechnological potential.

The study of the effect of saline and temperature in biomass and metabolites production in five different yeasts including *Vishniacozyma victoriae* CRUB 2064, *Debaryomyces hansenii* CRUB 2079; *Saccharomyces eubayanus* CRUB 1939, *Phaffia tasmanica* PYCC 6858 T; and *Mrakia* sp. CRUB 1272 showed that each strain exhibited a growth pattern consistent with its specific profile.

For the production of secondary metabolites, the accumulation of glycerol in *V. victoriae*, *Phaffia tasmanica*, *S. eubayanus,* and *Mrakia* sp. was the highest in the medium with 5% of NaCl. On the contrary, *D. hansenii* CRUB 2079 (polyextremotolerant) temperatures and salinity tested in this study did not affect final biomass production or glycerol accumulation, although temperature affected the growth rate. The production of glycerol in yeast is a very well-known mechanism for survival in extreme conditions mainly acting as a compatible solute against osmotic stress (Andreishcheva and Zvyagilskaya 1999). Although most of the works are centered on *S. cerevisiae* as a yeast model the role of glycerol in osmotolerance was observed for other yeasts, like *D. hansenii* (Andreishcheva and Zvyagilskaya 1999), this is the first report of glycerol accumulation for the other 3 species used in this work in at least one temperature condition, that not always is coincident with the best temperature observed for the growth. Pérez-Torrado et al. (2016) mention that *Saccharomyces* species show different strategies to survive under osmotic or cold-osmotic stressful conditions, where the balance of intracellular glycerol did not respond to one specific condition, which would indicate they are using different strategies to face environmental alterations. In this sense, our work contributes to establishing that intracellular glycerol concentration is probably a universal response among yeasts to cope with osmotic stress, although this response also depends on the culture temperature, and the best condition for each species is a priori unpredictable.

Surprisingly, *D. hansenii* is the only species used that did not show significant changes in glycerol accumulation. Khroustalyova et al (2001) showed that in this species there is a strong correlation between NaCl concentration and intracellular glycerol accumulation, and Prista et al (2005) demonstrated that this yeast possesses glycerol and sodium symport transporters to regulate the concentration of these solutes and ions in the intracellular medium in response to external osmotic pressure. Probably the osmotic conditions used in this work and the lack of extracellular glycerol in culture media, are related to the observation of the constant intracellular glycerol content, and the NaCl concentration, used in this study, that was selected to allow the growth of strains whose profiles were not necessarily halotolerant.

Regarding trehalose accumulation, we observed that strains *S. eubayanus* CRUB 1939 and *Phaffia tasmanica* PYCC 6858 ^{T} had a higher, in almost all tested conditions, compared to the other strains. It has been reported that trehalose is accumulated in yeasts that ferment sugars into ethanol, which provides protection against oxidative damage to proteins and lipids, extends lifespan, reduces generation of petites, and increases ethanol production (Eleutherio et al. 2015). Although the production of ethanol in *P. tasmanica* was not proved in this work, it is well established that crabtree and ethanol production occurs in the sister species *P. rhodozyma* (Reynders et al. 1997). On the other hand, trehalose acts as a protector in yeasts because it stabilizes the structure of the lipid bilayer and proteins, avoids protein aggregation, as well as prevents chemical changes in biomolecules (Feofliva et al., 2014; Jiang et al. 2018; Kosar et al. 2018). Based on these results could be interesting the use of *Phaffia* spp. or *S. eubayanus* for trehalose production. For the other species *V. victoriae* CRUB 2064*, D. hansenii* CRUB 2064, and *Mrakia* sp. CRUB 1272 trehalose accumulation was significantly higher at 5 °C without NaCl, several authors have mentioned that trehalose might have a protective role against the low temperatures stress but not at high NaCl concentration (Li et al. 2008; Tsuji 2016; Jiang et al. 2018). Our results are in agreement with those observations and suggest that for these species trehalose accumulation is probably a physiological adaptation for the growth at low temperatures.

When we analyze ergosterol accumulation, four of the studied strains have a basal production of this metabolite that increased under higher temperature and salinity culture conditions, ergosterol is a sterol molecule that is found in the cell membranes of fungi, including yeasts. This molecule plays an important role in maintaining the structural integrity and fluidity of the cell membrane, contributes to membrane rigidity and stability, and is involved in stress responses. Ergosterol has been found to have a protective role in yeasts against desiccation and osmotic stress (Dupont et al. 2011, 2012), these findings are consistent with the results obtained in this work. Moreover, Villareal et al. (2018), found similar results in Antarctic yeasts when cultures were grown at different temperatures, ergosterol production did not vary significantly, however, when NaCl was added ergosterol increased in the cell. Our work contributes to the hypothesis that ergosterol plays a protective role against desiccation and osmotic stress in different polyextremophilic yeasts (Dupont et al. 2011, 2012).

MGG is a species-specific trait in yeasts, therefore, its accumulation could only be detected in two of the five analyzed strains, *V. victoriae* CRUB 2064 and *Phaffia tasmanica* PYCC 6858 T (Moliné et al. 2014). In both strains, the higher production of this metabolite was observed in optimal conditions (with light and at 18 °C without NaCl), This finding would suggest that MGG does not have a role as an osmolyte as some authors had previously proposed for black fungi (Kogej et al. 2006) and is consistent with previous studies where the production of MGG in strains of *Phaffia rhodozyma* (Cystofilobasidiales) and *Dioszegia* sp. (Tremellales) decreased when NaCl was present in the culture medium (Moliné 2010; Moliné et al. 2014). Additionally, no accumulation of this metabolite was observed when cells grow at low temperatures indicating that this metabolite is not a response to resist cold shock in these species.

The production of metabolites upon stress in five strains of cold-adapted yeasts has shown us that the responses are not always universal, and although there are general tendencies, such as the greater accumulation of glycerol under osmotic stress, trehalose at low temperatures, ergosterol at high temperatures and salinity, and MGG under optimal growth conditions, these responses are not general and depend on the yeast strains. Thus, looking at the maximum biomass production under optimal growth conditions, it can be seen that the conditions associated with the highest metabolite production are under suboptimal conditions that trigger the production of stress-associated metabolites. To reconcile metabolite and biomass production, the study needs to be extended to other temperatures and salt concentrations, for example using Response Surface Methodology (RSM). This will make it possible to obtain results that can be used by the biotechnological industry.

This work contributes to the knowledge of non-conventional yeasts from cold environments, their growth profile, metabolites, and biomass production in different conditions. However, further studies with psychrophilic and extremotolerant yeasts are needed which will allow us to observe in more detail the effects of stress conditions on different metabolite production, especially those with application in different biotechnological processes.

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Data Availability Data will be available in https://ri.conicet.gov.ar/.

Declaration

Conflict of interest The authors declare no conflicts of interest.

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