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A Physiological Characterization in Controlled Bioreactors Reveals a Novel Survival Strategy for *Debaryomyces hansenii* at High Salinity

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Abstract

Debaryomyces hansenii is traditionally described as a halotolerant non-conventional yeast, and has served as a model organism for the study of osmo- and salt tolerance mechanisms in eukaryotic systems for the past 30 years. However, unravelling of *D. hansenii*'s biotechnological potential has always been difficult due to the persistent limitations in the availability of efficient molecular tools described for this yeast. Additionally, there is a lack of consensus and contradictory information along the recent years that limits a comprehensive understanding of its central carbon metabolism, mainly due to a lack of physiological studies in controlled and monitored environments. Moreover, there is little consistency in the culture conditions (media composition, temperature and pH among others) used by different groups, which makes it complicated when trying to get prevalent conclusions on behavioural patterns.

In this work, we present for the first time a characterization of *D. hansenii* in batch cultivations using highly controlled lab-scale bioreactors. Our findings contribute to a more complete picture of the central carbon metabolism and the external pH influence on the yeast's ability to tolerate high Na⁺ and K⁺ concentrations, pointing to a differential effect of both salts, as well as a positive effect in cell performance when low environmental pH values are combined with a high sodium concentration in the media. Finally, a novel survival strategy at very high salinity (2 M) is proposed for this yeast, as well as potential outcomes for its use in industrial biotechnology applications.

KEYWORDS: *Debaryomyces hansenii*, non-conventional yeast, osmotic stress, salt tolerance, batch fermentation, bioreactors

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Take away: High salt concentrations stimulate respiration in *Debaryomyces hansenii*.

Sodium exerts a stronger positive impact on cell performance than potassium. $\mu_{\max}$ is higher at a combination of low pH, high salt and high temperature. Concentrations of 2M salt result in slower growth but increased biomass yield. The positive effect of salts is enhanced at low glucose concentration.

1 | INTRODUCTION

Debaryomyces hansenii is described as a halophilic/halotolerant non-conventional yeast. It is globally present in seawater and has been isolated from soil, air, materials of plant and animal origin as well as from polar waters and ice from Antarctic and Arctic glaciers (Norkrans, 1966; Breuer & Harms, 2006; Gunde-Cimerman, Ramos, & Plemenitas, 2009). *D. hansenii* has been a model for the study of osmo- and salt tolerance mechanisms in eukaryotic cells over the last 30 years (Adler, Blomberg, & Nilsson, 1985; Prista, Almagro, Loureiro-Dias, & Ramos, 1997; Prista, Loureiro-Dias, Montiel, García, & Ramos, 2005). Potassium and sodium are crucial factors for *D. hansenii*'s growth in high salt environments, and its halotolerant nature has been fully confirmed by the fact that the presence of sodium in the medium protects the yeast cells against oxidative stress and additional abiotic stresses like extreme pH or high temperature (Almagro et al., 2000; Papouskova & Sychrova, 2007; Navarrete, Siles, Martínez, Calero, & Ramos, 2009).

D. hansenii's genome was completely sequenced in 2004 (Dujon et al., 2004), and although more than 6500 genes have been annotated since then, the molecular characterization of this yeast is still far from being fully known. The majority of these annotated genes are mainly associated with salt and osmotic stress tolerance mechanisms (Prista, Michán, Miranda, & Ramos, 2016). It has been described that *D. hansenii* has the highest coding capacity among yeasts (79.2% of the genome) with 6906 detected coding sequences (CDS) and a gene redundancy of almost 50% (Gènolevures online database, igenolevures.org/databases/).

As mentioned before, potassium and sodium fluxes (and their accumulation in cell organelles) play a key role in ion homeostasis and halotolerance in *D. hansenii*. Genes for K⁺ influx (*DhTRK1*, *DhHAK1*) were identified and studied at a molecular, transcriptional and protein level by different authors (Prista, González-Hernández, Ramos, & Loureiro-Dias, 2007; Martínez, Sychrova, & Ramos, 2011). Two different plasma membrane cation efflux systems (*DhEna1/2*, *DhNha1*) have also been described (Almagro, Prista, Benito, Loureiro-Dias, & Ramos, 2001; Velkova & Sychrova, 2006) as well as two intracellular Na⁺/H⁺ antiporters (*DhKha1* and *DhNhx1*) (García-Salcedo, Montiel, Calero, & Ramos, 2007; Montiel & Ramos, 2007). Very importantly, the role of glycerol production and accumulation in the osmotic stress response to high osmolarity was also fully established by Gustafsson & Norkrans in 1976, and further confirmed by Adler, Blomberg & Nilsson in 1985.

From a biotechnological point of view, *D. hansenii* is of great interest, due to its salt tolerant character (Gadanhó, Almeida, & Sampaio, 2003; Butinar, Zalar, Frisvad, & Gunde-Cimerman, 2005; Prista, Loureiro-Dias, Montiel, García, & Ramos, 2005; Ramos, Melero, Ramos-Moreno, Michán, & Cabezas, 2017), especially in food industry where it is used in the ripening process of sausages by production of exopeptidases, development of flavor characteristics, and production of cheeses among others (López Del Castillo-Lozano, Delile,

Spinnler, Bonnarne, & Landaud, 2007; Cano-García, Belloch, & Flores, 2014). Moreover, *D. hansenii* is known to respire a broad range of carbon substrates and produce mycocins against other yeast species, like *Candida* (Banjara, Nickerson, Suhr, & Hallen-Adams, 2016).

Nevertheless, only a few genes with high biotechnological relevance have been characterized so far in *D. hansenii*. For example, the monocarboxylic acid transporter gene *DhJEN1* (Casal, Paiva, Queirós, & Soares-Silva, 2008), and genes coding for xylitol reductase, xylose dehydrogenase and xylose/H⁺ transporter (Biswas, Datt, Aggarwal, & Mondal, 2013; Ferreira et al., 2013), have all been used for the production of second-generation bioethanol in the fermentation of pentoses by *Saccharomyces cerevisiae* (Breuer & Harms, 2006).

The difficulties when studying *D. hansenii*'s biotechnological potential have always been related to the limitations in the availability of highly efficient molecular tools described for this yeast (Prista, Michán, Miranda, & Ramos, 2016). There is also a lack of information and full understanding of its carbon metabolism and physiological characterization during biotechnological processes. On top of that, the large variation in culture conditions used (e.g. media composition, temperature, and pH) in previous studies impedes the thorough and conclusive understanding of this peculiar yeast's behaviour. To date, nobody has conducted an in-depth physiological analysis of this yeast in a controlled environment (e.g. using bioreactors), and there is a lack of consensus about its capacity to produce ethanol in high saline environments or its cell performance in the presence of high salt concentrations. While some studies point to a beneficial role of salts on *D. hansenii*'s performance (Almagro et al., 2000; Papouskova & Sychrova, 2007; Navarrete, Siles, Martínez, Calero, & Ramos, 2009; García-Neto, Cabrera-Orefice, Uribe-Carvajal, Kowaltowski, & Luévano-Martínez, 2017), others claim that sodium is detrimental in terms for the fitness of this yeast in general (Capusoni, Arioli, Donzella, Guidi, Serra, & Compagno, 2019; Sánchez, Calahorra, Ramírez, & Peña, 2018). In addition, the osmo- and halotolerant terminology are often used incorrectly, as there are some studies in which sodium is used for both purposes, despite the reported evidences about sodium and potassium triggering different effects. Potassium does not cause a toxicity response, whereas sodium has been described to have detrimental effects on cell growth at lower concentrations than potassium, pointing to some authors to recently claim *D. hansenii* not being halophilic but just halotolerant (Sánchez, Calahorra, Ramírez, & Peña, 2018), whilst several previous studies report *D. hansenii* as a halophilic yeast (González-Hernández, Cárdenas-Monroy, & Peña, 2004; Chao, Yen, & Ku, 2009; Martínez, Sychrova, & Ramos, 2011).

In relation to *D. hansenii*'s alcoholic fermentation capacity, the kinetics of cell inactivation in the presence of ethanol at 20%, 22.5% and 25% (v/v), has been measured by progressive sampling and viable cell counting, and used as an inference of the ethanol resistance status of different yeasts (Pina, Couto, & Hogg, 2004). *D. hansenii* PYCC 2968T (a.k.a. CBS 767, which we use in our present study) was found to be one of the most sensitive yeasts to ethanol in this study. Ethanol (or other polyols) production has only been reported when a mixture of sugars or complex substrates were used as carbon sources. For example, *D. hansenii* CCM1 941 was cultivated in xylose/galactose or xylose/glucose (Tavares, Duarte, Amaral-Collaco, & Gírio, 2008) for production of xylitol, although only ethanol and glycerol were produced for a xylose/glucose ratio above 30%. Another strain background, *D. hansenii* B-2, was grown in semi-synthetic banana peel-yeast extract-peptone broth for ethanol production, where 40% (w/v) of glucose was fermented to 5.8% ethanol (Brooks, 2008). In

2009, Calahorra, Sánchez & Peña observed the activation of glucose fermentation by salts in *D. hansenii* Y7426. In that work, the authors incubated yeast cells in media containing 40mM glucose, then extracted proteins which were then used to measure the ethanol production by the enzyme extract. Only marginal ethanol production was reported though, within the range of micromoles per gram of glucose. However, more recent work performed by García-Neto, Cabrera-Orefice, Uribe-Carvajal, Kowaltowski, & Luévano-Martínez in 2017, indicates otherwise: that the presence of high salts increases *D. hansenii*'s respiratory activity, in the same strain (Y7426). Overall, *D. hansenii* has not been shown to produce ethanol during growth on glucose as a sole carbon source.

In this work, we present for the first time a physiological characterization of *D. hansenii* during batch cultivations in highly monitored and controlled bioreactors using glucose as carbon source. *D. hansenii*'s carbon metabolism and the external pH influence on its capacity to tolerate high Na⁺ and K⁺ concentrations are also shown. Finally, *D. hansenii*'s ethanol production capability in the presence of salts is further discussed, and a novel survival strategy at high saline environments is suggested.

2 | MATERIAL AND METHODS

2.1 | Strain and culture conditions

The *Debaryomyces hansenii* strain CBS767 (PYCC2968; Prista, Almagro, Loureiro-Dias, & Ramos, 1997; Navarrete, Siles, Martínez, Calero, & Ramos, 2009) was used in this study. For the micro-fermentation experiments and growth assay on plate, IBT22 and IBT26 (CBS1101) strains were also used (from IBT culture collection of fungi from DTU, Denmark). Glycerol stocks containing sterile 30% glycerol (Sigma-Aldrich, Germany) were used to maintain the strain, and were stored at -80°C.

Yeast extract Peptone Dextrose (YPD) medium plates with 2% agar, were used for growing the cells from the cryostocks at 28°C. For the pre-cultures of yeast cells, synthetic complete medium was used (6.7 g/L Yeast Nitrogen Base w/o amino acids, from Difco, plus 0.79 g/L complete supplement mixture, from Formedium). Separately sterilized 2% D-(+)-glucose monohydrated (VWR Chemicals, Germany) was added to the medium, and pH was adjusted to 6.0 with NaOH. All the solutions were autoclaved at 121°C for 20 min. Cells were incubated in 500 mL baffled Erlenmeyer shake flasks (culture volume 100 mL) at 28°C, 150 rpm for at least 24 hours.

For the growth curves in low glucose conditions, *D. hansenii* pre-cultures were prepared as specified in the above paragraph. From those pre-cultures, cells were grown in the same medium but with 0.2% of glucose, and with or without 1M/2M of NaCl or KCl, in order to get their growth profile. Initial OD₆₀₀ in the flask was 0.1 and samples were taken during eight days of cultivation at 28°C, 150 rpm.

2.2 | Growth assay on plate

Three *D. hansenii* strains (CBS767, IBT22 and IBT26) were pre-cultured for at least 24 h in synthetic complete medium (pH 6) at 28°C as specified in "Strain and culture conditions" section, and cell suspensions were prepared from those pre-cultures. Cell drops at OD₆₀₀ 0.1 and serial dilutions (1:10, 1:100) were spotted on YNB plates (pH 6) with or without KCl or NaCl at different concentrations (0.5, 1 and 2M). Cell growth was monitored at 28°C for 144

h or 240 h (when higher salt concentrations were used). Pictures were taken using an in-house build digital camera set-up (Nikon D90 SLR camera).

2.3 | Microscopy

D. hansenii cells were grown on synthetic complete medium (pH 6) with or without KCl or NaCl (0.5, 1 and 2M) at 28°C as specified in “Strain and culture conditions” section. Undiluted cell samples were taken and photographed at the microscope (BA310E & camera 580INT, In Vitro DK) after 51 h of cultivation.

2.4 | Bioreactor cultivations

Batch cultivations were performed in biological replicates (between 2-7 per condition) in 1.0 L Biostat Qplus bioreactors (Sartorius Stedim Biotech, Germany). The temperature was controlled at 28°C and pH was maintained at 6.0 (when desired) by the automatic addition of 2M NaOH / 2M H₂SO₄, and measured by pH sensors (Model EasyFerm Plus K8 160, Hamilton). The volumetric flow rate (aeration) was set at 1 vvm and the stirring was constant at 600 rpm. Dissolved oxygen (DO) concentration was also measured by DO sensors (Model OxyFerm FDA 160, Hamilton). The working volume in the vessel was 0.5 L, using exactly the same medium composition as in the pre-culture, containing either 2% or 0.2% of glucose when required. To study the effect of salt in *D. hansenii* cells, NaCl or KCl (PanReac Applichem, ITW Reagents) were added to the medium before autoclavation. The bioreactors were inoculated with 24 h inoculum from the pre-culture to get an initial OD₆₀₀ of 0.05-0.1. Samples for dry weight, optical density and HPLC were taken after the CO₂ reached 0.1% and until stationary phase.

2.5 | Micro-fermentation experiments

All micro-fermentations were carried out in BioLector[®] I and II systems (m2p Labs, Germany) using 48-well FlowerPlate[®] (MTP-48-B, m2p Labs, Germany) with a working volume of 1.5 mL. Synthetic complete medium (Yeast nitrogen base 6.7 g/L, Complete Supplement Mixture 0.79 g/L, D-(+)-Glucose monohydrated 2%) at different pH (4, 6 and 8) was used, and different temperatures were tested (26, 28 and 30°C). The pH was adjusted with NaOH, and potassium phthalate monobasic ≥99.5% (20.4 g/L, Sigma-Aldrich, Germany) was added to the media and used as a pH buffer for the experiments. Three *D. hansenii* strains (CBS767, IBT22 and IBT26) were pre-cultured in YNB medium (pH 6) at 28°C for at least 24 h, washed with ddH₂O and inoculated in the corresponding wells at an initial OD₆₀₀ 0.1. The programmed settings were as follows: filter; biomass (gain 10 and 3, respectively for the two BioLector[®] systems), humidity; on (>85% using ddH₂O), temperatures; 26, 28, and 30 °C, oxygen supply; 20.85% (atmospheric air), agitation speed; 1000 rpm. The biomass formation was monitored for at least 70 h.

The light scattering data obtained from the BioLector[®] I and II systems were analyzed using R version 3.6.1 (The R Foundation for Statistical Computing).

2.6 | Metabolite analysis

The concentrations of glucose, glycerol and ethanol were measured by High Performance Liquid Chromatography (Model 1100-1200 Series HPLC System, Agilent Technologies, Germany). The injection volume was 20 µl, the eluent 5mM H₂SO₄ and the flow rate was set at 0.6 mL/h. The temperature of a Bio-Rad Aminex HPX-87H column was kept at 60°C. A

standard solution containing glucose (20 g/L), glycerol (2 g/L) and ethanol (20 g/L) was used (Sigma-Aldrich, Germany) for exo-metabolites concentration determination.

2.7 | Off-gas and dissolved oxygen measurements

CO₂ and O₂ concentrations were continuously analyzed in real time by mass spectrometry coupled to the off-gas line (model Prima PRO Process MS, Thermo Scientific, UK). From the off-gas CO₂ emission data, given in percentage, the maximum specific growth rate was calculated. Off-gas CO₂ and O₂ emission data were also used to determine the Carbon Dioxide Evolution Rate (CER), the Oxygen Uptake Rate (OUR) and the Respiratory Quotient (RQ).

Dissolved oxygen values were measured by DO sensors (Model OxyFerm FDA 160, Hamilton) as previously described in “Bioreactor cultivations” section.

2.8 | Analytical procedures

Specific growth rates in the different growth conditions were calculated based on the optical density (OD₆₀₀) and emitted CO₂ values.

Yield coefficients and carbon balances were used to describe the metabolite, by-products and biomass formation by the yeast cells, and were calculated based on the DW, accumulated CO₂ and HPLC data. The average minimal formula CH_{1.79}O_{0.50}N_{0.20} for yeast dry cell biomass composition was used for the calculations as proposed by Roels in 1983. The specific glucose consumption rates were calculated based on the logarithmic method proposed by Görgens, Van Zyl, & Knoetze in 2005.

2.9 | Statistical analysis

Statistical analysis was performed with Microsoft Excel® 2016 (version 1903, 32-bit, USA). All values are represented as averages ± 95% confidence interval of independent biological replicate cultures. For regression analysis, the coefficient of determination (r²) was used to determine the statistical significance of the fit, where a value above 95% was considered statistically significant.

3 | RESULTS

3.1 | *D. hansenii* growth rate under the effect of high salt concentrations

Specific growth rates were calculated based on the optical density profiles and the volumetric CO₂-production rates under the different growth conditions tested (Figure 1). In general terms, a higher maximum specific growth rate was observed when a higher salt concentration was present in the media, except for concentrations of 2M salt, compared to control conditions. Sodium exhibited a significantly stronger positive impact than potassium, as the highest growth rate was reached in the presence of 0.5-1M NaCl. Similar observations were made for KCl, where concentrations of 0.5M gave slightly higher values than 1M, both resulting in a higher growth rate compared to control, although not as high as those for NaCl. On the other hand, a decrease in specific growth rate was observed when the concentration of salts was above 1M, and up to 2M (Figure 1, tables). However, even at concentrations of both sodium and potassium up to 1.5M, the growth rate values are still significantly higher than in the control conditions (no salts added).

Surprisingly, at 2M NaCl the growth rate is lower, but still very close to control conditions, although a comparatively long lag phase was observed (exponential growth phase starts around 30-35 hours in the control vs. 40-45 hours for 2M NaCl, as inferred from the CO₂ profiles in Figure 1). In contrast, the addition of 2M KCl resulted in a lower $\mu_{\max}$ compared to the 2M NaCl and also lower than the control. Microscope samples were taken, randomly, from the liquid cultures at increasing salt concentrations and also from control cultures (no salts added), and no differences were observed in cell size or morphology between the different conditions (Supp.Info Figure S1).

3.2 | Biomass yield on substrate and biomass titers show a differential effect among K⁺ and Na⁺ and an enhanced positive effect of salts when glucose levels are low

The observed decrease in the specific growth rate above 1M of salt seemed to be compensated by a slightly higher biomass yield upon glucose, as was observed for the dry weight measurements during the cultivation time, although only for the addition of potassium (Table 1). The final biomass titers were not significantly different though, with the exception of the 2M sodium which were slightly higher by comparison (Supp.Info Figure S2).

Regarding the specific glucose consumption rate, *D. hansenii* showed higher consumption in the presence of NaCl or KCl than in control conditions, and 1M was observed to be the optimal concentration. Glucose consumption rates began to decrease once the concentration of salts used was above 1M up to 1.5M (Table 1), while at 2M NaCl the specific consumption rates were lower than the control. The rates were higher for sodium than for potassium at lower concentrations (0.5M - 1M - 1.25M), however the opposite was observed at higher concentrations (uptake rates were higher in the presence of potassium).

To further investigate the effect of salts on the specific glucose uptake rates, *D. hansenii* was grown in low glucose conditions (0.2%), preliminarily in shake flasks and later by using bioreactors, to further confirm our findings in flasks.

In the shake flask experiments, we observed that cells growing in the presence of 1M KCl adapted much faster to the nutrient limitation and were able to grow at a significantly higher rate compared to the other conditions tested (Supp.Info Figure S3). This effect was also observed in the presence of 1M NaCl, but at a lower level. The presence of 2M of either salt in the medium had no detrimental effect for the cells, which still grew at a similar growth rate compared to the control.

Interestingly, the presence of 2M of NaCl resulted in almost 3 times higher OD₆₀₀ value than the control conditions. In this particular circumstance (high salts and low glucose), the effect of potassium seemed to be beneficial in terms of growth rate, but sodium seemed to have a better effect in cell performance in the long run, since the growth rate was slower but the final biomass concentration was much higher in return (Supp.Info Figure S3).

Subsequently, we conducted the same type of cultures (0.2% glucose) in bioreactors without pH regulation (thus mimicking the previous flask conditions). Our previous observations in flasks cultivations were confirmed and additional interesting facts were seen. The carbon dioxide evolution rates (CER) exhibited a much faster adaptation (Figure 2a), evidenced by a shorter lag phase, when cells were grown in the presence of 1M NaCl or KCl as previously seen in flasks too. We also obtained significantly higher specific glucose uptake rates and, surprisingly, much higher growth rates at 1M NaCl or KCl than compared with the other

conditions (Figure 2b). In contrast, CO₂ and biomass yields were higher at concentrations of 2M. Additional data showing the timeline of DW, OD₆₀₀ and glucose consumption, are provided in Supp.Info Figure S4.

As shown in Figure 2a, the CER profiles were very similar between control conditions and cells grown at 2M KCl, only for 2M NaCl the CO₂ production peak seemed lower. However when we looked at the CO₂ yields on glucose (Figure 2b), they were higher for both salts at 2M in comparison to 1M and also with the control. The biomass yields at 2M were also higher than for 1M, and much higher than the control. Altogether, this proved an enhanced positive effect of the presence of high salt concentration when glucose is scarce: moderate levels of salt (1 M) resulted in higher glucose consumption rates and higher specific growth rates, while higher concentration of salts (2M) resulted in higher biomass yields and CO₂ yields, at the cost of a lower growth rate - a metabolic trade-off between growing as fast as possible versus growing as much as possible.

It is worth mentioning that the final OD values were higher for the control in bioreactor experiments than in shake flasks. Although we observed a higher biomass when cells were grown in 2M NaCl in both controlled batch (bioreactors) and shake flasks, the cell growth in control conditions (no salt added) was arrested at a lower OD in flasks than in bioreactors, while the difference in total biomass among all conditions was less significant in bioreactors (Supp.Info Figure S3). This is not surprising, and simply illustrates the importance of using well stirred reactors in physiology studies.

3.3 | Dissolved oxygen concentration and RQ levels confirm a fully respiratory metabolism

Dissolved oxygen (DO) levels during bioreactor cultivations decreased faster when Na⁺ or K⁺ was present in the medium, where lower DO levels were observed at increasing salt concentrations, thus suggesting a higher oxygen demand (Figure 3a and 3b, upper graphs). This confirmed a higher metabolic activity at increasing salt concentrations, which might suggest a fully respiratory metabolism stimulated by the presence of (high) salt in the medium.

The Respiratory Quotient (RQ) values, calculated over the entire course of the culture, were below or equal to 1 during the exponential growth phase, but never above this value (Figure 3a and 3b, lower graphs). This proved a full respirative process, hence the absence of fermentation, confirming that *D. hansenii* was not producing ethanol from glucose in our conditions. As a final confirmation of the absence of a fermentative process, our off-gas data and the HPLC analysis showed no trace of ethanol neither in the gas phase nor in the liquid broth samples (Table 1).

It is worth mentioning that in some of the tested conditions the dissolved oxygen levels got very close to 0. This does not mean that cells were suffering from oxygen limitation. The cells did not appear to be deprived from oxygen at any point (due to the airflow inlet throughout the whole bioreactor cultivation) as can be seen (Figure 3a and 3b, lower graphs) by the very stable RQ value (RQ = 1) which coincided with the period of higher oxygen utilization. Thus, the lower dissolved oxygen values simply illustrate a much higher oxygen utilization by the cells, but never oxygen deprivation (which would show an RQ > 1).

3.4 | Additional limiting factors affect cell performance in non-controlled cultivation environments

In order to test the influence of external pH regulation in the halotolerant / halophilic behavior of *D. hansenii*, parallel bioreactor cultivations in 2% glucose were run, in which no pH control was set, and extracellular pH levels were measured on-line in real time. It was observed that, when no pH control was exerted, the CO₂ profiles of *D. hansenii* maintained a plateau phase which could not be seen in pH-controlled fermentations, both in control conditions (Figure 4a) or under the effect of salts (Figure 4b and 4c). The changes in the external pH over time are shown in Supp.Info Figure S5 for the different conditions tested.

The maximum specific growth rates ($\mu_{\max}$) increased when 1M NaCl or KCl were used without pH regulation, suggesting a potential summative effect of low pH and high salt concentrations. Sodium seemed to have a higher positive impact on growth when compared to potassium. The specific growth rates decreased once the concentration of both salts approached 2 M, as also observed in pH-controlled experiments, although there was an even lower growth rate compared to the control conditions in contrast to when pH was controlled (Table S1 at Supp.Info).

Interestingly, the aforementioned plateau in the CO₂ profiles was not observed for non-pH controlled bioreactors run with glucose limiting conditions (0.2%), where sharper and well defined peaks were seen in the profiles (Figure 2a). Here, a faster pH decrease occurred in optimal growth conditions, corresponding to 1M salt (either NaCl or KCl) in the medium (Supp.Info Figure S6), compared to the control. This supports a combined effect of low pH and increasing salts to be very beneficial for overall *D. hansenii*'s performance, as shown in Figure 2b.

3.5 | Spot-tests and micro-fermentation screening analyses confirm the advantages of high salinity, especially sodium, for the optimal proliferation of *D. hansenii*'s strains

In a follow up round of experiments, we aimed to confirm our observations in liquid media for all the conditions tested (including controlled bioreactors and shake flask cultivations), that is: a faster growth at medium-high salinity (1M salts), and slower rate but longer proliferation capacity to higher biomass densities at higher salinity (up to 2M). To that end, we performed spot tests on solid media plates with increasing salt concentrations (Figure 5), this time including two other *D. hansenii* strains (IBT22, IBT26) than the model CBS767 to check if our observations were strain dependent or applicable to isolates from other international collections.

As shown in the figure, we confirmed that the presence of NaCl resulted more beneficial than KCl, and that moderate-to-high concentrations (0.5 – 1M) of sodium allow for a faster proliferation in the plates compared to the control. High concentrations (2M) resulted in a slower proliferation pace. However, in the long run (240 hours of cultivation), we observed that the yeast proliferation capacity remained unaltered, 2M sodium plates showing similar growth than the control.

Finally, complementary tests were performed in controlled micro-fermentors (BioLector®) including the other *D. hansenii* strains (IBT22, IBT26), to assess the effect of salt in combination with other abiotic parameters such as pH (4, 6, and 8) and temperature (26, 28, and 30 °C). This micro-fermentation system was used for the screening of combinations of

these parameters under controlled conditions, and the visualization of these results presented as surface-response plots (Figure 6).

We observed that the three *D. hansenii* strains showed different behaviour in the absence of salt in the culture medium. For example, IBT22 showed a more constant behaviour in terms of growth at all the temperatures tested, while CBS767 was clearly more sensitive to the combination of 30 °C and acid pH (4). IBT26 grew poorly at pH 4 compared with the other two, however showed its higher growth rate at pH 6 and 26 °C, similarly to CBS767 but unlike IBT22, whose maximum growth rate was reached at pH 8 and 28 °C. Nevertheless, once 1M salt was present we clearly observed how the cell growth at the different conditions showed a very similar tendency for all the three strains: higher growth rates at pH 4 and lower ones at pH 8, all the three showing their maximum growth rate with the combination of acid pH and high temperature (Figure 6). Another important observation was that, as early seen in the 1L bioreactors, there was a positive correlation between high Na⁺ and low pH in terms of cell growth. We also noticed an interesting fact: in the absence of sodium, the combination of low pH and high temperature gave a lower growth rate for all the three strains tested, however the addition of 1M NaCl turned that combination (pH 4 and 30 °C) into the best condition in terms of growth rate for the three strains. The poorest culture condition suddenly became the best, simply by the addition of sodium.

4 | DISCUSSION

The results obtained in our study indicate that high salt concentrations are indeed needed by *D. hansenii* for optimal levels of cell performance. Especially in the case of NaCl, our results seem to confirm the halophilic behavior of this particular yeast. An interesting finding is that NaCl exhibits a more significant positive impact than KCl, already evidenced in the spot-tests in solid media, but especially clear from the optimal growth rates obtained from the bioreactor experiments in 1M NaCl, closely followed by those obtained in 1M KCl. The positive impact of salt is even higher when the glucose source is scarce (0.2 %).

The longer lag phase observed when the yeasts were grown in the presence of 2M NaCl may have led to the previous conclusion by other studies that *D. hansenii*'s growth is inhibited by high sodium concentrations (Capusoni, Arioli, Donzella, Guidi, Serra, & Compagno, 2019). This conclusion was probably due to the use of different media, different yeast strains, and more importantly, because the experiments were performed in non-controlled conditions. In our study, growth on 2M salts in non-controlled conditions might indeed mistakenly point to a subtle growth deficiency as observed in Figures 4b and 4c. We observed a slightly higher biomass yields in normal carbon (2% glucose) media and much higher biomass yields in nutrient-limiting conditions (0.2% glucose) compared to control conditions with no salt, as shown in Figure 2b, that none of the previous studies observed. Our spot-test assays with increasing salinity in solid media plates confirmed that the presence of up to 1M salt (and particularly sodium) results in a faster proliferation compared to control plates. Although the presence of 2M sodium causes a slower proliferation pace, after 240 hours the yeast had grown to similar levels as the control, hence there was overall no detrimental effect on biomass generation caused by high sodium concentrations. The cell growth was not inhibited: they simply grew slower.

Considering all of the above, we propose this as a survival strategy of *D. hansenii* to prevail in drying environments (e.g. during periods of drought). While other microbial species chose, as a survival strategy, to enter a dormant state or sporulate when environmental salinity levels rise (as a consequence of the lack of water), *D. hansenii* reacts by increasing its growth rate at moderate-high salinity levels (over 1M is already considered toxic for other yeast and bacterial species, so they have difficulties to proliferate (Yan, Marschner, Cao, Zuo, & Qin, 2015)). The resulting increase in glucose uptake rate (as our data suggests) enables *D. hansenii* to get the most of the available carbon for its survival and limit resources for its competitors. Later, when the salt concentration in the media increases, these yeast cells slow their growth rate while still proliferating to overpopulate the area, thus changing the metabolic strategy from growing as fast as possible to growing as much as possible. This suggested strategy would be in accordance with the latest publications indicating that *Debaryomyces* are the most represented species in thawing Arctic glacier samples and coastal environments (Butinar, Strmole, & Gunde-Cimerman, 2011; Jaques, Zenouche, Gunde-Cimerman, & Casaregola, 2014).

During the bioreactor runs, the dissolved oxygen levels in the culture medium decreased faster in presence of higher salt concentrations, suggesting a higher respiratory activity stimulated by salts, and RQ values together with HPLC and off-gas MS analyses further evidenced the absence of fermentation. A previous study by Calahorra, Sánchez & Peña (2009) reported that the presence of high salts activates a fermentative process in *D. hansenii*, although this work was performed using a different *D. hansenii* strain (Y7426) in other culture conditions, and their measurements correspond to ethanol production using enzyme extracts from previously cultured cells. However, a follow up study performed by García-Neto, Cabrera-Orefice, Uribe-Carvajal, Kowaltowski & Luévano-Martínez (2017) using the same strain (Y7426) already contradicted these observations by reporting that the presence of salt activates respiration, and not fermentation. Our results align well with the latter study, confirming that no ethanol production occurred by our strain either, hence concluding that *D. hansenii* is not a Crabtree positive yeast.

Our data from bioreactors without pH control pointed to additional limiting factors during the cultivation, based on the CO₂ production profiles, compared to pH controlled cultivations, hence indicating that shake flask experiments (non-controlled environments) are not the ideal setup to obtain accurate conclusions about physiological and/or metabolic parameters. Consequently, we suggest that the conclusions obtained by this means in previous works must be taken cautiously. We also proved that it is not appropriate to randomly choose Na⁺, K⁺ or other cationic salts in order to study osmotolerance or halotolerance in general: our results showed that there are differences exerted by either NaCl or KCl, as already suggested by Martinez, Sychrova, & Ramos (2011) and Martínez, Luna, & Ramos (2012).

A very recent study, conducted by Ramos-Moreno, Ramos, & Michán (2019), interestingly hypothesized about a link between salt stress and oxidative stress response in *D. hansenii*. They cultured this yeast in the presence of high salt concentrations, reporting an increase in the expression of ROS detoxification genes (sodium showing a higher effect than potassium). Our findings align very well with their experimental results, complementing their observations by adding evidence of higher oxygen utilization when salt is present, especially NaCl. A higher respiratory metabolism causes the activation of oxidative stress responsive genes, which would confirm the link between salt stress and the oxidative stress that Ramos-

Moreno, Ramos, & Michán (2019) suggest. In addition, both Ramos-Moreno, Ramos, & Michán (2019) and our results confirm earlier studies pointing to a protective effect of NaCl against oxidative damage, such as that caused by H₂O₂ (Navarrete, Siles, Martínez, Calero, & Ramos, 2009). In that case, the presence of NaCl would activate oxidative stress defenses as described above, thus they would be already active and therefore protective against the further damage caused by increased levels of hydrogen peroxide.

Last but not least, an important conclusion from our study based on the comparative analysis using controlled micro-fermentation systems, is that the benefits of salt were observed for all the different *D. hansenii* strains tested, confirming that the positive effect of high sodium is not strain dependent. The definitive evidence about the crucial positive impact of sodium on *D. hansenii*'s cell performance was obtained from the micro-fermentation analysis too. For all the strains tested, the worst culture conditions rendering the lowest growth rates (a combination of low pH and higher temperature) became the best of all, with the highest growth rates, when 1M of sodium was present in the cultivation medium. This positive correlation in terms of cell growth between low pH and the presence of high salt was first suggested by Almagro *et al.* (2000) for the strain *D. hansenii* CBS767. Our results not only confirm their hypothesis, but moreover show that this effect does not seem to be strain dependent, as we found the same positive response for the other strains included in our study (IBT22 and IBT26). Similarly, our results also agree with a study performed by Papouskova & Sychrova (2007) which reported an increased capacity of *D. hansenii* to grow at higher temperatures when salt is present. Other studies had described *D. hansenii* as acid sensitive, suggesting that low pH was stressing for this yeast and therefore negatively impacting cell growth (Turk, Montiel, Zigon, Plemenitas & Ramos, 2007). Our results in controlled micro-fermentors also confirmed this in the absence of salt, however the addition of 1M NaCl significantly improved cell growth at pH 4, once more proving the benefits of this salt in *D. hansenii*'s performance.

All in all, our results shed light upon the behaviour of *D. hansenii* in controlled bioreactors, presenting this peculiar non-conventional yeast as a strain which is able to perform very well in “standard” cultivation conditions (no stress added) but whose performance gets significantly improved when environmental conditions get harsh: high salinity / osmotic pressure, media acidification and nutrient scarcity, all in combination. This undoubtedly confers to *D. hansenii* an incredibly strong potential for industrial production setups: those are the conditions that the microbial cells encounter upon large scale bioproduction processes (meaning vessels of 1000 litre or above), and which can significantly limit their performance in industrial bioreactors (Takors, 2012). Therefore, having such a strain with the aforementioned behaviour, and more importantly, obtaining sufficient information for understanding it (and, consequently, being able to take advantage of that knowledge) is of paramount interest for advancing in the field of cell factory design for industrial bioprocesses, and therefore for the biotech industry overall.

One of the strongest outcomes of our current study is the possibility of using *D. hansenii* for industrial bioprocesses in culture media containing relatively high salt concentrations, as recently suggested by Navarrete & Martinez (2020). On one hand, there is no need of using pure water sources, which significantly decreases the production costs as one could take advantage of desalination effluents, or even the use of sea water for the media composition, while still increasing the production yields. On the other hand, using saline environments has

another strong advantage which is the reduced risk of contaminations, hence sterilization costs would also be significantly reduced (Navarrete, Jacobsen, Martinez & Procentese, 2020).

5 | CONCLUSION

Altogether, our study reveals the beneficial role of salts, and more particularly sodium, in the cell performance of *D. hansenii*. Our findings suggest the need to further investigate how sodium and potassium influence the cell metabolism at a molecular level. It is clear that salts are not just tolerated in *D. hansenii*, but they play a crucial role in its survival strategy, which is currently underestimated. Further research, including a global expression analysis by RNAseq in steady-state continuous bioreactor cultivations, will shed light upon what are the intracellular mechanisms that trigger such metabolic changes, and how the discrimination between sodium and potassium occurs to trigger the different behavioural patterns described within this study. It will also be interesting from a biotechnological point of view, to identify of molecular elements that could potentially be responsive to the presence of salts, as our observations suggest that there can be molecular switches which react to the presence or absence of sodium and/or potassium in the environment, triggering a specific metabolic response.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

JLM conceived the project. CN, ATF, LRM and MRK designed and performed the experiments. CN, ATF and MRK analyzed the data. CN and JLM wrote the manuscript. All authors read, commented and approved the manuscript.

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(a)

Maximum specific growth rates ($\mu_{\max}$).		
Conditions	$\mu_{\max}$ - OD ¹	$\mu_{\max}$ - CO ₂ ¹
Control	0.103±0.006	0.105±0.004
0.5M NaCl	0.162±0.013	0.185±0.007
1M NaCl	0.170±0.005	0.176±0.000
1.25M NaCl	0.168±0.006	0.165±0.005
1.5M NaCl	0.150±0.008	0.143±0.017
2M NaCl	0.106±0.005	0.101±0.000

Note: Data shown are mean values ± 95% confidence interval of a number of replicates.

¹ The maximum specific growth rates (h⁻¹) on glucose determined by OD₆₀₀ and off-gas CO₂.

(b)

Maximum specific growth rates ($\mu_{\max}$).		
Conditions	$\mu_{\max}$ - OD ¹	$\mu_{\max}$ - CO ₂ ¹
Control	0.103±0.006	0.105±0.004
0.5M KCl	0.169±0.002	0.179±0.004
1M KCl	0.153±0.014	0.165±0.001
1.25M KCl	0.131±0.002	0.162±0.004
1.5M KCl	0.119±0.037	0.119±0.050
2M KCl	0.086±0.009	0.092±0.003

Note: Data shown are mean values ± 95% confidence interval of a number of replicates.

¹ The maximum specific growth rates (h⁻¹) on glucose determined by OD₆₀₀ and off-gas CO₂.

FIGURE 1 Maximum specific growth rates and carbon dioxide evolution rate (CER) profiles from batch cultivations with sodium (a) or potassium (b) chloride. Maximum specific growth rates determined by optical density (OD) and off-gas CO₂ emission data from batch cultivations of *Debaryomyces hansenii* in synthetic complete media containing 2% glucose at 28°C and pH 6 with varying concentrations of sodium/potassium chloride are shown in the tables. The CER profiles are based on off-gas CO₂ emission data over time from one replicate for each condition and are representative of their associated replicate(s).

FIGURE 2 Carbon dioxide evolution rate (CER) profiles and yield coefficients, specific glucose consumption and maximum specific growth rates from batch cultivations under limiting glucose conditions and without pH regulation. Off-gas CO₂ emission data from batch cultivations of *Debaryomyces hansenii* in synthetic complete media containing 0.2% of glucose at 28°C and initial pH value of 6 with varying concentrations of sodium/potassium chloride are shown in the figure (a). The CER profiles are based on off-gas CO₂ emission data over time from one replicate for each condition and are representative of their associated duplicate. Yield coefficients and maximum specific growth rates from batch cultivations of *Debaryomyces hansenii* at different salt concentrations are shown in the table. Additionally, the specific glucose consumption rates during the exponential phase calculated based on the logarithmic method are presented (b). The cells were grown in synthetic complete medium containing 0.2% of glucose and with or without salt at different concentrations, at 28°C and initial pH of 6. Data shown are mean values ± 95% confidence interval of duplicates.

(a)

(b)

FIGURE 3 Dissolved oxygen (DO) and respiratory quotient (RQ) profiles from batch cultivations with sodium (a) or potassium (b) chloride. Dissolved oxygen (%) levels measured over time (h) in synthetic complete media containing 2% glucose at 28°C and pH 6 with varying concentrations of sodium/potassium chloride, are represented in the figure. The DO profiles are based on one replicate for each condition and are representative of their associated replicate(s). The RQ profiles are based on OUR (oxygen uptake rate) and CER (CO₂ emission rate) values calculated from off-gas O₂ consumption and off-gas CO₂ emission data from one replicate for each condition and are representative of their associated replicate(s).

FIGURE 4 Graphical comparison of CER (CO₂ emission rate) profiles with and without pH regulation for batch cultivations with varying concentrations of salts. The CER profiles are based on off-gas CO₂ emission data over time from one replicate for each condition and are representative of their associated replicate(s). The cells were grown in synthetic complete media containing 2% glucose at 28°C with and without pH regulation (pH 6 with regulation). The cells were either grown without salts (a), NaCl (b) or KCl (c).

FIGURE 5 Growth assay of *D. hansenii* strains CBS767, IBT22 and IBT26 in YNB plates (pH 6) control and supplemented with different concentrations of KCl or NaCl. Cells were grown in flasks without salt for 24 h, and cell suspensions were prepared from those cultures. Cell drops at OD₆₀₀ 0.1 and serial dilutions (1:10, 1:100) were spotted on the plates. Growth was monitored for 144 h, or 240 h when higher salt concentrations were used.

FIGURE 6 Maximum specific growth rates ($\mu_{\max} \text{ h}^{-1}$) from micro-fermentations of *D. hansenii* strains CBS767, IBT22, and IBT26 in YNB control medium and supplemented with 1M NaCl (both with 2% glucose) as a function of pH and temperature ($^{\circ}\text{C}$). The surface-response plots were generated using R.

TABLE 1 Yield coefficients and specific glucose consumption rates from batch cultivations.

Conditions	Y_{SC}^1	Y_{SX}^1	Y_{SE}^1	Y_{SG}^1	C-balance ²	SGCR (r_s) ³
Control	0.63±0.01	0.35±0.00	ns ⁴	ns ⁴	0.98±0.01	0.120±0.004
0.5M NaCl	0.57±0.03	0.32±0.02	ns	ns	0.90±0.05	0.157±0.001
1M NaCl	0.63±0.07	0.35±0.04	ns	ns	0.98±0.11	0.163±0.012
1.25M NaCl	0.60±0.02	0.34±0.01	ns	ns	0.94±0.04	0.159±0.002
1.5M NaCl	0.63±0.10	0.35±0.06	ns	ns	0.98±0.16	0.133±0.014
2M NaCl	0.63±0.00	0.36±0.00	ns	ns	0.99±0.00	0.098±0.014
0.5M KCl	0.61±0.00	0.34±0.00	ns	ns	0.95±0.00	0.153±0.009
1M KCl	0.68±0.05	0.38±0.03	ns	ns	1.05±0.08	0.159±0.012
1.25M KCl	0.63±0.05	0.35±0.03	ns	ns	0.98±0.08	0.148±0.004
1.5M KCl	0.68±0.04	0.38±0.02	ns	ns	1.06±0.06	0.144±0.001
2M KCl	0.66±0.14	0.37±0.08	ns	ns	1.02±0.21	0.138±0.001

Note: Yield coefficients from batch cultivations of *Debaryomyces hansenii* at different salt concentrations are shown in the table. Additionally, the specific glucose consumption rates during the exponential phase calculated based on the logarithmic method are presented. The cells were grown in synthetic complete medium with or without salt at different concentrations, at 28°C and pH 6. Data shown are mean values ± 95% confidence interval of 2-7 biological replicates per condition.

¹ Yield of CO₂ (C), Biomass (X), Ethanol (E), and Glycerol (G) from Glucose (S) in cmol/cmol.

² Carbon balance on a C-mol basis, where a value of 1 indicate a closed carbon balance.

³ Specific Glucose consumption rate in cmol/cmol/h based on logarithmic method.

⁴ ns: Not statistically significant. The amount of ethanol and glycerol was neglectable in all the conditions tested.