

1 **High-throughput screening of a *Debaryomyces hansenii* library for**
2 **potential candidates with improved stress tolerance and wider carbon**
3 **utilisation capabilities**

4 Anne Sofie B. Dyerberg¹, Clara Navarrete¹ and José L. Martínez^{1*}

5 **1Department of Biotechnology and Biomedicine, Technical University of Denmark (DTU).**
6 **Søltofts Plads, Building 223. 2800 Kgs. Lyngby, Denmark.**

7 *corresponding author: jlmr@dtu.dk

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9 **Abstract**

10 Industrial biotechnology comprises the manufacturing of bulk chemicals and high-value end-
11 products from renewable feedstocks, thus it presents a valuable aspect in the present transition from
12 traditional- resource demanding manufacturing to sustainable solutions. The non-conventional yeast
13 *Debaryomyces hansenii* encompasses halotolerant characteristics that ensures its use in industrial
14 applications, and hence, its industrial importance. For this purpose, a comprehensive and holistic
15 understanding of its behaviour and response to abiotic stresses is essential. Through high-
16 throughput screening methods, using advanced robotics and automation devices, the present study
17 enlightens intraspecies behavioural characteristics of novel *D. hansenii* strains in response to
18 sodium, as well as their ability to tolerate abiotic stress in semi-controlled micro-fermentations and
19 spot-test studies. A significantly improved performance under those abiotic stresses was observed
20 under the presence of 1M NaCl. Moreover, a positive and summative effect on growth was also
21 found in pH 4 and high salt content. Our results align with previous findings suggesting the
22 halophilic (and not just halotolerant) behaviour of *D. hansenii*, which is now extensive to all the *D.*
23 *hansenii* strains included in this study. Strain-specific differential responses to the presence of
24 sodium were also observed, with some strains exerting a more notable induction by the presence of
25 salt than the standard strain (CBS767). Furthermore, our study provides indications of the use of *D.*
26 *hansenii* in industrial bioprocesses based on lignocellulosic biomass and non-lignocellulosic
27 feedstocks.

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38 **Introduction**

39 Industrial biotechnology comprises the ability to manufacture bulk chemicals and high-value
40 products from renewable feedstocks. Rapid increase in innovative industrial biotech tools have
41 reduced cost of production, thus creating competitive advantages for the production of biomass-
42 derived products and making them commercially successful (Li et al. 2014). However, several
43 hurdles still need to be addressed to fully realise and obtain the comprehensive potential of
44 industrial bioprocessing (Erickson, Nelson, and Winters 2012). Enabling high yield and reduced
45 manufacturing maintenance, including reduced sterilisation processes, remain a current obstacle in
46 manufacturing biotechnological products and it reflects the often-costly processes. Deficiency of
47 cost-effective solutions decelerate the diversion towards a bio-based economy, hence continued
48 research in optimum hosts and processes is needed to maximise commercial success and
49 competitive methods (Li et al. 2014). Saline environments permit reduced sterilisation costs due to
50 their natural barrier toward microbial contaminants, for which the high salinity is toxic. Therefore,
51 using saline environments in industrial bioprocesses offer the potential of minimising operational
52 costs (Navarrete et al. 2020b).

53 The hemiascomycetous non-conventional yeast *Debaryomyces hansenii* encompasses physiological
54 properties that have generated an increasing interest in the yeast (Prista et al. 2016). Its unique and
55 prominent halophilic/halotolerant characteristics ensure its industrial applications, and hence, its
56 industrial importance. It is described to be able to tolerate levels up to 4.11 M of sodium (Chao,
57 Yen, and Ku 2009). However, growth inhibition has been observed at concentrations exceeding 2M
58 NaCl (Navarrete et al. 2020a; Capusoni et al. 2019). Additionally, when *D. hansenii* is grown in 1M
59 NaCl, protective and non-detrimental effects have been observed. Presence of stress factors,
60 including extreme pH conditions, oxidative stress or different temperatures, are proved to have less
61 detrimental effect when in co-existence with NaCl (Prista et al. 2016). At high temperatures with
62 severe growth inhibitions, presence of salt has also appeared to stimulate growth of the yeast.
63 Additionally, stress by extreme pH has shown to be relieved by the presence of 0.25M NaCl
64 (Almagro et al. 2000). Similar results were obtained by other authors, observing a survival strategy
65 of *D. hansenii* in high salinity environments (Butinar, Strmole, and Gunde-Cimerman 2011;
66 Navarrete et al. 2020a).

67 Furthermore, *D. hansenii* can utilize a broad spectrum of carbon substrates, generally catabolised
68 through respiratory metabolism (Nobre, Lucas, and Leão 1999; Palma et al. 2007). Industrial
69 substrates often contain a variety of lignocellulosic biomass and non-lignocellulosic feedstocks,
70 which are composed of mixtures of hexoses and pentoses (Navarrete et al. 2020b). Hence, *D.*
71 *hansenii*'s ability to assimilate different carbon sources encourages further research about its
72 potential applications in industrial bioprocesses (Prista et al. 2016).

73 In 2016, Prista *et al.* reviewed the main characteristics of *D. hansenii* that makes the use of this
74 yeast interesting for food and biotechnology applications. Moreover, there is a demand for studies
75 of wild *D. hansenii*-strains isolated from natural habitats, to increase the understanding of this yeast,
76 while enabling identification of novel capabilities with biotechnological importance (Prista et al.
77 2016). A study conducted by Navarrete *et al.* (2020) demonstrated a beneficial role of salts, with
78 sodium exerting a more significant effect, on the cell performance of *D. hansenii*, validating the role
79 of salt in the yeast's survival and establishing its halophilic behaviour. Simultaneously, their results
80 suggested a positive and summative effect of acidic environment and high salt on the cell growth
81 (Navarrete et al. 2020a).

82 The present study aims to establish an intraspecies behavioural characterisation of novel *D. hansenii*
83 strains, through high-throughput screening methods using advanced robotics and automation
84 devices. Micro-fermentation experiments are used to investigate the different strains' capacity to
85 tolerate high sodium concentrations and pH oscillations. Furthermore, carbon assimilation and
86 growth inhibition induced by side-products from conventional feedstocks, is investigated using
87 advanced screening robotics in solid media.

88

89 **Materials and Methods**

90 **Strains and Culture Conditions**

91 The strain library was obtained from the IBT culture collection of fungi from the Technical
92 University of Denmark.

93 All strains were obtained from cryostocks (16% glycerol, Sigma-Aldrich, Germany) and were
94 grown on yeast extract peptone dextrose (YPD) medium plates (10 g/L yeast extract, 20 g/L peptone,
95 20 g/L dextrose) containing 2% agar and at 28°C.

96 Strains were cultivated in synthetic complete solid medium (6.7 g/L Yeast Nitrogen Base (YNB)
97 w/o amino acids (Difco), 0.79 g/L complete supplement mixture (Formedium), 20 g/L dextrose) or
98 YPD solid medium containing 2% agar. The pH was adjusted to 6.0 with NaOH. When needed,
99 NaCl was added at the desired concentration (1M, 2M and 3M). All media were autoclaved at 121°C
100 for 20 min. Separately filtered carbon source (glycerol, D-xylose, D-(+)-sucrose, D-arabinose, D-
101 galactose, D-lactose, D-(+)-maltose, D-(+)-glucose) was added to the media to a final concentration
102 of 2%. Inhibitors, furfural (1, 2, 4 g/L), hydroxy-methyl furfural (HMF) (0.5, 1, 2 g/L), vanillin
103 (0.1, 0.2, 0.4 g/L), and methanol (1, 2, 4, 6 % v/v) were separately sterilized as well and added to
104 the media when desired. Unless otherwise stated, cultures were incubated at 28°C.

105

106 **Robustness Assay**

107 The strain library was spotted from liquid to solid YNB medium, containing 2% carbon source and
108 with/wo salt, using the ROTOR HDA (Singer Instruments, UK). When YPD solid medium was
109 used, growth inhibitors in the desired concentrations were added, also in the presence or absence of
110 salt.

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112 **Microscopy Analysis**

113 Qualitative cell growth was monitored after 48 h and 144 h using an in-house build digital camera
114 setup (Nikon D90 SLR camera) or the PhenoBooth instrument (Singer Instruments, UK).

115

116 **High-throughput Micro Fermentations**

117 Strains were pre-cultured in flasks containing YNB medium (pH 6), at 28°C and 250 r.p.m. for at
118 least 24 h. Then, the cells were washed with ddH₂O and re-inoculated in a 48-well FlowerPlate®
119 (MTP-48-B, m2p Labs, Germany) in YNB at pH 4 or 6, and with or w/o 1 M NaCl. The working
120 volume in the plate was 1.5 mL at an initial OD₆₀₀ of 0.1. The fermentations were performed in the

121 BioLector® I (m2p Labs, Germany), adjusted to the following settings: temperature; 28°C, filter;
122 biomass (gain 10), humidity; on (>85% via ddH₂O), oxygen supply; 20.85% (atmospheric air) and
123 agitation speed; 1000 r.p.m. Light scattering units (excitation wavelength of 620 nm) were
124 continuously monitored for at least 70 h. Data obtained from the micro-fermentation experiments
125 were analysed using R version 4.0.3 (The R Foundation for Statistical Computing).

126

127 **Statistical Analysis**

128 ***Heat Maps of Spot Tests***

129 Strain's growth on plates with or without 1M NaCl, was examined using the PhenoBooth
130 instrument (Singer Instruments, UK) and belonging software. Following a uniform distribution,
131 heat maps were generated based on standard scores (z-scores). Z-scores were obtained by (i) means
132 of the control colony sizes, calculated on those strains cultivated in YPD plates containing
133 inhibitors in the desired concentration (duplicates; n = 2), (ii) for each strain, measuring the distance
134 to the mean on strains cultivated on the corresponding plates supplemented with 1M NaCl. Z-scores
135 were illustrated in heat-maps with a purple to yellow spectra, corresponding to negative to positive
136 values.

137

138 ***Analysis of Growth Rate***

139 Background-subtracted light scattering units (LSU) were calculated by average measurements of
140 uninoculated wells. Specific growth rates were calculated from the background-subtracted LSU and
141 represented as the mean of the replicates, including a standard deviation. Variations in maximum
142 specific growth rates (μ_{\max} (h⁻¹)) were assessed with an analysis of variance (ANOVA test) using
143 R (R Core Team 2020; Champely 2020). Local growth rates were estimated for each background-
144 subtracted LSU by fitting a linear regression in rolling windows of six neighbouring measurement
145 points, equivalent to estimating growth rates over a span of 18 minutes (Rugbjer et al. 2018;
146 Wickham et al. 2020; Zeileis and Grothendieck 2005). A moving regression based on a locally
147 estimated scatterplot smoothing was applied to obtain scatterplot smoothing and to obtain a pattern
148 in the progression of local growth rate. A confidence interval of 0.95 was included. The
149 computation of local growth rates and the moving regression were constructed using (R Core Team
150 2020).

151

152 **Results**

153 ***Carbon Utilisation and Robustness Indications of *D. hansenii****

154 To assess robustness and investigate the carbon source utilisation and the effect of sodium in the
155 different *D. hansenii* strains, we conducted a high-throughput spot-screening on selected carbon
156 sources (Figure 1). While all strains (n=61) were able to utilise the majority of carbon sources
157 investigated, weakened and varying growth was observed on both arabinose and xylose (on control
158 plates and when supplemented with 1M NaCl) (Figure 1). In accordance with previous results
159 obtained by Nobre et al. (1999), co-presence of medium-high salinity (1M sodium) and xylose or
160 arabinose, indicates a tendency of improved carbon utilisation, and hence improved proliferation
161 (Nobre, Lucas, and Leão 1999). Respectively, 1.7- and 8.2 percent points (pp) increase in strains

162 able to grow on xylose or arabinose, were observed after 144 h of cultivation between control plates
163 (no sodium) and plates supplemented with 1M sodium (data not shown).

164 The percentage of carbon source assimilation by *D. hansenii* was estimated as the presence of
165 growth after 48 h, and calculated from the spot-screening in YNB plates (Table 1). In the absence of
166 salt, *D. hansenii* was successfully able to use all the sugars tested, being arabinose and xylose the
167 ones with the lower estimated assimilation (78.7% and 67.2% respectively). The addition of 1M
168 NaCl to the medium, seemed to improve the assimilation of arabinose but not xylose.

169 These results support the general assumptions and indications of non-inhibitory and non-detrimental
170 effects of sodium on *D. hansenii* (Prista et al. 2016), while demonstrating that the yeast's behaviour
171 is not strain dependent, but it is extended intraspecies.

172 **Proliferation Capacity of *D. hansenii* at Higher Salinity**

173 The general non-detrimental effect of 1M sodium observed in the spot-screenings for carbon
174 utilisation (Figure 1 and Table 1), was further confirmed when we investigated the effect of higher
175 concentrations of sodium (2M and 3M) on *D. hansenii*'s performance. Whilst 3M of NaCl
176 detrimentally affected all isolates of *D. hansenii*, only slower growth was observed in the presence
177 of 2M, as previously reported by Navarrete et al. (2020) (data not shown).

178

179 **Effect of Sodium on Cell Proliferation Capacity in the Presence of Inhibitory Compounds**

180 To address the potential use of waste-derived feedstocks in bioprocesses with *D. hansenii*, we
181 analysed co-presence of abundant inhibitory by-products from pre-treatment of both lignocellulosic
182 and non-lignocellulosic materials (Kim 2018), and in the presence of sodium (Figure 2). Indications
183 of a protective effect of sodium on the proliferation of *D. hansenii*, was observed when 2 g/L of
184 furfural or 1 g/L of HMF were added to the medium. After 48 hours of cultivation, the colonies
185 exhibited better growth on plates with both the inhibitor and salt, when compared to plates only
186 containing the inhibitor. Similar results were obtained when half and double concentrations of
187 furfural and HMF were used, and after 48 hours of cultivation (z-scores > 0) (Figure 2).

188 While analysis of co-presence of sodium and the inhibitors methanol or vanillin also portrayed a
189 non-detrimental and non-inhibitory effect of sodium, the supplement of 1M sodium did not appear
190 to have a convincing engaging effect on cell proliferation after 48 hours of cultivation (Figure 2).
191 This lack of effect was also observed at a lower concentration of methanol (1% (v/v)), where z-
192 scores were distributed around zero and thus, suggesting little to no difference in the diameter span.
193 4% (v/v) of methanol seemed to affect cell proliferation of *D. hansenii* similarly to the observed
194 effect at 2% (v/v), regardless the presence of 1M sodium in the medium (data not shown). At 6%
195 (v/v) of methanol, a shift towards positive z-scores was observed after 48 h of cultivation. Thus,
196 implying the presence of 1M of NaCl to have an enhancing effect on the cell proliferation capacity
197 of *D. hansenii* at higher concentrations of methanol (Figure 2). On the other hand, the simultaneous
198 presence of sodium and 0.1-0.2 g/L of vanillin, appeared to hamper cell proliferation. When colony
199 sizes of *D. hansenii* cultivated on plates containing vanillin were compared to those on plates
200 supplemented with 1M NaCl, z-scores were found to be negative. A varying tendency in cell
201 growth was observed at 0.4 g/L of vanillin (Figure 2).

202

203 **Micro-fermentation Screening Analysis to Assess Effect of Sodium under Different Abiotic
204 Parameters**

205 A selection of the initial *D. hansenii* library was then analysed in the micro-fermentation screening
206 analysis. Strains were selected based on (1) ability to utilise a broad spectrum of carbon sources, (2)
207 substratum from which they were isolated, (3) capacity to grow in the presence of inhibitors

208 To address intraspecies behavioural trends of *D. hansenii* in 1M sodium and under different pHs (4
209 and 6), we studied biomass formation in simultaneous micro-fermentations experiments. The
210 applied setup allowed for a semi-controlled environment and real-time monitoring of the growth of
211 the selected strains. Scattered light signal, which is used as an indicator for biomass formation, may
212 actually cause growth deviations, due to i.e. morphological changes associated with heterogeneity
213 in the cell division cycle. Additionally, high cell densities frequently cause incorrect high
214 measurements of biomass through the course of the bioprocess. These deviations are non-
215 correctable (Kunze et al. 2014). Therefore, only maximum specific growth rates restricted to the
216 exponential growth phase were used to compare growth between strains (Table 2).

217 Differences in growth were observed in both the presence and absence of 1M sodium in the media.
218 At pH 6, addition of sodium did not throw any conclusive results, as some strains showed an
219 increased in growth while others did not.

220 Previous studies have shown synergistic and protective effect of NaCl when other stress factors are
221 present. Specifically, a positive correlation between available Na^+ and acidic environments is
222 proved to enhance cell growth, in comparison to non-saline, acidic environments (Almagro et al.
223 2000; Navarrete et al. 2020a). On the other hand, and although a general differential growth was
224 observed intraspecies at pH 4, a more conclusive positive effect on biomass formation was observed
225 when 1M sodium was present (Table 2). Additionally, IBT27 strain reached the highest maximum
226 specific growth rate in all the conditions studied. Importantly, these results, in accordance with
227 previous published work, indicate a synergistic and protective effect of low pH and high salt on the
228 cell growth of *D. hansenii* (Almagro et al. 2000; Navarrete et al. 2020a; Prista et al. 2016), and it is
229 not strain-dependent.

230

231 **Local Growth Rates Measured During the Course of the Simultaneous Micro-fermentations**

232 In order to study the dynamics of the strains' fitness over time and to understand the influence of
233 the abiotic parameters on their stability, we measured local growth rates throughout the micro-
234 fermentation experiments. Generally, a stable biomass formation was observed in all the strains,
235 when 1M NaCl was present in the media (Figure 3). Under non-acidic cultivation (pH 6) and
236 without sodium present, some strains were able to reach higher maximum specific growth rates (e.g.
237 strain IBT155, IBT364, and IBT493). However, a more pronounced decrease in local growth rate
238 was observed over time in these specific cases, resulting in a lower average local growth rates.
239 When cultivated in the presence of sodium, strains with higher average maximum specific growth
240 rates, did now show an abrupt decrease in the local growth rate (e.g. strain CBS767, IBT22, and
241 IBT81) (Figure 3). Growth-coupled production by selecting for cells with higher fitness is a
242 desirable target for strain design (Dinh et al. 2018). In conclusion, higher fitness of the strains was
243 not observed to be correlated with growth stability in the absence of sodium and at pH 6.

244 When the strains were cultivated at acidic pH (pH 4) and in the presence of sodium, a more stable
245 progression in growth throughout the course of the experiments was observed (Figure 3).
246 Interestingly, these conditions also promoted higher maximum specific growth rates for all the

247 strains, when compared to pH 4 without sodium (Table 2). Once more, the observed improvement
248 in growth indicates that the synergistic and protective effect of low pH and high sodium on cell
249 growth, is not strain dependent, but a general tendency within the *D. hansenii* species (Almagro et
250 al. 2000; Navarrete et al. 2020a; Prista et al. 2016).

251

252 Discussion

253 The global societal transition from chemical, conventional production of bulk- and high value
254 molecules to sustainable solutions is dependent on innovative bio-based production. Developing
255 cost-efficient solutions, will help to obtain the maximal potential of industrial biotechnology, and
256 thus, to accelerate the transition to a more sustainable processes (Erickson, Nelson, and Winters
257 2012). The non-conventional yeast *D. hansenii* presents some important physiological properties
258 that have generated an increased interest in this yeast as a promising host in bioprocesses (Prista et
259 al. 2016). Its unique and prominent halotolerant characteristics ensure its importance in industrial
260 applications (Chao, Yen, and Ku 2009; Prista et al. 2016).

261 While the halotolerant behaviour of *D. hansenii* was already established for several authors
262 (Lépingle et al. 2000; Souciet et al. 2009), recent studies have revealed the salt-including and
263 halophilic behaviour of this yeast (Navarrete et al. 2020a; Prista et al. 2016). Similar conclusions
264 are obtained from the present study. Medium-high concentration of sodium (1M) does not show a
265 detrimental effect on the carbon assimilation capacity of any of the strains included in this study.
266 From our results, *D. hansenii*'s ability to assimilate a broad spectrum of carbon sources (Nobre,
267 Lucas, and Leão 1999) can be now extrapolated to other strains of the same species (Figure 1). A
268 varying growth was observed in the presence of arabinose and xylose in the different strains tested,
269 which is in alignment with previous work reporting slower and weaker growth of *D. hansenii* when
270 assimilating pentoses (Nobre, Lucas, and Leão 1999; Palma et al. 2007; Pereira et al. 2014)
271 Moreover, an increase in the capacity to assimilate pentoses was observed with the addition of 1M
272 sodium, highlighting again the halophilic behaviour of *D. hansenii* strains.

273 A very recent study conducted by Navarrete *et al.* (2020), demonstrated an increased growth in the
274 presence of up to 1M NaCl when compared to control conditions (no salt). Therefore, non-
275 detrimental and non-inhibitory effects of sodium on the proliferation capacity of *D. hansenii* were
276 described by the authors. Despite the different applied method in this study, our results from the
277 high-throughput spot-screenings support previous observations by Navarrete *et al.* (2020). An
278 improved growth was observed when *D. hansenii* strains were cultivated in the presence of 1M
279 sodium (Figure 1).

280

281 Increased Stability of *D. hansenii* Strains when Cultivated in Acidic Culture Conditions

282 Whereas mutation-prone cells secure adaptation by shifting environmental changes for the evolving
283 population, each underlying mechanism presents a probable threat to the stability of the introduced
284 synthetic system. Evolving sub-cultures of non-producers, is an increasing issue in long-term
285 cultivation cell factory strains (Hewitt and Nienow 2007). Generally, an improved stability of the
286 library is observed when sodium is present in the culture media, during the micro-fermentations
287 (Figure 3). Importantly, the stability is even more profound when cells are cultivated in acidic
288 culture conditions. In addition, an initial higher maximum specific growth rate was observed for all
289 the strains in pH 4 and medium supplemented with 1M sodium (Table 2). A summative effect of

290 acidic environments and high salt content has previously shown to have a positive influence on cell
291 growth (Almagro et al. 2000; Navarrete et al. 2020). Our results further sustained previous
292 observations as all the strains of *D. hansenii* tested in this study showed higher maximum specific
293 growth rates in acidic environments supplemented with sodium (Table 2). Subsequently, more
294 stable local growth rate profiles were also observed at low pH and in the presence of sodium
295 (Figure 3).

296 Interestingly, strain IBT27, which showed broad carbon source assimilation and high resistance to
297 inhibitors, especially in the presence of 1M NaCl, also reached the highest maximum specific
298 growth rate at low pH and high sodium (Figure 1 and Table 1). Consequently, IBT27 can be
299 considered as one of the best candidates among the other tested strains. However, at pH 6 and with
300 1M NaCl, the maximum specific growth rate was lower compared to that with no sodium added.
301 Therefore, our results align very well with previous work showing an improved performance of *D.*
302 *hansenii* in environments under the presence of abiotic stresses, and a positive effect on growth in
303 acidic environments with high sodium content (Almagro et al. 2000; Navarrete et al. 2020a).
304 Additionally, we present strong indications of strain-specific responses to the presence of sodium,
305 with some strains responding much better to high sodium than the standard strain (CBS767).

306 The use of high throughput technology facilitates the screening of a range of different parameters in
307 a reasonable short period of time, while significantly contributing to understand the complexity of
308 bioprocesses in general. In addition, the type of analysis used in this work allows the detection of
309 significant differential behaviour between strains at a colony-growth level. Hence, this study
310 provides sufficient prove to be employed in future screenings and identification of optimal
311 candidates of engineered *D. hansenii* cells, e.g. to express a product of interest.

312

313 ***D. hansenii*' Response to Stresses derived from Lignocellulosic Biomass and Non- 314 lignocellulosic Feedstocks**

315 Abundant and renewable lignocellulosic biomass has high potential as a substitute for fossil
316 resources to produce bioprocessed chemicals and materials, while eluding less available and more
317 expensive feedstocks (Li et al. 2017). However, pre-treatment of lignocellulosic biomass yields
318 several inhibitory by-products, making the use of lignocellulosic biomass as feedstock problematic
319 in some cases (Navarrete et al. 2020b). Inhibitory compounds from biomass pre-treatment processes
320 include furan derivatives compounds (including furfural and HMF) and phenolic compounds, such
321 as vanillin (Ask et al. 2013). We found sodium to relieve the abiotic stress caused by the presence
322 of different concentrations of furfural and HMF (Figure 2). This reinforces previously results
323 suggesting that sodium does not have a toxic effect on *D. hansenii* cells, and being able to protect
324 the yeast cells against additional abiotic stress factors (Ramos-Moreno, Ramos, and Michán 2019;
325 Navarrete et al. 2020a).

326 Furan derivatives were found to severely affect the redox metabolism of *S. cerevisiae* while lower
327 inhibition of the energy metabolism was observed (Ask et al. 2013). A study conducted by Ramos-
328 Moreno et al. (2019) demonstrated that expression of genes related to oxidative stress were induced
329 in *D. hansenii* by high salt concentrations (with sodium enabling a better induction than potassium).
330 Hence, suggesting a direct link between high salt concentrations and ROS detoxification. Moreover,
331 a very recent study conducted by Navarrete et al. (2020) demonstrated an increased respirative
332 activity of *D. hansenii* in the presence of salts, especially sodium. The ability of sodium to enhance
333 the respiratory metabolism and to induce expression of ROS detoxification genes (Ramos-Moreno,
334 Ramos, and Michán 2019; Navarrete et al. 2020a) may explain why the proliferation capacity of *D.*

335 *hansenii* strains is inhibited by furan derivatives, when no sodium is available, but improved when
336 sodium is present.

337 On the contrary, the simultaneous presence of sodium and vanillin, known to inhibit the activity of
338 the DNA-dependent protein kinase catalytic subunit, appears to have an opposite effect, resulting in
339 hampered cell proliferation in *D. hansenii* strains (Figure 2). However, depending on the
340 lignocellulosic biomass composition and pretreatment used, a vast range of vanillin concentrations
341 can be present, from 1-26 mM (Ito et al. 2020). Therefore, the effect of higher concentration of
342 vanillin should be further investigated in order to get a better picture about the inhibitory impact of
343 vanillin on *D. hansenii* strains. In addition, the observed summative effect of sodium on cell
344 proliferation in the presence of furfural and HMF should be also further investigated, including both
345 furan derivatives and phenolic compounds.

346 An improved proliferation capacity was also observed at high concentrations of methanol (Figure
347 2). The results obtained from the spot-test suggest a robust behaviour of all *D. hansenii* strains, with
348 some of them outperforming the average.

349 Finally, our study sheds light on the behavioural patterns of novel *D. hansenii* strains in semi-
350 controlled micro-fermentations and spot-test analysis, which contribute to establish the fundamental
351 knowledge about this non-conventional yeast. A successful implementation of industrial scale
352 bioprocesses is restricted by limited access to cost efficient solutions (Li et al. 2014). From the
353 inhibitors study presented in this work, it was found that lignocellulosic biomass and non-
354 lignocellulosic feedstocks could be used in *D. hansenii*-based industrial bioprocesses, which will
355 decrease the process costs while increasing the potential use of this yeast in the market.
356 Furthermore, culture media or water supply containing high salt concentration (including sea-water)
357 as well as open continuous bioprocesses could be used in industrial set-ups, due to the halophilic
358 character of this yeast, and so bypass the need for extensive sterilisation processes.

359

360 Conclusion

361 The use of HT-technology to monitor the different behavioural patterns of several *D. hansenii*
362 strains was successfully used in the present study. Besides, our findings align very well with
363 previous published work of this yeast.

364 An improved performance in the presence of abiotic stresses was observed when 1M NaCl was
365 added to the media. Significantly, a positive and summative effect on *D. hansenii* strains' fitness
366 was observed when pH 4 and high sodium was used. Moreover, our results support previous reports
367 describing a protective effect of sodium on *D. hansenii*'s growth, further expanded to the use of
368 inhibitors that are released from pre-treatment of lignocellulosic on non-lignocellulosic feedstocks.
369 This reveals the great potential of *D. hansenii* in different industrial applications and bioprocesses.

370 Importantly, while some level of differentiation was observed within the different strains tested, the
371 halophilic behaviour and sodium-induced optimal performance of *D. hansenii* can be now extended
372 to all strains included in this study.

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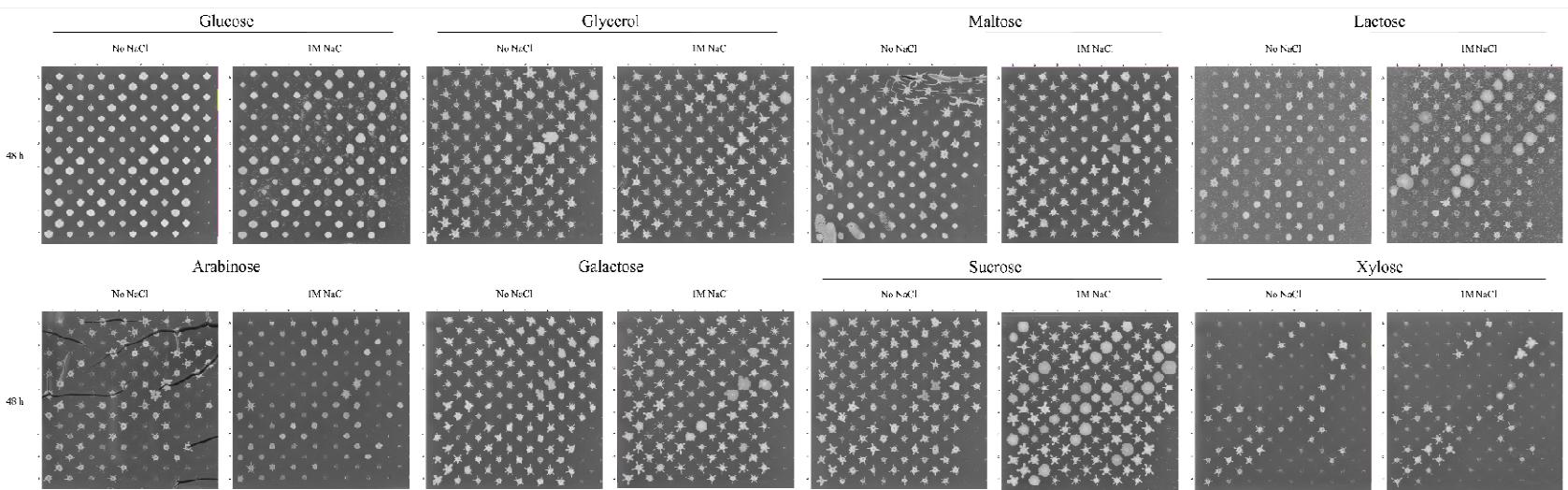


Figure 1. High-throughput spot-screening of carbon assimilation and effect of sodium on *Debaryomyces hansenii*. Cells were grown in YNB plates with or without 1M NaCl, and captures were obtained after 48h of cultivation. Position A1 contains *D. hansenii* CBS767 (a full map of all positions and strains can be found in Supplementary Table 1).

Table 1. Carbon source utilization and effect of sodium on *Debaryomyces hansenii*, calculated from the spot-screening in YNB plates. Percentage of assimilation of carbon source was estimated as presence of growth after 48 h (n=2).

Carbon source	No NaCl	1M NaCl
Glucose	100%	100%
Glycerol	100%	100%
Maltose	100%	100%
Lactose	100%	100%
Arabinose	78.7%	85.2%
Galactose	100%	100%
Sucrose	100%	100%
Xylose	67.2%	60.7%

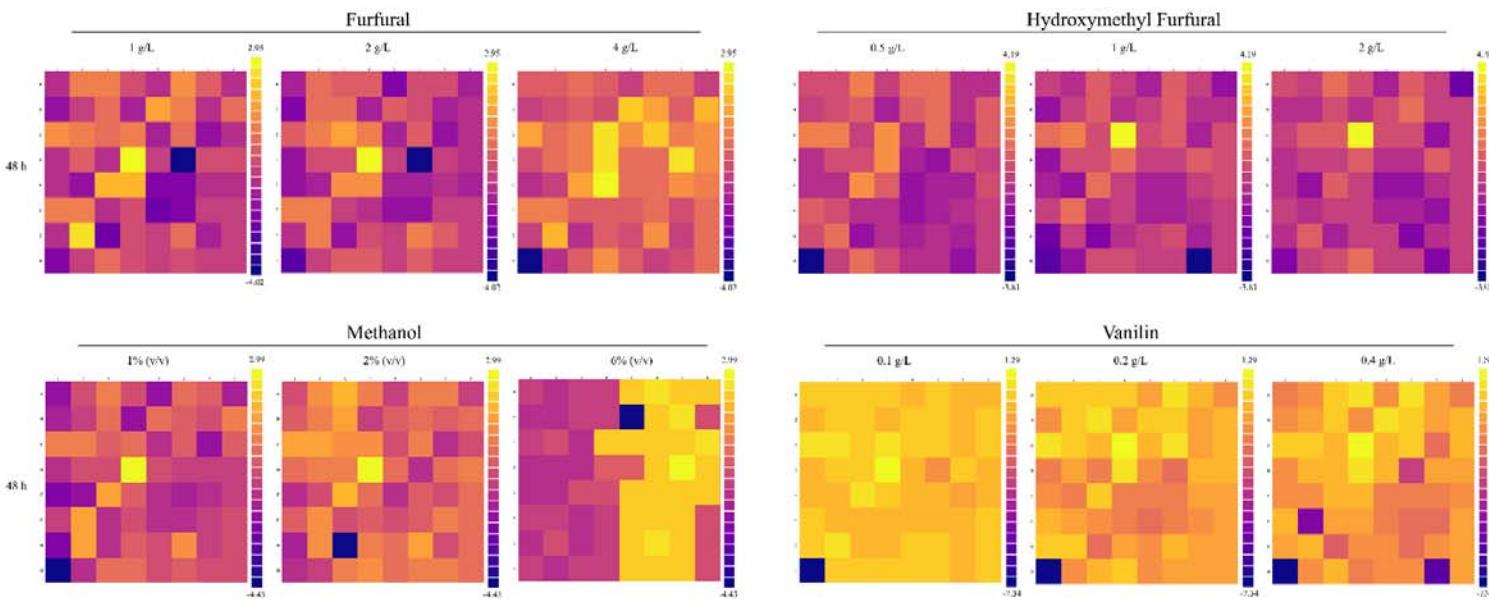
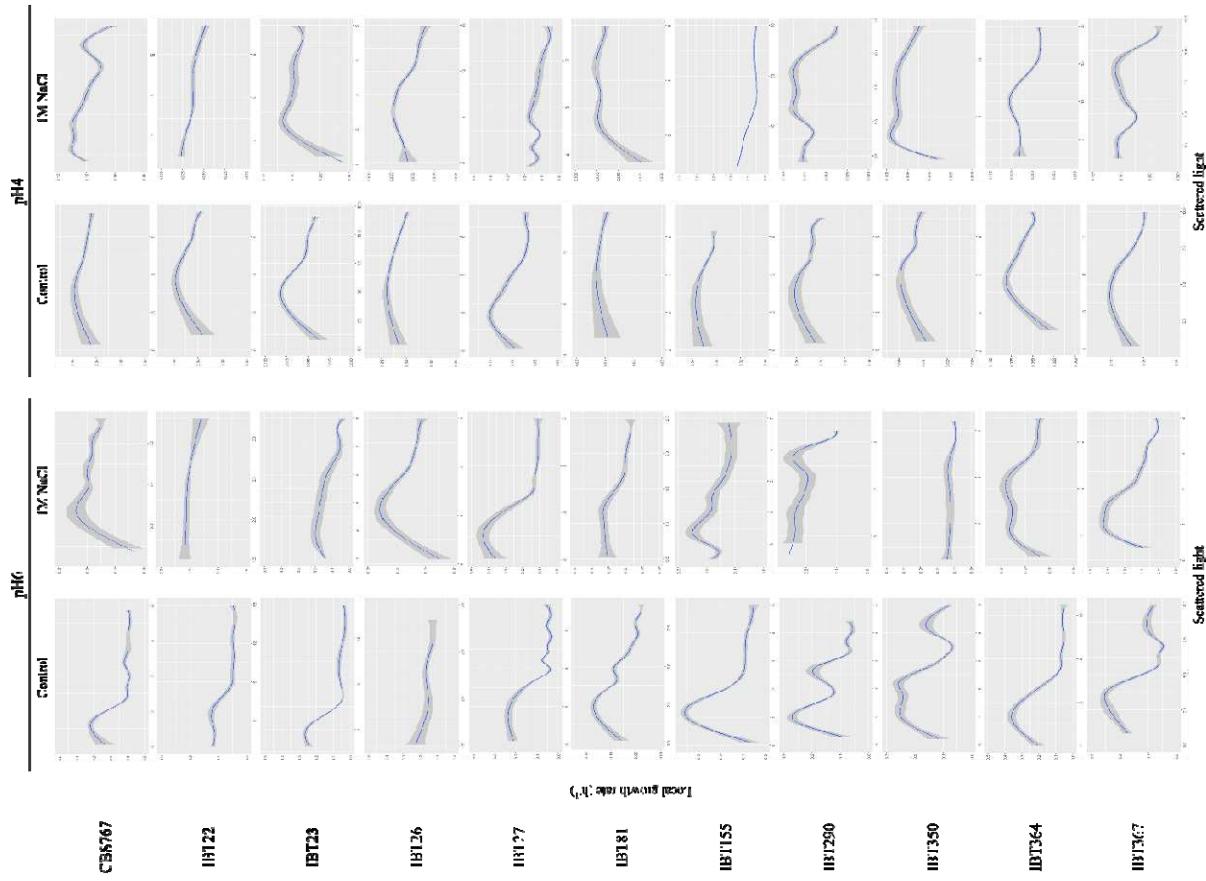


Figure 2. Heat-maps portraying z-scores of *Debaryomyces hansenii* strains (n=61) on 1M NaCl YNB plates (pH 6) including; furfural (1-, 2-, 4 g/L), hydroxymethyl furfural (HMF) (0.5-, 1-, 2 g/L), methanol (1-, 2-, 4% (v/v)), and vanillin (0.1-, 0.2-, 0.4 g/L). Colony size of the corresponding *D. hansenii* strains on YNB plates (pH 6) including the aforementioned inhibitors constitute the plate mean. Heat-maps were obtained after 48h of cultivation using the PhenoBooth instrument and software. Differences in colony size are illustrated as a result of estimated z-score between the control plates (no sodium added) and plates containing inhibitors. Position A1 corresponds to *D. hansenii* CBS767 (a full map of all positions and strains can be found in Supplementary Table 1. A purple to yellow spectra corresponds to negative to positive z-scores and hence, worse to better capability to grow in the studied conditions.

Table 2. Maximum specific growth rates (μ_{max} (h⁻¹)) from micro-fermentations of selected *Debaryomyces hansenii* strains. Values were determined by light scattering units (LSU) obtained from batch cultivations in complete synthetic medium containing 2% glucose at 28 °C and with initial pH of 4 or 6 (without pH regulation). (n=2).

Strain	Condition	pH6 μ_{max} (h ⁻¹)	pH6 (1M NaCl) μ_{max} (h ⁻¹)	pH4 μ_{max} (h ⁻¹)	pH4 (1M NaCl) μ_{max} (h ⁻¹)
CBS767		0.226	0.231	0.060±0.009	0.102
IBT22		0.126±0.029	0.130±0.021	0.049±0.003	0.050±0.006
IBT23		0.210±0.020	0.187±0.031	0.081±0.001	0.086±0.018
IBT26		0.146±0.019	0.227±0.018	0.064±0.025	0.069±0.017
IBT27		0.308±0.036	0.412±0.029	0.131±0.010	0.155±0.014
IBT81		0.097±	0.243±0.024	0.042	0.054±0.010
IBT155		0.299±0.006	0.146±0.073	0.017	0.027±0.001
IBT290		0.238±0.001	0.195±0.010	0.015±0.002	0.082±0.003
IBT350		0.251±0.020	0.145±0.043	0.032±0.001	0.059
IBT364		0.353±0.010	0.278±0.038	0.013±0.002	0.072±0.004
IBT367		0.271±0.010	0.385±0.023	0.075±0.012	0.091±0.010
IBT427		0.203±0.026	0.180±0.008	0.099±0.004	0.141
IBT444		0.280±0.034	0.302	0.071±0.001	0.074±0.004
IBT447		0.151	0.249±0.004	0.051±0.004	0.061
IBT449		0.234±0.062	0.235±0.008	0.070±0.007	0.084±0.001
IBT493		0.238±0.002	0.184±0.002	0.085±0.002	0.085±0.009
IBT510		0.135±0.022	0.059±0.019	0.049	0.070
IBT600		0.139±0.034	0.213±0.007	0.048±0.011	0.060
IBT608		0.107±0.025	0.087±0.010	0.054±0.011	0.062
IBT627		0.299±0.004	0.231±0.041	0.077±0.007	0.088±0.021



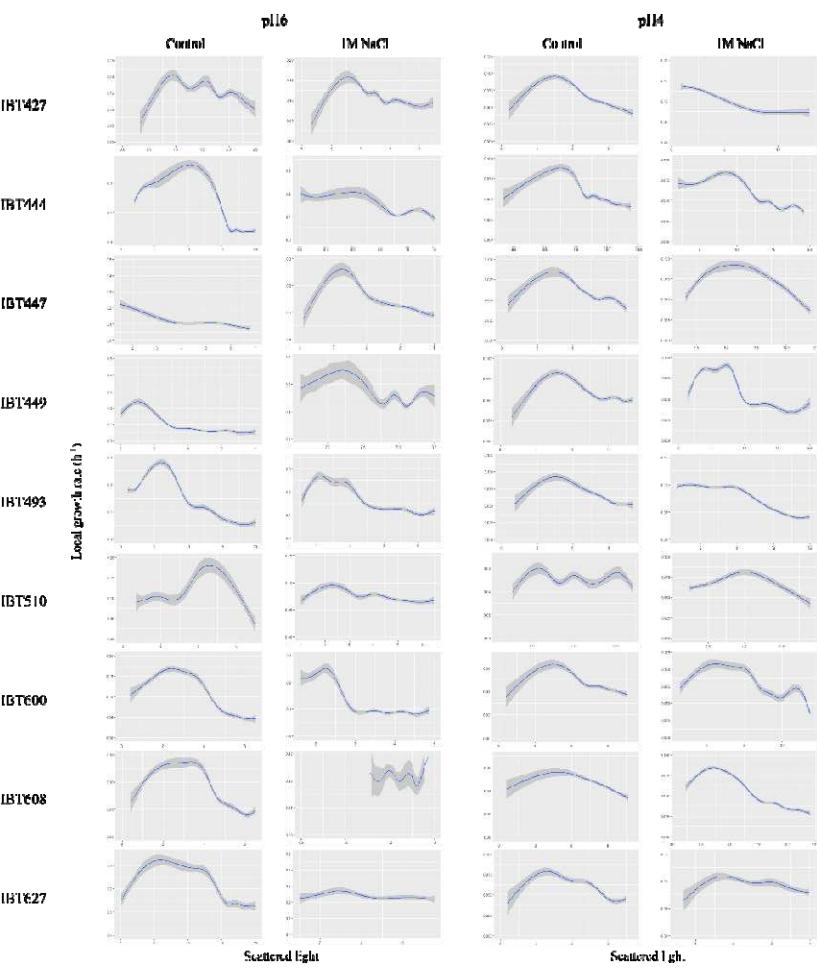


Figure 3. Local growth rates for *Debaryomyces hansenii* strains in YNB medium (pH 6 and pH 4) supplemented with 1M NaCl. Local growth rates were measured throughout the course of the micro-fermentation experiments. Smoothed conditional means were calculated using locally estimated scatterplot smoothing. Light grey areas represent confidence interval of 0.95 ($n = 2$).

able 1

	1	2	3	4	5	6	7	8
A	CBS767	IBT600	IBT104	IBT512	IBT553	IBT425	IBT307	IBT351
B	IBT284	IBT608	IBT155	IBT508	IBT554	IBT427	IBT332	IBT481
C	IBT286	IBT293	IBT22	IBT298	IBT352	IBT428	IBT445	IBT288
D	IBT287	IBT294	IBT23	IBT299	IBT355	IBT430	IBT447	IBT290
E	IBT482	IBT296	IBT26	IBT462	IBT364	IBT441	IBT449	IBT292
F	IBT493	IBT297	IBT627	IBT472	IBT367	IBT444	IBT459	
G	IBT494	IBT81	IBT510	IBT513	IBT390	IBT300	IBT340	
H	IBT507	IBT27	IBT511	IBT514	IBT423	IBT301	IBT350	