bioRxiv preprint doi: https://doi.org/10.1101/2020.01.10.901843; this version posted January 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

A Physiological Characterization in Controlled Bioreactors Reveals a Novel Survival Strategy for *Debaryomyces hansenii* at High Salinity and Confirms its Halophilic Behavior.

Clara Navarrete[1], August T. Frost[1], Laura Ramos-Moreno[2] and José L. Martínez[1*]

[1]Technical University of Denmark, Department of Biotechnology and Biomedicine, Søltofts Plads Building 223, 2800 Kgs. Lyngby, Denmark

[2]University of Córdoba, Department of Microbiology, Campus de Rabanales, Edificio Severo Ochoa, 14071 Córdoba, Spain

*Corresponding author: Section of Synthetic Biology (DTU Bioengineering), Department of Biotechnology and Biomedicine, Technical University of Denmark, Søltofts Plads Building 223, 2800 Kgs. Lyngby, Denmark. Tel: +45 45 25 26 30; E-mail: jlmr@dtu.dk

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

Abstract

Debaryomyces hansenii is traditionally described as a halotolerant non-conventional yeast, being the model organism for the study of osmo- and salt tolerance mechanisms in eukaryotic systems for the past 30 years.

However, unraveling 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 controversy about the diversity in the culture conditions (media composition, temperature and pH among others) used by different groups, which makes it complicated when trying to get significant conclusions and behavioral patterns.

In this work, we present for the first time a complete physiological characterization of *D. hansenii* in batch cultivations, in highly instrumented and 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 ability to tolerate high Na^+ and K^+ concentrations. Finally, the controversial halophilic/halotolerant character of this yeast is further clarified.

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 Artic glaciers (Norkrans 1966; Breuer and Harms 2006; Gunde-Cimerman *et al*. 2009). *D. hansenii* has been a model for the

bioRxiv preprint doi: https://doi.org/10.1101/2020.01.10.901843; this version posted January 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

37 study of osmo- and salt tolerance mechanisms in eukaryotic cells over the last 30 years (Adler *et*
38 *al*. 1985; Prista *et al*. 1997, 2005). Potassium and sodium are crucial factors for yeast growth in
39 high salt environments, and its halotolerant nature has been fully confirmed by the fact that the
40 presence of sodium in the medium protects the yeast cells against oxidative stress and additional
41 abiotic stresses like extreme pH or high temperature (Almagro *et al*. 2000; Papouskova and
42 Sychrova 2007; Navarrete *et al*. 2009).

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

50 As mentioned before, potassium and sodium fluxes (and their accumulation in cell organelles)
51 play a key role in ion homeostasis and halotolerance in *D. hansenii*. Genes for K^+ influx
52 (*DhTRK1*, *DhHAK1*) were identified and studied at a molecular, transcriptional and protein level
53 by different authors (Prista *et al*. 2007; Martinez *et al*. 2011). Two different plasma membrane
54 cation efflux systems (DhEna1/2, DhNha1) have also been described (Almagro *et al*. 2001;
55 Velkova and Sychrova 2006) as well as two intracellular Na^+/H^+ antiporters (DhKha1 and
56 DhNhx1) (Garcia-Salcedo *et al*. 2007; Montiel and Ramos 2007). Very importantly, the role of
57 glycerol production and accumulation in the osmotic stress response to high osmolarity was also
58 fully established by Gustafsson and Norkrans in 1976, and further confirmed by Adler *et al*. in
59 1985.

60 From a biotechnological point of view, *D. hansenii* is considered of great interest, due to its salt
61 tolerant character (Gadanho *et al*. 2003; Butinar *et al*. 2005; Prista *et al*. 2005; Ramos *et al*. 2017
62 among others), especially in food industry where it is used in the ripening process of sausages by
63 production of exopeptidases, development of flavor characteristics, and production of cheeses
64 among others (Lopez Del Castillo-Lozano *et al*. 2007; Cano-Garcia *et al*. 2014). Moreover, *D.*
65 *hansenii* is known to respire a broad range of carbon substrates and produce mycocins against
66 other yeast species, like *Candida* (Banjara *et al*. 2016).

67 Nevertheless, only a few genes with high biotechnological relevance have been characterized so
68 far in *D. hansenii*. It is the case of *DhJEN1*, coding for a monocarboxylic acid transporter (Casal
69 *et al*. 2008), and genes coding for xylitol reductase, xylose dehydrogenase and xylose/H^+
70 transporter (Biswas *et al*. 2013; Ferreira *et al*. 2013), all of them used for the production of
71 second-generation bioethanol in the fermentation of pentoses by *Saccharomyces cerevisiae*
72 (Breuer and Harms 2006).

73 The difficulties when studying *D. hansenii*'s biotechnological potential have always been related
74 to the limitations in the availability of highly efficient molecular tools described for this yeast
75 (Prista *et al*. 2016). There is also a lack of information and full understanding of its carbon
76 metabolism and physiological characterization during biotechnological processes.

bioRxiv preprint doi: https://doi.org/10.1101/2020.01.10.901843; this version posted January 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

77 On top of that, the large variation in culture conditions used (e.g. media composition,
78 temperature, and pH) in previous studies impedes thorough understanding and solid conclusions
79 on this peculiar yeast's behavior. To date, nobody has conducted an in-depth physiological
80 analysis of this yeast in a controlled environment (e.g. using bioreactors), and there is a lack of
81 consensus about its capacity to produce ethanol in high saline environments or its cell
82 performance in the presence of high salt concentrations. While some studies point to a benefitial
83 role of salts on *D. hansenii's* performance (Almagro *et al*. 2000; Papouskova *et al*. 2007;
84 Navarrete *et al*. 2009; Garcia-Neto *et al*. 2017), other claim that sodium is detrimental in terms
85 for the fitness of this yeast in general (Capusoni *et al*. 2019; Sánchez *et al*. 2018). In addition, the
86 osmo- and halotolerant terminology are often used wrongly, as there are some studies in which
87 sodium is used for both purposes, despite there are reported evidences about sodium and
88 potassium triggering a different effect. Potassium does not trigger a toxicity response, whereas
89 sodium has been described to have detrimental effects on cell growth at lower concentrations
90 than potassium, pointing to some authors to recently claim *D. hansenii* not being halophilic but
91 just halotolerant (Sánchez *et al*. 2018), whilst several previous studies report *D. hansenii* as an
92 halophilic yeast (Gonzalez-Hernandez *et al*. 2004; Chao *et al*. 2009; Martinez *et al*. 2011).

93 In relation to *D. hansenii*'s alcoholic fermentation capacity, the kinetics of cell inactivation in the
94 presence of ethanol at 20%, 22.5% and 25% (v/v), have been measured by progressive sampling
95 and viable counting, and used as an inference of the ethanol resistance status of different yeasts
96 (Pina *et al.* 2004). *D. hansenii* PYCC 2968T (a.k.a. CBS 767, which we use in our present study)
97 was found to be one of the most sensitive yeasts to ethanol in this study. Ethanol (or other
98 polyols) production has been only reported when a mix of sugars or complex substrates are used
99 as carbon source. For example, *D. hansenii* CCMI 941 was cultivated in xylose/galactose or
100 xylose/glucose (Tavares *et al.* 2008) for production of xylitol, although only ethanol and glycerol
101 were produced for a xylose/glucose ratio above 30%. Another strain background, *D. hansenii* B-
102 2, was grown in semi-synthetic banana peel-yeast extract-peptone broth for ethanol production,
103 where 40% (w/v) of glucose was fermented to 5.8% of ethanol (Brooks, 2008). In 2009,
104 Calahorra *et al.* stated the activation of glucose fermentation by salts in *D. hansenii* Y7426. In
105 this work, the authors incubated yeast cells in media containing 40mM of glucose, and
106 afterwards they made a protein extraction and measured the ethanol production *in vitro*, by the
107 enzyme extract. Only marginal ethanol production was reported though, within the range of
108 micromoles per gram of glucose. However a most recent work performed by Garcia-Neto *et al.*
109 in 2017, indicates otherwise: that the presence of high salts increases *D. hansenii*'s respiratory
110 activity, in the same strain (Y7426). Overall, no ethanol production by *D. hansenii* has been
111 shown during the growth process using glucose as a sole carbon source.

112 In this work, we present for the first time a complete physiological characterization of *D.*
113 *hansenii* during batch cultivations in highly instrumented and controlled bioreactors. *D.*
114 *hansenii*'s carbon metabolism and the external pH influence on the yeast capacity to tolerate high
115 Na^+ and K^+ concentrations are also shown. Finally, its capability of ethanol production and the
116 controversial halophilic/halotolerant character of this yeast is further discussed, and a novel
117 survival strategy at high saline environments suggested.

bioRxiv preprint doi: https://doi.org/10.1101/2020.01.10.901843; this version posted January 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

118

119 **Materials and Methods**

120 **Strain and culture conditions**

121 The *Debaryomyces hansenii* strain CBS767 (PYCC2968; Prista *et al*. 1997; Navarrete *et al*.
122 2009) was used in this study. Glycerol stocks containing sterile 30% glycerol (Sigma-Aldrich,
123 Germany) were used to maintain the strain, and were preserved at -80□C.

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

131 For the growth curves in low glucose conditions, *D. hansenii* pre-cultures were prepared as
132 specified in the above paragraph. From those pre-cultures, cells were grown in the same medium
133 but with 0.2% of glucose, and with or without 1M/2M of NaCl or KCl, in order to get their
134 growth profile. Initial OD_{600} in the flask was 0.1 and samples were taken during eight days of
135 cultivation at 28°C, 150 rpm.

136 **Bioreactor cultivations**

137 Batch cultivations were performed in biological replicates (between 2-7 per condition) in 1.0 L
138 Biostat Qplus bioreactors (Sartorius Stedim Biotech, Germany). The temperature was controlled
139 at 28°C and pH was maintained at 6.0 (when desired) by the automatic addition of 2M NaOH /
140 2M H_2SO_4, and measured by pH sensors (Model EasyFerm Plus K8 160, Hamilton). The
141 volumetric flow rate (aeration) was set at 1 vvm and the stirring was constant at 600 rpm.
142 Dissolved oxygen concentration was also measured by DO sensors (Model OxyFerm FDA 160,
143 Hamilton). The working volume in the vessel was 0.5 L, using exactly the same medium
144 composition as in the pre-culture, containing either 2% or 0.2% of glucose when required. To
145 study the effect of salt in *D. hansenii* cells, NaCl or KCl (PanReac Applichem, ITW Reagents)
146 were added to the medium before autoclavation. The bioreactors were inoculated with 24 h
147 inoculum from the pre-culture to get an initial OD_{600} of 0.05-0.1. Samples for dry weight, optical
148 density and HPLC were taken after the CO_2 percentage values reached 0.1 and until stationary
149 phase.

150 **Metabolite analysis**

151 The concentrations of glucose, glycerol, acetate and ethanol were measured by High
152 Performance Liquid Chromatography (Model 1100-1200 Series HPLC System, Agilent
153 Technologies, Germany). The injection volume was 20 µl, the eluent 5mM H_2SO_4 and the flow
154 rate was set at 0.6 mL/h. The temperature of a Bio-Rad Aminex HPX-87H column was kept at
155 60°C. A standard solution containing glucose (20 g/L), glycerol (2 g/L), acetate (2 g/L) and

bioRxiv preprint doi: https://doi.org/10.1101/2020.01.10.901843; this version posted January 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

156 ethanol (20 g/L) was used (Sigma-Aldrich, Germany) for exo-metabolites concentration
157 determination.

158 **Off-gas and dissolved oxygen measurements**

159 CO_2 and O_2 concentrations were continuously analyzed in real time by mass spectrometry
160 coupled to the off-gas line (model Prima PRO Process MS, Thermo Scientific, UK). From the
161 off-gas CO_2 emission data, given in percentage, the maximum specific growth rate was
162 calculated. Off-gas CO_2 and O_2 emission data were also used to determine the Carbon Dioxide
163 Evolution Rate (CER), the Oxygen Uptake Rate (OUR) and the Respiratory Quotient (RQ).

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

166 **Analytical procedures**

167 Specific growth rates in the different growth conditions were calculated based on the optical
168 density (OD_{600}) and emmited CO_2 values.

169 Yield coefficients and carbon balances were used to describe the metabolite, by-products and
170 biomass formation by the yeast cells, and were calculated based on the DW, accumulated CO_2
171 and HPLC data. The average minimal formula $CH_{1.79}O_{0.50}N_{0.20}$ for yeast dry cell biomass
172 composition was used for the calculations as proposed by Roels in 1983. The specific glucose
173 consumption rates were calculated based on the logarithmic method proposed in Görgens *et al.*
174 in 2005.

175 **Statistical analysis**

176 Statistical analysis was performed with Microsoft Excel® 2016 (version 1903, 32-bit, USA). All
177 values are represented as averages ± 95% confidence interval of independent biological replicate
178 cultures. For regression analysis, the coefficient of determination (r^2) was used to determine the
179 statistical significance of the fit, where a value above 95% was considered statistically
180 significant.

181

182 **Results**

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

184 Specific growth rates were calculated based on the optical density and the volumetric CO_2-
185 production rates under the different growth conditions tested (Fig. 1). In general terms, a higher
186 maximum specific growth rate was observed when a higher salt concentration is present in the
187 media, except for concentrations of 2M of either salt, compared to control conditions. Sodium
188 exhibit a significantly stronger positive impact than potassium, as the highest growth rate was
189 reached in the presence of 1M NaCl (similar observations were made for KCl, where
190 concentrations of 1M resulted in a higher growth rate compared to control, although lower than
191 for NaCl). On the other hand, a decrease in specific growth rate is observed when the
192 concentration of salts is above 1M, and up to 2M (Fig. 1, table). However, even at concentrations

bioRxiv preprint doi: https://doi.org/10.1101/2020.01.10.901843; this version posted January 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

193 of both sodium and potassium up to 1.5M, the growth rate values are still significantly higher
194 than in control conditions (no salts added). Surprisingly, at 2M NaCl the growth rate is lower,
195 but still very close to control conditions, although a prolonged lag phase is observed in
196 comparison (exponential growth phase starts around 30-35 hours in the control vs. 40-45 hours
197 for 2M NaCl, as inferred from the CO_2 profiles in Fig.1). In contrast, the addition of 2M KCl
198 results in a lower μ_{max} compared to the 2M NaCl and also lower than the control, reinforcing the
199 fact that NaCl still exerts some beneficial effect in *D. hansenii*'s cell performance overall.

200 The same conclusion can be inferred if we observe the carbon dioxide evolution rate (CER) of *D.*
201 *hansenii* in the presence of salts over time (Fig. 1). The profiles show a higher CO_2 production
202 (that can be translated into higher glucose consumption, hence a higher metabolic rate) when the
203 cells grow in the presence of NaCl or KCl compared to control conditions with no salt. Once
204 again, this effect begins to decrease when the salt concentrations are over 1.25M, nevertheless
205 still higher than compared to the control conditions, except for 2M in which the production rate
206 is lower than the control for both salts (Fig. 1).

207 **Biomass yield on substrate and biomass titers show a differential effect among K^+ and Na^+**

208 The observed decrease in the specific growth rate from above 1M of salt seems to be
209 compensated by a slightly higher biomass yield upon glucose, as was observed for the dry weight
210 measurements during the cultivation time, although only for the addition of potassium (Table 1).
211 The final biomass titers were not significantly different though, with the exception of the 2M
212 sodium which were slightly higher in comparison (Suppl. Fig. S1).

213 When the specific glucose consumption rate was calculated, *D. hansenii* showed higher rates of
214 consumption in the presence of NaCl or KCl than in control conditions, and again this effect
215 started to revert once the concentration of salts used was above 1M up to 1.5M, although still
216 higher values than those observed in the control (Table 1), while at 2M NaCl the specific
217 consumption rates were lower than the control. The specific glucose consumption values
218 observed were higher for sodium than for potassium at lower concentrations (1M – 1.25M), as
219 seen before for the specific growth rate, however the opposite is observed at higher
220 concentrations (uptake rates are higher in the presence of potassium).

221 To further investigate the effect of salts on the specific glucose uptake rates, *D. hansenii* was
222 grown in low glucose conditions (0.2%), preliminarily in shake flasks and later by using
223 bioreactors, to further confirm the findings in flasks. In the shake flask experiments, it was
224 observed that cells growing in the presence of 1M KCl adapted much faster to the nutrient
225 limitation and were able to grow at a significantly higher rate compared to the other conditions
226 tested (Suppl. Fig. S2). This effect was also observed in the presence of 1M NaCl, but at a lower
227 level. Moreover, the presence of 2M of either salt in the medium had no detrimental effect for
228 the cells, which still grew at a similar growth rate compared to the control. Strikingly, the
229 presence of 2M of NaCl resulted in almost 3 times higher biomass concentration than the control
230 conditions. In this particular circumstance (high salts and low glucose), the effect of potassium
231 seems to be benefitial in terms of growth rate, but sodium seems to have a better effect in cell
232 performance in the long run, since the growth rate is slower but the final biomass concentration

bioRxiv preprint doi: https://doi.org/10.1101/2020.01.10.901843; this version posted January 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

233 is much higher in return (Suppl. Fig. S2). All together seem to confirm, without a doubt, the
234 halophilic character of *D. hansenii* in one hand, and the differential effect of Na^+ and K^+ salts, in
235 the other.

236 Based on these results in shake-flasks, and in order to obtain a more complete dataset that would
237 support such observations, we then conducted the same type of batch cultures with a reduced
238 initial glucose concentration (0.2%) in bioreactors without pH regulation (thus mimicking the
239 previous flask conditions). Our previous observations in flasks cultivations were confirmed,
240 however interesting differences were observed. The carbon dioxide evolution rates (CER) exhibit
241 a much faster adaptation (Fig. 2A), evidenced by a shorter lag phase, when cells growh in the
242 presence of 1M of either salt (NaCl or KCl), which confirms our observations in flasks. Further
243 analyses showed significantly higher specific glucose uptake rates and, surprisingly, much higher
244 growth rates at 1M of NaCl or KCl, than compared with the rest of the conditions (Fig. 2B). In
245 contrast, CO_2 yields and biomass yields are higher at concentrations of 2M, being 2M NaCl the
246 highest values of all conditions compared. Additional data showing the timeline of DW, OD_{600}
247 and glucose consumption, is shown in Suppl. Fig. S3.

248 As observed in the graph, the CER profiles are very similar between control conditions and cells
249 growing at 2M KCl, only for 2M NaCl the CO_2 production peak seems lower (Fig. 2A), however
250 when we look at the CO_2 yields on glucose, they are higher for both salts at 2M in comparison to
251 1M and also with the control. The biomass yields at 2M are also higher than the 1M
252 concentration, and much higher than the control, as shown in Figure 2B. Altogether, this
253 confirms our observations in the shake flasks, pointing that on reduced glucose conditions, the
254 positive effect of the presence of high salt concentration is even more acused. Moderate levels of
255 salt (1 M) result in higher glucose consumption rates, and higher specific growth rates, while
256 higher concentration of salts (2M) result in higher biomass yields and CO_2 yields, at the cost of a
257 lower growth rate. A metabolim switch that can be described as: from growing as fast as possible
258 to growing as much as possible. We also further confirmed that sodium exert a more positive
259 impact than potassium, once again.

260 It is worth mentioning that the observed final OD values were higher for the control in bioreactor
261 experiments than in shake flasks. Still we observed a higher biomass when cells are growing in
262 2M NaCl, as we also saw in the shake flasks, however cell growth in control conditions was
263 arrested at a lower OD in flasks compared to bioreactors, so the difference in total biomass is less
264 significant for the latter (Suppl. Fig. S2). This is not surprising though, and simply illustrates the
265 importance of using well stirred reactors in physiology studies.

266 **Dissolved oxygen concentration and RQ levels confirm a fully respiratory metabolism,**
267 **discarding a fermentative process in *D. hansenii* in our experimental conditions**

268 Dissolved oxygen levels during bioreactor cultivations were observed to decrease faster when
269 Na^+ or K^+ is present in the medium, being those levels lower while the concentration of salt
270 increases, suggesting a higher oxygen demand (Fig. 3). This confirms a higher metabolic activity
271 at increasing salt concentrations, which again points to the halophilic character of *D. hansenii*,

bioRxiv preprint doi: https://doi.org/10.1101/2020.01.10.901843; this version posted January 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

272 and might already suggest a fully respiratory metabolism, regardless of the presence or absence
273 of (high) salt in the cultivation media.

274 The Respiratory Quotient (RQ) values calculated over the entire course of the culture, show to be
275 below or equal to 1 during the exponential growth phase, but never above this value (Fig. 3), this
276 further supports the absence of fermentation, and therefore confirms that *D. hansenii* is not
277 producing ethanol from glucose in our conditions, not even in the presence of high salts, as
278 reported by Calahorra *et al*. (2009). As a final confirmation of the absence of a fermentative
279 process, our off-gas data and the HPLC analysis show no trace of ethanol neither in the gas phase
280 nor in the liquid broth samples (Table 1).

281 Although it worths mentioning that this previous work (Calahorra *et al.* 2009) was performed
282 using a different *D. hansenii* strain in other culture conditions, and their measurements
283 correspond to ethanol production using enzyme extracts from previously cultured cells, a follow
284 up study performed by García-Neto *et al.* (2017) contradicted such observation using the same
285 strain. Our observations align well with the latter study, confirming that no ethanol production is
286 occurring in our strain either, thus further proving that *D. hansenii* is crabtree negative.

287 **Additional limiting factors affect cell performance in non-controlled cultivation**
288 **environments**

289 In order to test the influence of external pH regulation in the halotolerant / halophilic behavior of
290 *D. hansenii*, parallel bioreactor cultivations in normal glucose (2%) were run, in which no pH
291 control was set, and extracellular pH levels were measured on-line in real time. It was observed
292 that, when no pH control was exerted, the CO_2 profiles of *D. hansenii* evidenced a long
293 mantained plateau phase which cannot be seen in pH-controlled fermentations, both in control
294 conditions and under the effect of salts (Fig. 4). The changes in the external pH over time, for the
295 different conditions tested, are also shown in Supplementary Fig. S4.

296 This led us to determine, that no meaningful conclusion about metabolic patterns or behavior can
297 be made, with a high degree of accuracy, in non-controlled cultivation environments: whatever
298 conclusion made is undoubtedly linked to other limiting factors. This means, that previous
299 studies reporting such conclusions, whose data was obtained from non-controlled environments
300 (such as shake flasks, for example) must be considered cautiously.

301 If we have a look at the maximum specific growth rates under no pH regulation, it can be
302 observed an increase in the μ_{max} when 1M NaCl and KCl are used. This points to a potential
303 summative effect of low pH and high salt concentrations and, once again, sodium seems to have
304 a higher positive impact on growth when compared to potassium. This had been already
305 suggested by Almagro *et al* (2000), so our results further confirm this observation. The specific
306 growth rates decrease once the concentration of both salts is close to 2 M, as also observed in
307 pH-controlled experiments, although this time there is a lower growth rate compared to the
308 control conditions, than when pH control is set in the bioreactors (Table S1 at Supp. material).

309 Interestingly, the previously described plateau in the CO_2 profiles is not observed for non-pH
310 controlled bioreactors run with glucose limiting conditions, where sharper and well defined

bioRxiv preprint doi: https://doi.org/10.1101/2020.01.10.901843; this version posted January 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

311 peaks can be seen in the CO_2 emission profiles (Figure 2A). Here, a faster pH decrease occurs in
312 optimal growth conditions, corresponding to 1M salt added (either NaCl or KCl) to the media
313 (Suppl. Fig. S5), compared to the control. This further supports the summative effect of low pH
314 and increasing salts, to be very beneficial for overall *D. hansenii*'s performance, as already
315 shown in Figure 2B.

316 About the type of limitation that is occuring, and that generates the long-plateau phase seen in
317 the CO_2 emission profiles at normal glucose (2%), and why we do not observe such limitation in
318 limited sugar (0.2%) we cannot elaborate further with the data that we have. This shall need to be
319 addressed by future, and more specific, experimental studies.

320 **Discussion**

321 The results obtained during this study, indicate that high salt concentrations in the culture media
322 are indeed needed for optimal levels of cell performance in *Debaryomyces hansenii*. Especially
323 in the case of NaCl, our results seem to finally confirm the halophilic behavior of this particular
324 yeast: sodium does not exert detrimental effects over *D. hansenii*, but on the contrary. Another
325 interesting finding is that NaCl exhibit a more significant positive impact than KCl, as the
326 optimal growth rates are reached in the presence of 1M of NaCl, closely followed by the values
327 reached at 1M of KCl. This effect is even higher when the glucose source is scarce, as shown in
328 the limited carbon (0.2%) bioreactor experiments.

329 There is, however, a longer lag phase when the yeast are grown in the presence of 2M NaCl,
330 both seen in normal (2%) and limited (0.2%) carbon, which may have led to the previous
331 conclusion by several other studies, performed in non-controlled environments, that the cell
332 growth is affected by high sodium concentrations (Capusoni *et al.*, 2019). Although these
333 previous conclusions can of course be due to the fact that those studies have been performed
334 using different media and another yeast strain different than ours, and in non-controlled
335 conditions (we actually see in our study that 2M salts in non-controlled conditions can indeed
336 result in growth defficiency, as observed in Fig. 4B and 4C), our data additionally show that the
337 decrease in growth rate is not that dramatic at high salts (2M), and moreover, we also observed a
338 slightly higher biomass yield in normal carbon, a much higher biomass yields in nutrient-limiting
339 conditions compared to control conditions, as shown in Figure 2B, that the previous studies did
340 not observe. We propose this as a survival strategy of *D. hansenii* to prevail in drying
341 environments (e.g. during periods of drought). While other microbial species chose, as surviving
342 strategy, to enter a dormant state or sporulate while environmental salinity levels are increasing
343 (as a consequence of the lack of water), *Debaryomyces* reacts by increasing μ_{max} at moderate-
344 high salinity levels (over 1M is already considered toxic for other yeast and bacterial species, so
345 they have difficulties to proliferate (Yan et al., 2015)), hence increasing the glucose uptake rate
346 (as our data reveals) thus ensuring getting the most of the available carbon for its survival in
347 detriment to its competitors, and later when the salt concentration in the media keeps on
348 increasing, slowing down the growth rate while still proliferating to overpopulate the area,
349 changing the metabolic strategy from growing as fast as possible to growing as much as possible.
350 This suggested strategy would be in accordance with the latest publications indicating that

bioRxiv preprint doi: https://doi.org/10.1101/2020.01.10.901843; this version posted January 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

351 *Debaryomyces* are the most represented species in thawing arctic glacier samples and coastal
352 environments (Butinar *et al*. 2011; Jaques *et al*. 2014).

353 The dissolved oxygen levels in presence of higher salt concentration are decreasing faster in the
354 medium, which suggests a higher metabolic activity. RQ values throughout the whole cultivation
355 period remain below or equal at a value of 1 (reached constantly during the exponential phase),
356 clearly supporting that there is no fermentative process, as stated previously. Finally, no ethanol
357 is observed neither in the exometabolite analysis by HPLC, nor in the analysis in the off-gas by
358 MS, therefore *D. hansenii* is herewith confirmed a crabtree negative yeast.

359 To conclude, our data without pH control in the bioreactor vessels indicate additional limiting
360 factors during the cultivation, based on the CO_2 production profiles, compared with pH
361 controlled cultivations, evidenced by a long plateau phase, proving that shake flask experiments
362 with non-controlled environment are not the ideal setup to obtain accurate conclusions about
363 physiological and/or metabolic parameters, therefore we also suggest that previous studies
364 providing conclusions obtained by this means, must be taken cautiously. It is also worth
365 mentioning that it is not appropriate to choose randomly Na^+ salts or K^+ salts in order to study
366 osmotolerance or halotolerance in general, as our results show that there is a clear differential
367 effect exerted by either NaCl or KCl, as already suggested by Martinez *et al*. (2011 and 2012).

368 All in all, our results shed light upon the behaviour of *D. hansenii* in controlled bioreactor
369 conditions, presenting this peculiar non-conventional yeast as a strain which is able to perform
370 very well in "standard" cultivation conditions (no stress added) but whose performance gets
371 significantly improved when environmental conditions get harsh: high salinity / osmotic
372 pressure, media acidification and nutrient scarcity, all in combination. This undoubtedly confers
373 to *D. hansenii* an incredibly strong potential for industrial production setups: those are the
374 conditions that, upon large scale bioproduction processes (meaning vessels of 1000 liter or
375 above) the microbial cells will encounter throughout the cultivation process, and that are limiting
376 the suitable performance of microbes in bioreactors (Takors, R. 2012). Therefore, having such a
377 strain with the abovementioned behavior, and more importantly, obtaining sufficient information
378 for understanding it (and, consequently, being able to take advantage of that knowledge) is of
379 paramount interest for advancing in the field of cell factory design for industrial bioprocesses,
380 and therefore for the biotech industry overall.

381 One of the strongest outcomes of our current study is the possibility of using *D. hansenii* in
382 culture media containing relatively high salt concentrations, for industrial bioprocesses. On one
383 hand, there is no need of using pure water sources, which significantly decreases the production
384 costs as one could take advantage of desalination effluents, or even use directly sea water for the
385 media composition, while still increasing the production yields (salinity will not affect *D.*
386 *hansenii*, but will improve its cell performance, as shown by our research). On the other hand,
387 using saline environments has another strong advantage which is the reduced risk of
388 contaminations, hence sterilization costs would also be significantly reduced.

389

390 **Conclusion**

bioRxiv preprint doi: https://doi.org/10.1101/2020.01.10.901843; this version posted January 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

391 Altogether, our findings reveal the beneficial role of salts, and more particularly sodium, in the
392 cell performance of *Debaryomyces*, and open the need to further investigate how sodium and
393 potassium influence the cell metabolism at a molecular level. It is clear that salts are not just
394 tolerated in *D. hansenii*, but they play a crucial role in its survival strategy, to date
395 underestimated. The presence of salts is needed for optimal cell performance. Further research,
396 including a global expression analysis by RNAseq in steady-state continuous bioreactor
397 cultivations, will shed light upon what are the intracellular mechanisms that trigger such
398 metabolic changes, and how the discrimination between sodium and potassium occurs to trigger
399 the different behavioral patterns described within this study. It will also be interesting from a
400 biotechnological point of view, the identification of molecular elements that could potentially be
401 responsive to the presence of salts, as our observations suggest that there are undoubtedly
402 molecular switches which react to the presence or absence of sodium and/or potassium in the
403 environment, triggering a specific metabolic response.

404

405 **Acknowledgements**

406 We acknowledge the Novo Nordisk Fonden, within the framework of the Fermentation Based
407 Biomanufacturing Initiative, for supporting this work. LRM received the fellowship “Ayuda de
408 Movilidad Internacional para el Fomento de Tesis con Mención Internacional” from the
409 University of Córdoba (Spain), to carry out her research work at DTU Bioengineering for three
410 months. Prof. José Ramos (University of Córdoba, Spain) and Dr. Markus Bisschops (TU Delft,
411 The Netherlands) are deeply acknowledged for critical reading of this manuscript. The authors
412 would also like to thank the Fermentation Core at DTU Bioengineering, and Tina Johansen as
413 well as Martin Nielsen for their technical support.

414

415 **Author’s contributions**

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

419

420 **Competing interests**

421 The authors declare that they have no competing interests.

422

423 **References**

424 Adler L, Blomberg A, Nilsson A. Glycerol metabolism and osmoregulation in the salt-tolerant
425 yeast *Debaryomyces hansenii*. *J Bacteriol* 1985;**162**: 300-06.

bioRxiv preprint doi: https://doi.org/10.1101/2020.01.10.901843; this version posted January 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

426 Almagro A, Prista C, Castro S *et al*. Effects of salt on *Debaryomyces hansenii* and
427 *Saccharomyces cerevisiae* under stress conditions. *Int J Food Microbiol* 2000;**56**: 191-97.

428 Almagro A, Prista C, Benito B *et al*. Cloning and expression of two genes coding for sodium
429 pumps in the salt-tolerant yeast *Debaryomyces hansenii*. *J Bacteriol* 2001;**183**: 3251-55.

430 Banjara N, Nickerson KW, Suhr MJ *et al*. Killer toxin from several food-derived *Debaryomyces*
431 *hansenii* strains effective against pathogenic *Candida* yeasts. *Int J Food Microbiol* 2016;**222**: 23-
432 29.

433 Biswas D, Datt M, Aggarwal M *et al*. Molecular cloning, characterization, and engineering of
434 xylitol dehydrogenase from *Debaryomyces hansenii*. *Appl Microbiol Biotechnol* 2013;**97**: 1613-
435 23.

436 Breuer U, Harms H. *Debaryomyces hansenii* – an extremophilic yeast with biotechnological
437 potential. *Yeast* 2006;**23**: 415-37.

438 Brooks AA. Ethanol production potential of local yeast strains isolated from ripe banana peels.
439 *Afr J Biotechnol* 2008;**7**: 3749-52.

440 Butinar L, Strmole T, Gunde-Cimerman N. Relative incidence of *Ascomycetous* yeasts in Arctic
441 coastal environments. *Microb Eco*l 2011;**61**: 832-43.

442 Butinar L, Zalar P, Frisvad JC *et al*. The genus Eurotium: members of indigenous fungal
443 community in hypersaline waters of salterns. *FEMS Microbiol Ecol* 2005;**51**: 155-66.

444 Calahorra M, Sánchez NS, Peña A. Activation of fermentation by salts in *Debaryomyces*
445 *hansenii*. *FEMS Yeast Res* 2009;**9**: 1293-301.

446 Cano-Garcia L, Belloch C, Flores M. Impact of *Debaryomyces hansenii* strains inoculation on
447 the quality of slow dry-cured fermented sausages. *Meat Sci* 2014;**96**: 1469-77.

448 Capusoni C, Arioli S, Donzella S *et al*. Hyper-osmotic stress elicits membrane depolarization
449 and decreased permeability in halotolerant marine *Debaryomyces hansenii* strains and in
450 *Saccharomyces cerevisiae*. *Front Microbiol* 2019;**10**: 64.

451 Casal M, Paiva S, Queiros O *et al*. Transport of carboxylic acids in yeasts. *FEMS Microbiol Rev*
452 2008;**32**: 974-94.

453 Chao HF, Yen YF, Ku MS. Characterization of a salt-induced DhAHP, a gene coding for an
454 alkyl hydroperoxide reductase, from the extremely halophilic yeast *Debaryomyces hansenii*.
455 *BMC Microbiol* 2009;**9**:182.

456 Dujon B, Sherman D, Fischer G *et al*. Genome evolution in yeasts. *Nature* 2004;**430**: 35-44.

457 Ferreira D, Nobre A, Silva ML *et al*. XYLH encodes a xylose/H^+ symporter from the highly
458 related yeast species *Debaryomyces fabryi* and *Debaryomyces hansenii*. *FEMS Yeast Res*
459 2013;**13**: 585-96.

bioRxiv preprint doi: https://doi.org/10.1101/2020.01.10.901843; this version posted January 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

460 Gadanho M, Almeida JM, Sampaio JP. Assessment of yeast diversity in a marine environment in
461 the south of Portugal by microsatellite-primed PCR. *Antonie Van Leeuwenhoek* 2003;**84**: 217-27.

462 García-Neto W, Cabrera-Orefice A, Uribe-Carvajal S *et al*. High osmolarity environments
463 activate the mitochondrial alternative oxidase in *Debaryomyces hansenii*. *PLOS One* 2017;
464 12(1):e0169621.

465 Garcia-Salcedo R, Montiel V, Calero F *et al*. Characterization of *DhKHA1*, a gene coding for a
466 putative Na(+) transporter from *Debaryomyces hansenii*. *FEMS Yeast Res* 2007;**7**: 905-11.

467 Gonzalez-Hernandez JC, Cardenas-Monroy CA, Peña A. Sodium and Potassium transport in the
468 halophilic yeast *Debaryomyces hansenii*. *Yeast* 2004;**21**:403-412.

469 Görgens JF, Van Zyl WH, Knoetze JH. Reliability of methods for the determination of specific
470 substrate consumption rates in batch culture. *Biochem Eng J* 2005;**25**: 109-12.

471 Gunde-Cimerman N, Ramos J, Plemenitas A. Halotolerant and halophilic fungi. *Mycol Res*
472 2009;**113**: 1231-41.

473 Gustafsson L, Norkrans B. On the mechanism of salt tolerance: production of glycerol and heat
474 during growth of *Debaryomyces hansenii*. *Arch Microbiol* 1976;**110**: 177-83.

475 Jacques N, Zenouche A, Gunde-Cimerman N et al. Increased diversity in the genus
476 *Debaryomyces* from Arctic glaciers samples. *Antonie Van Leeuwenhoek* 2015;**107**: 487-501.

477 Lopez Del Castillo-Lozano M, Delile A, Spinnler HE *et al*. Comparison of volatile sulphur
478 compound production by cheese-ripening yeasts from methionine and methionine–cysteine
479 mixtures. *Appl Microbiol Biotechnol* 2007;**75**: 1447-54.

480 Martinez JL, Sychrova H, Ramos J. Monovalent cations regulate expression and activity of the
481 Hak1 potassium transporter in *Debaryomyces hansenii*. *Fungal Genet Biol* 2011;**48**: 177-84.

482 Martinez JL, Luna C, Ramos J. Proteomic changes in response to potassium starvation in the
483 extremophilic yeast *Debaryomyces hansenii*. *FEMS Yeast Res* 2012;**12**: 651-61.

484 Montiel V, Ramos J. Intracellular Na and K distribution in *Debaryomyces hansenii*: cloning and
485 expression in *Saccharomyces cerevisiae* of *DhNHX1*. *FEMS Yeast Res* 2007;**7**: 102-09.

486 Navarrete C, Siles A, Martínez JL *et al*. Oxidative stress sensitivity in *Debaryomyces hansenii*.
487 *FEMS Yeast Res* 2009;**9**: 582-90.

488 Norkrans B. Studies on marine occurring yeasts: growth related to pH, NaCl concentration and
489 temperature. *Arch Mikrobiol* 1966;**54**: 374-92.

490 Papouskova K, Sychrova H. The co-action of osmotic and high temperature stresses results in a
491 growth improvement of *Debaryomyces hansenii* cells. *Int J Food Microbiol* 2007;**118**: 1-7.

492 Pina C, Couto JA, Hogg T. Inferring ethanol tolerance of *Saccharomyces* and non-
493 *Saccharomyces* yeasts by progressive inactivation. *Biotechnol Lett* 2004;**26**: 1521-27.

bioRxiv preprint doi: https://doi.org/10.1101/2020.01.10.901843; this version posted January 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

494 Prista C, Almagro A, Loureiro-Dias MC *et al*. Physiological basis for the high salt tolerance of
495 *Debaryomyces hansenii*. *Appl Environ Microbiol* 1997;**63**: 4005-09.

496 Prista C, Loureiro-Dias MC, Montiel V *et al*. Mechanisms underlying the halotolerant way of
497 *Debaryomyces hansenii*. *FEMS Yeast Res* 2005;**5**: 693-701.

498 Prista C, Gonzalez-Hernandez JC, Ramos J *et al*. Cloning and characterization of two K^+
499 transporters of *Debaryomyces hansenii*. *Microbiology* 2007;**153**: 3034-43.

500 Prista C, Michán C, Miranda IM *et al*. The halotolerant *Debaryomyces hansenii*, the Cinderella
501 of non-conventional yeasts. *Yeast*. 2016;**33**: 523-33.

502 Ramos J, Melero Y, Ramos-Moreno L *et al*. *Debaryomyces hansenii* strains from Valle de Los
503 Pedroches iberian dry meat products: isolation, identification, characterization, and selection for
504 starter cultures. *J Microbiol Biotechnol* 2017;**27**:1576-85.

505 Roels JA. Energetics and kinetics in biotechnology. Amsterdam; New York: Elsevier Biomedical
506 Press, 1983, 1-330.

507 Sánchez NS, Calahorra M, Ramírez J *et al*. Salinity and high pH affect energy pathways and
508 growth in *Debaryomyces hansenii*. *Fungal Biol* 2018;**122**: 977-90.

509 Takors R. Scale-up of microbial processes: impacts, tools and open questions. *J Biotechnol*
510 2012;**160**:3-9.

511 Tavares JM, Duarte LC, Amaral-Collaco MT *et al.* The influence of hexoses addition on the
512 fermentation of D-xylose in *Debaryomyces hansenii* under continuous cultivation. *Enzyme*
513 *Microb Technol* 2000;**26**: 743-47.

514 Velkova K, Sychrova H. The *Debaryomyces hansenii NHA1* gene encodes a plasma membrane
515 alkali-metal-cation antiporter with broad substrate specificity. *Gene* 2006;**369**: 27-34.

516 Yan N, Marschner P, Cao W *et al*. Influence of salinity and water content on soil
517 microorganisms. *Int Soil and Water Conservation Research* 2015;**3**:316-323.

518

519

520

521

522

523

524

525

526

bioRxiv preprint doi: https://doi.org/10.1101/2020.01.10.901843; this version posted January 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

527 **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	0.98±0.01	0.120±0.004
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	0.36	ns	ns	0.99	0.098±0.014
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
2M KCl	0.66±0.14	0.37±0.08	ns	ns	1.02±0.21	0.138±0.001

528 Yield coefficients from batch cultivations of *Debaryomyces hansenii* at different salt concentrations
529 are shown in the table. Additionally, the specific glucose consumption rates during the exponential
530 phase calculated based on the logarithmic method are presented. The cells were grown in
531 synthetic complete medium with or without salt at different concentrations, at 28°C and pH 6.
532 Data shown are mean values ± 95% confidence interval of a number of replicates.
533 [1]Yield of CO_2 (C), Biomass (X), Ethanol (E), and Glycerol (G) from Glucose (S) in cmol/cmol.
534 [2]Carbon balance on a C-mol basis, where a value of 1 indicate a closed carbon balance.
535 [3]Specific Glucose consumption rate in cmol/cmol/h based on logarithmic method.
536 [4]ns: Not significant. The amount of ethanol and glycerol was neglectable.
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552

bioRxiv preprint doi: https://doi.org/10.1101/2020.01.10.901843; this version posted January 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

553 **Figure legends**

554

555 **Figure 1.** Maximum specific growth rates and carbon dioxide evolution rate (CER) profiles from
556 batch cultivations with sodium (**A**) or potassium (**B**) chloride. Maximum specific growth rates
557 determined by optical density (OD) and off-gas CO_2 emission data from batch cultivations of
558 *Debaryomyces hansenii* in synthetic complete media at 28°C and pH 6 with varying
559 concentrations of sodium/potassium chloride are shown in the tables. The CER profiles are based
560 on off-gas CO_2 emission data over time from one replicate for each condition and are
561 representative of their associated replicate(s).

562 **Figure 2.** Carbon dioxide evolution rate (CER) profiles and yield coefficients, specific glucose
563 consumption and maximum specific growth rates from batch cultivations under limiting glucose
564 conditions and without pH regulation. Off-gas CO_2 emission data from batch cultivations of
565 *Debaryomyces hansenii* in synthetic complete media containing 0.2% of glucose at 28°C and
566 initial pH value of 6 with varying concentrations of sodium/potassium chloride are shown in the
567 figure (**A**). The CER profiles are based on off-gas CO_2 emission data over time from one
568 replicate for each condition and are representative of their associated duplicate. Yield
569 coefficients and maximum specific growth rates from batch cultivations of *Debaryomyces*
570 *hansenii* at different salt concentrations are shown in the table. Additionally, the specific glucose
571 consumption rates during the exponential phase calculated based on the logarithmic method are
572 presented (**B**). The cells were grown in synthetic complete medium containing 0.2% of glucose
573 and with or without salt at different concentrations, at 28°C and initial pH of 6. Data shown are
574 mean values ± 95% confidence interval of duplicates.

575 **Figure 3.** Dissolved oxygen (DO) and respiratory quotient (RQ) profiles from batch cultivations
576 with sodium (**A**) or potassium (**B**) chloride. Dissolved oxygen (%) levels measured over time (h)
577 in synthetic complete media at 28°C and pH 6 with varying concentrations of sodium/potassium
578 chloride, are represented in the figure. The DO profiles are based on one replicate for each
579 condition and are representative of their associated replicate(s). The RQ profiles are based on
580 OUR and CER values calculated from off-gas O_2 consumption and off-gas CO_2 emission data
581 from one replicate for each condition and are representative of their associated replicate(s).

582 **Figure 4.** Graphical comparison of CER profiles with and without pH regulation for batch
583 cultivations with varying concentrations of salts. The CER profiles are based on off-gas CO_2
584 emission data over time from one replicate for each condition and are representative of their
585 associated replicate(s). The cells were grown in synthetic complete media at 28°C with and
586 without pH regulation (pH 6 with regulation). The cells were either grown without salts (**A**),
587 NaCl (**B**) or KCl (**C**).

Figure 1

A)

Maximum specific growth rates (μ_{max}).

Conditions	μ_{max} - OD[1]	μ_{max} - CO_2[1]
Control	0.103±0.006	0.105±0.004
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

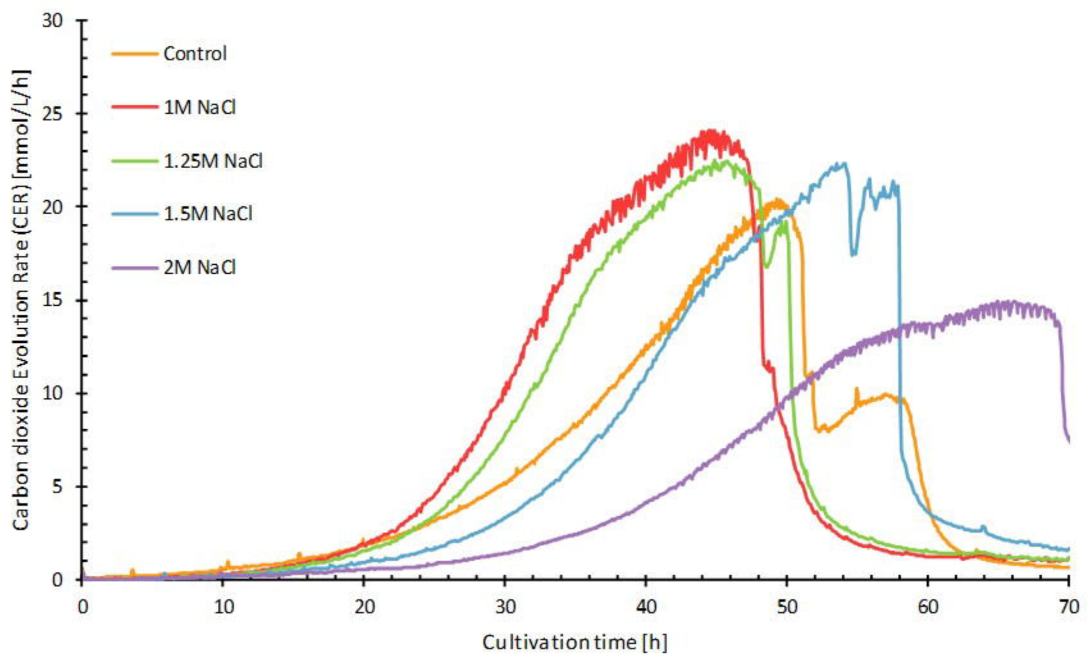
Note: Data shown are mean values ± 95% confidence interval of a number of replicates.
[1] The maximum specific growth rates (h^{-1}) on glucose determined by OD_{600} and off-gas CO_2.

B)

Maximum specific growth rates (μ_{max}).

Conditions	μ_{max} - OD[1]	μ_{max} - CO_2[1]
Control	0.103±0.006	0.105±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 OD_{600} and off-gas CO_2.



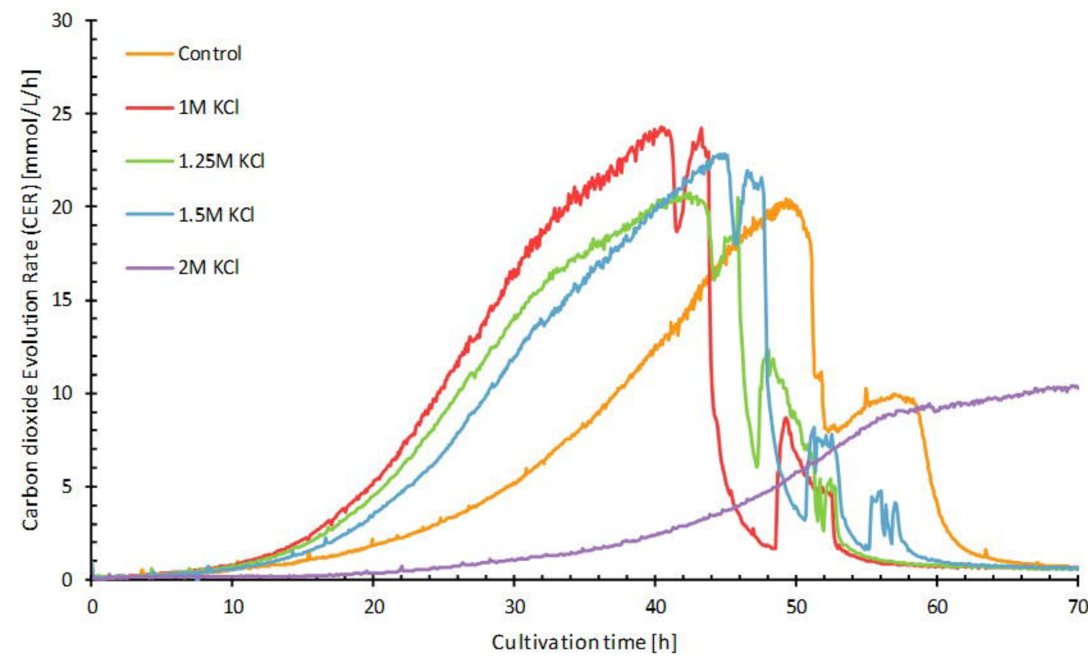
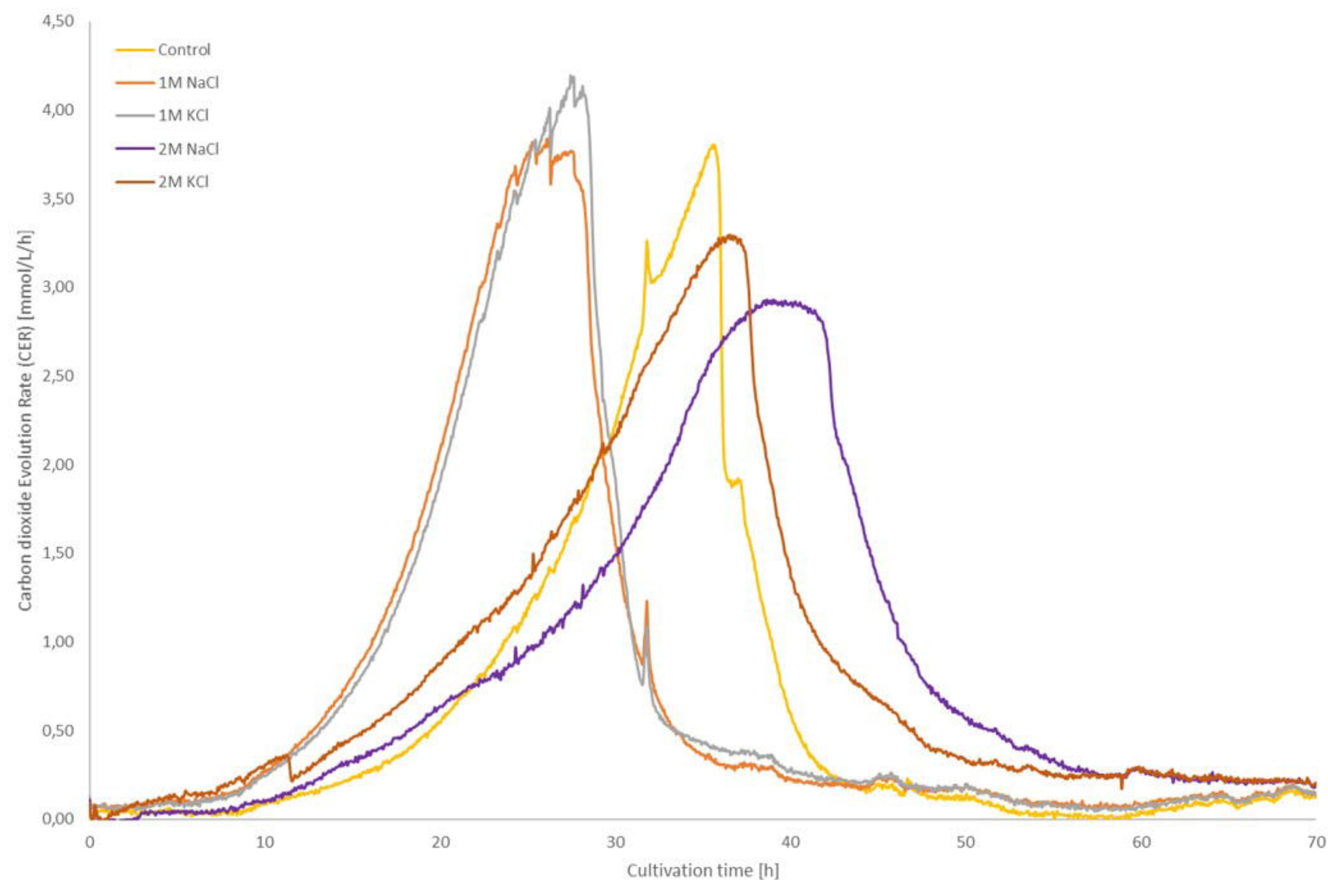


Figure 2

A)



B)

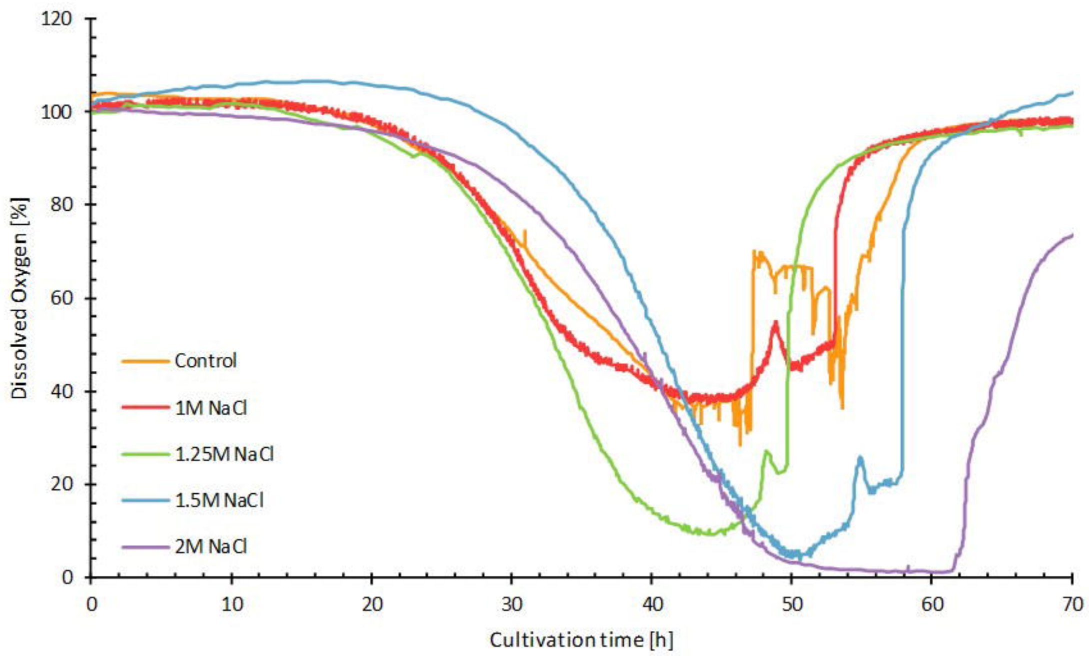
Yield coefficients, specific glucose consumption and maximum specific growth rates from batch cultivations in glucose limiting conditions and without pH regulation.

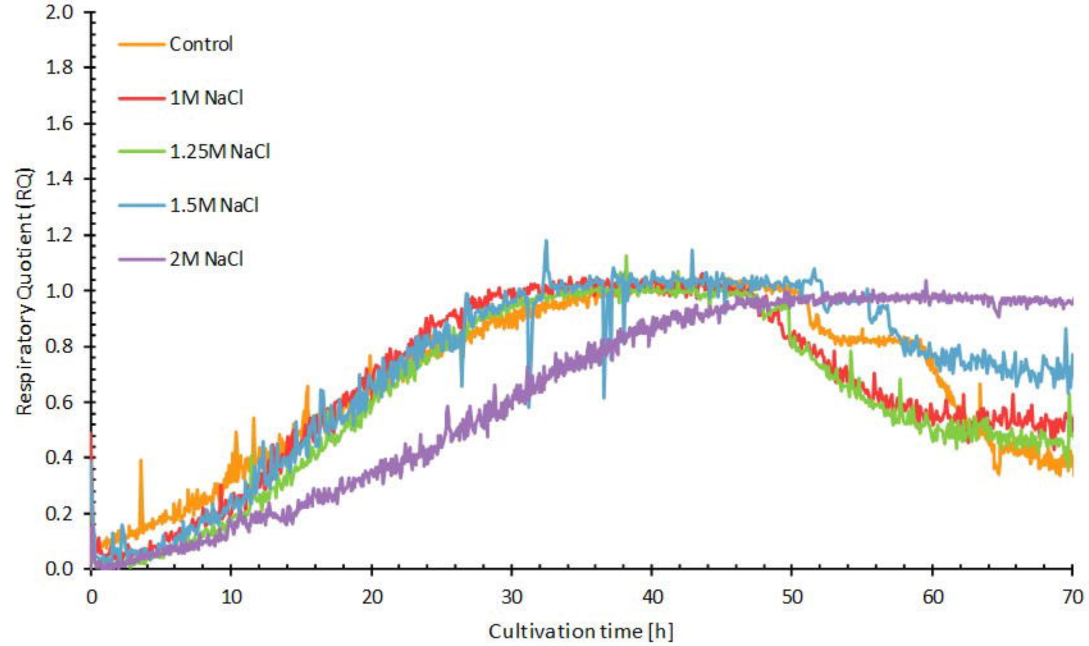
Conditions	Y_{SC}[1]	Y_{SX}[1]	SGCR (r_s)[2]	μ_{max}[3]
Control	0.37±0.06	0.21±0.03	0.16±0.02	0.177±0.024
1M NaCl	0.53±0.03	0.30±0.02	0.38±0.13	0.214±0.008
1M KCl	0.51±0.07	0.29±0.04	0.46±0.03	0.214±0.005
2M NaCl	0.61±0.03	0.34±0.02	0.13±0.01	0.200±0.009
2M KCl	0.65±0.05	0.36±0.03	0.14±0.00	0.167±0.028

[1] Yield of CO_2 (C) and Biomass (X) from Glucose (S) in cmol/cmol.
[2] Specific Glucose consumption rate in cmol/cmol/h based on logarithmic method.
[3] The maximum specific growth rates (h^{-1}) on glucose determined by off-gas CO_2.

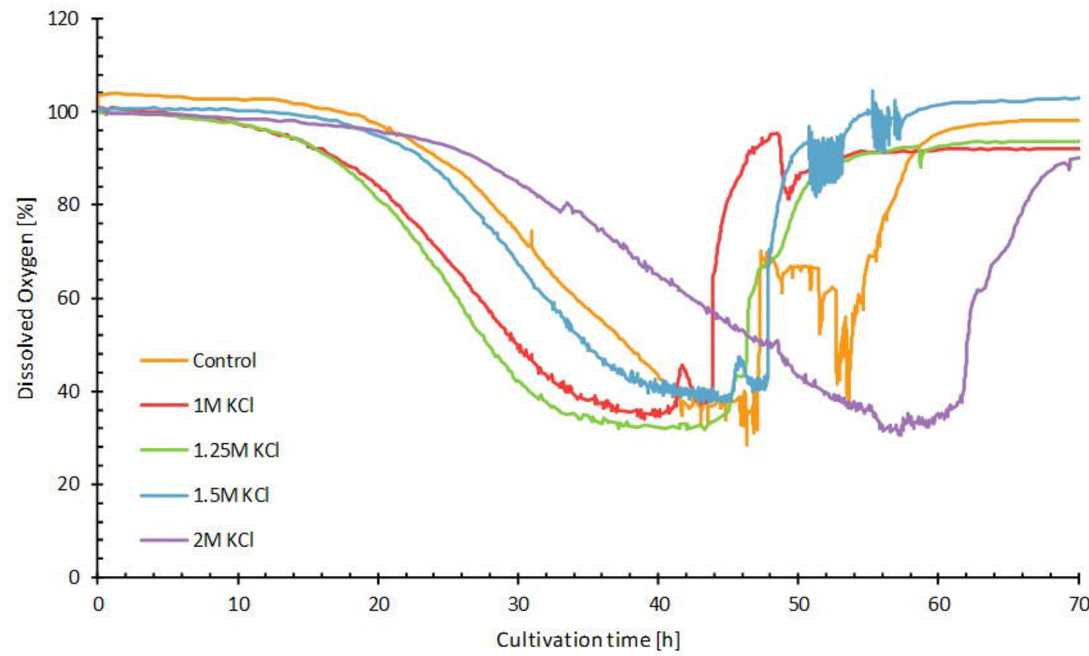
Figure 3

A)





B)



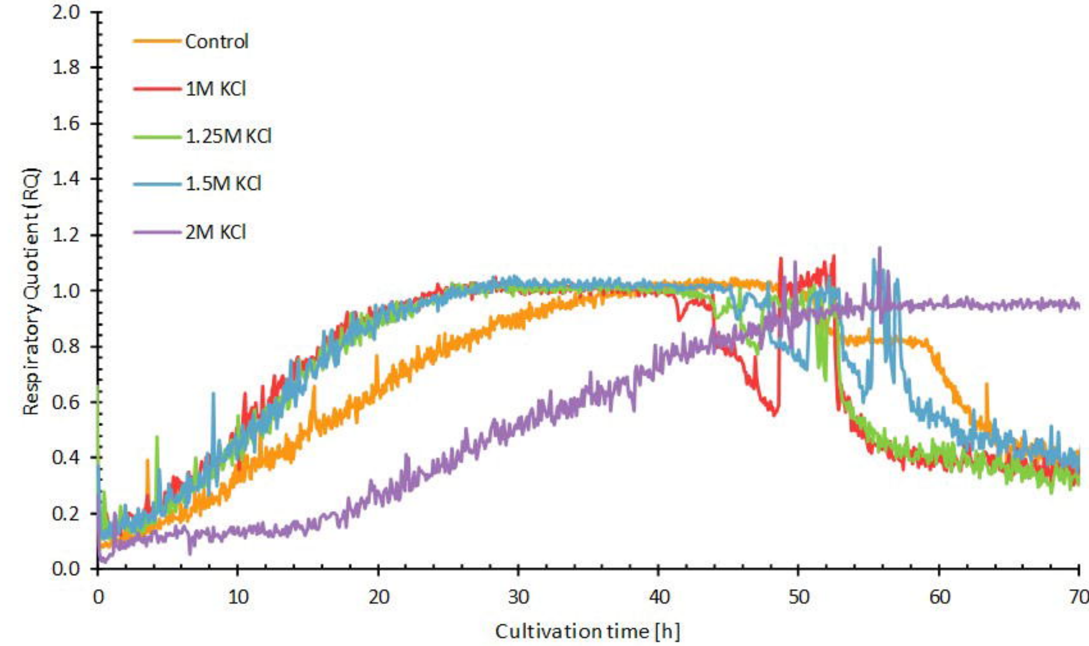
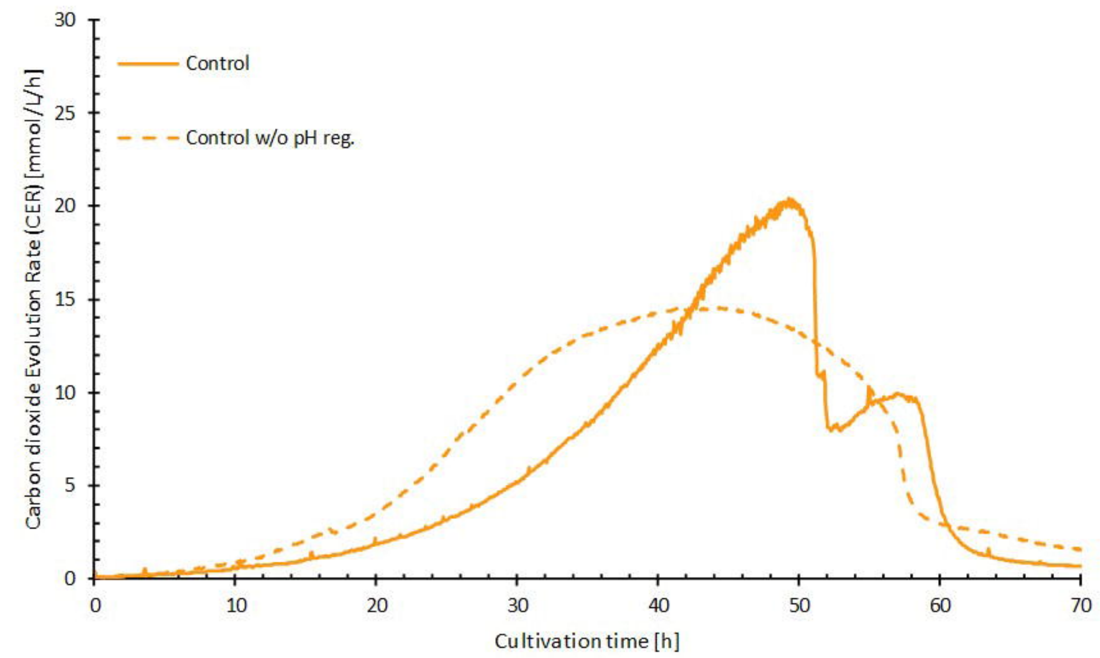
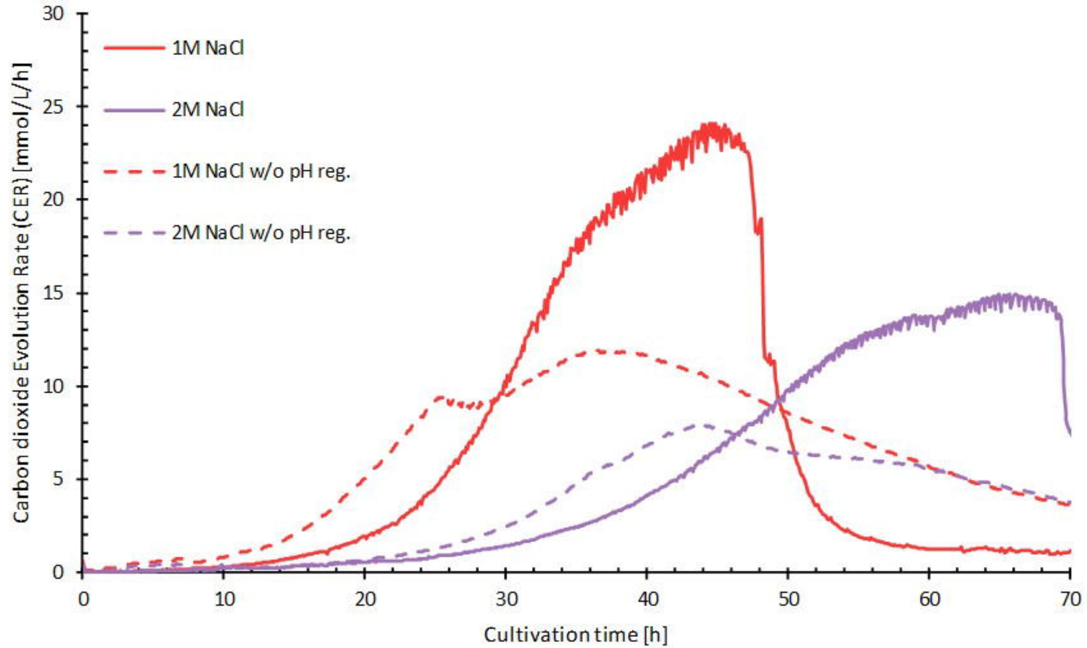


Figure 4

A)



B)



C)