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1 Accelerostat study in conventional and microfluidic bioreactors to assess the key role of
2 residual glucose in the dimorphic transition of *Yarrowia lipolytica* in response to
3 environmental stimuli

4

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13

14

15 **Abstract**

16 *Yarrowia lipolytica*, with a diverse array of biotechnological applications, is able to grow as
17 ovoid yeasts or filamentous hyphae depending on environmental conditions. This study has
18 explored the relationship between residual glucose levels and dimorphism in *Y. lipolytica*.
19 Under pH stress conditions, the morphological and physiological characteristics of the yeast
20 were examined during well-controlled accelerostat cultures using both a 1L-laboratory scale
21 and a 1mL-microfluidic bioreactor. The accelerostat mode, via a smooth increase of dilution
22 rate (D), enabled the cell growth rate to increase gradually up to the cell wash-out ($D \geq \mu_{\max}$
23 of the strain), which was accompanied by a progressive increase in residual glucose
24 concentration. The results showed that *Y. lipolytica* maintained an ovoid morphology when
25 residual glucose concentration was below a threshold value of around 0.35-0.37mg L⁻¹.
26 Transitions towards more elongated forms were triggered at this threshold and progressively
27 intensified with the increase in residual glucose levels. The effect of cAMP on the dimorphic
28 transition was assessed by the exogenous addition of cAMP and the quantification of its
29 intracellular levels during the accelerostat. cAMP has been reported to be an important
30 mediator of environmental stimuli that inhibit filamentous growth in *Y. lipolytica* by
31 activating the cAMP-PKA regulatory pathway. It was confirmed that the exogenous addition
32 of cAMP inhibited the mycelial morphology of *Y. lipolytica*, even with glucose concentrations
33 exceeding the threshold level. The results suggest that dimorphic responses in *Y. lipolytica* are
34 regulated by sugar signaling pathways, most likely via the cAMP-PKA dependent pathway.

35

36 **Keywords:** accelerostat; residual glucose; cAMP; dimorphic transition; microfluidic
37 bioreactor; *Yarrowia lipolytica*

38

39 **ABBREVIATIONS**

40 cAMP, cyclic adenosine monophosphate; cAMP-PKA, cAMP-dependent protein kinase;
41 cFDA, carboxyfluorescein diacetate; D, dilution rate (h⁻¹); DO, dissolved oxygen; DCW, dry
42 cell weight; HPLC, haute performance liquid chromatography; HPIC, haute performance
43 ionic chromatography; MAPK, mitogen-activated protein kinase; PDMS,
44 polydimethylsiloxane; PI, propidium iodide

45 **Introduction**

46 The yeast *Yarrowia lipolytica* has generated considerable interest for biotechnological
47 applications due to both its versatility towards carbon source utilization [1-5] and its
48 proficiency in producing a broad spectrum of valuable metabolites [6-11]. Nevertheless, *Y.*
49 *lipolytica* is known to undergo metabolic and dimorphic transitions in response to
50 environmental fluctuations which can lead to difficulties in scale up of bioprocesses [12].

51 In previous work [13, 14], the dynamic behavior of *Y. lipolytica* was described in response to
52 pH and dissolved oxygen (DO) fluctuations in well-controlled bioreactor cultures. It was
53 demonstrated that in batch culture, *Y. lipolytica* undergoes dimorphic transition in response to
54 both pH and DO fluctuations. In contrast, *Y. lipolytica* maintains its yeast-like form (ovoid) in
55 chemostat culture conditions at pH7 at all tested dilution rates (from 0.03 h⁻¹ to 0.20 h⁻¹), and
56 also at pH5.6 with fluctuations at pH7 [14]. In the case of DO perturbations, chemostat
57 cultures with anoxic periods and at 2 % DO (0.15 mg L⁻¹ DO concentration), did not engender
58 mycelial transition. However, filamentation was observed under conditions where limiting O₂
59 transfer provided only 80% of the cell requirement in the presence of a residual glucose
60 excess. In this particular condition, the system switched from a glucose-limited to an O₂ -
61 limited chemostat culture with an increase in residual glucose concentration and the onset of
62 filamentation [13]. This data suggested a possible impact of residual glucose level on the
63 signaling pathways regulating dimorphic responses in *Y. lipolytica*, but a delayed effect of the
64 onset of O₂ limitation could not be completely ruled out. Indeed, among the rare studies
65 carried out under well-controlled conditions, the effect of a low DO concentration (< 0.13 mg
66 L⁻¹) was evaluated under chemostat at 0.032h⁻¹ dilution rate and observed filamentous cells
67 [15] . On increasing the DO concentration, a transition to yeast-like cells was observed. From
68 their results, the authors concluded that there was a direct link between DO limitation and
69 dimorphic transition [15]. As previously described [13], their study was carried out under
70 lipid-producing conditions (N- limitation) and not during the biomass propagation phase. No
71 information on the residual concentration of the C-source was provided, making comparison
72 between the two studies difficult.

73 It is established that regulation of the dimorphic transition in *Y. lipolytica* is based on the
74 signal transduction pathways involving both mitogen-activated protein kinase (MAPK) and
75 the cyclic-AMP dependent protein kinase A (cAMP-PKA) [16-18]. These pathways operate
76 in opposite directions during the yeast-to-mycelium transition: the MAPK pathway is needed

77 for mycelial growth while the PKA pathway is required for growth in the yeast-like form [16-
78 18]. Specifically, increasing intracellular cAMP levels inhibited the mycelial growth of *Y.*
79 *lipolytica* [18, 19]. The cAMP concentration can be increased either by the activation of
80 adenylate cyclase or by the entry of exogenous nucleotides into the cell [18]. Several genes
81 involved in dimorphism have been isolated and characterized, including the Rho family
82 among others. These genes are not only involved in dimorphism, but also in a variety of other
83 cellular activities, such as cell wall organization and biogenesis and membrane trafficking
84 [16, 20-22]. Proteins implicated in the yeast-to-mycelium transition have also been identified
85 and characterized in depth recently in order to unravel the regulatory mechanisms involved in
86 the dimorphic shift [23].

87 However, to date, a potential relationship between the level of residual glucose and the
88 regulation of the dimorphic transition in *Y. lipolytica* has not been reported and the link
89 between glucose signaling and morphogenesis has only been deciphered for the pathogenic
90 *Candida albicans* [24, 25] and *Saccharomyces cerevisiae* [26, 27]. The glucose-sensing and -
91 signaling mechanisms in yeasts have been well-described, but mainly for *S. cerevisiae*,
92 *Kluyveromyces lactis* and *C. albicans* [28-36], where glucose uptake is a complex process
93 involving different types of transporters and multiple parallel signaling pathways. Three
94 different types of glucose signaling pathways are involved, each playing a distinctive but
95 interacting role: (i) the Rgt2/Snf3 glucose induction pathway, (ii) the Snf1/Mig1, glucose
96 repression pathway, and (iii) the Ras-cAMP-activated kinase (PKA) pathway. Depending on
97 the amount of glucose present in the medium, specific transporters would be expressed and
98 specific signaling pathways induced or repressed. However, in *Y. lipolytica*, sugar
99 assimilation is still poorly understood with only a recent study focusing on the
100 characterization of hexose transporters [37].

101 Here, the impact of residual glucose concentrations on the induction of the dimorphic
102 transition in response to pH stress has been investigated. In order to modulate the residual
103 glucose concentration under stress conditions, well-controlled accelerostat approaches using a
104 conventional lab-scale reactor and a microfluidic reactor were implemented. The accelerostat
105 strategy was chosen in order to increase gradually the residual glucose concentration in the
106 medium as the dilution rate approached the maximum specific growth rate of the strain (μ_{\max}).
107 In addition, the role of cAMP was investigated based on the quantification of intracellular
108 cAMP and the continuous feeding of cAMP during the accelerostat cultures. The dynamic

109 behavior of *Y. lipolytica* based on quantitative physiological and morphological
110 characterization under accelerostat conditions is reported.

111 **Materials and Methods**

112 **Microorganism, media and growth conditions**

113 The strain used was the wild-type *Y. lipolytica* W29 (ATCC® 20460™). Culture conditions
114 and medium composition were performed as previously reported [14]. When necessary,
115 cAMP sodium salt (Sigma-Aldrich, Saint-Quentin Fallavier, France) was dissolved in water,
116 sterilized by filtration and added to the sterile media at a concentration of 25 mM.

117 **Laboratory-scale 1L bioreactor cultures**

118 Batch, glucose-limited continuous and accelerostat cultures were performed in a 1.6 L
119 stainless-steel stirred tank bioreactor with a working volume of 1 L (BIOSTAT® Bplus,
120 Sartorius, Germany) (**Figure 1A**). Reactor equipment and configuration, as well as inoculum
121 preparation steps were as previously described [14]. The temperature was regulated at 28°C
122 and the pH at 5.6 and pH6.5 by addition of 2M KOH (VWR Chemicals, Fontenay-sous-Bois,
123 France). The antifoam polypropylene glycol (PPG) (Sigma-Aldrich, France) was added
124 periodically (pulse-based addition) to maintain a nearly constant concentration (1 mL L⁻¹) in
125 the bioreactor.

126 **Microfluidic 1 mL bioreactor cultures**

127 Perfused, glucose-limited continuous and accelerostat cultures were performed in single-use 1
128 mL microbioreactor chips (Pharyx Inc., Woburn, MA, USA) (Figure 1B). Detailed
129 description of the design is provided in previous reports [38-40]. The chips were sterilized by
130 γ -radiation (14 KGy). The medium bottles and feed lines were autoclaved separately. The
131 microreactor was equipped with optical density, dissolved oxygen (DO), pH and temperature
132 probes. The growth chamber comprised three interconnected 500 μ L sections, of which only
133 two were full at any time to ensure both the 1000 μ L working volume and the mixing. Gas
134 exchange was ensured by gaseous diffusion across the polydimethylsiloxane (PDMS)
135 membrane. Heating was performed at the base of the device using a resistive heating element.
136 Control and monitoring were performed using MBS_Dashboard software package (Pharyx
137 Inc., Woburn, MA, USA).

138 Inoculum cultures were prepared as previously described previously [13, 14]. 1 mL of diluted
139 inoculum (5% v/v) was directly injected inside the empty chamber. Temperature was
140 regulated at 28°C. pH was maintained at the set-point (pH5.6 and 6.5) and regulated by
141 addition of 1mM NaHCO₃ (Sigma-Aldrich, France) via peristaltic metering valves. Samples
142 for offline analysis were collected via one output port connected to the growth chamber.

143 **Continuous cultivations: chemostat and accelerostat**

144 Continuous cultures were initiated either by batch (1L-bioreactor) or perfusion (microfluidic
145 bioreactor) in order to reach the suitable biomass concentration ($\approx 5\text{ g L}^{-1}$). Transitions to
146 continuous mode were carried out at dilution rates (D) of 0.12 h⁻¹ and 0.15 h⁻¹ for the 1L-
147 laboratory scale bioreactor and the 1mL-microfluidic bioreactor, respectively. Steady-state
148 phases were considered as reached after at least 5 residence times and then characterized
149 during two further residence times.

150 After characterization of the steady-state phase (D 0.12 h⁻¹/0.15 h⁻¹, pH5.6), the pH was
151 adjusted to 6.5, because at pH7 the medium exhibited slight precipitation. Although the
152 presence of mineral crystals was not an issue in the conventional lab-scale bioreactor, it could
153 be a critical point in the microfluidic device. Indeed, the feed and sampling lines are very thin
154 (1.6 mm internal diameter) and are susceptible to clogging by crystals during fermentation.
155 When the steady-state at pH6.5 and at dilution rate D 0.12 h⁻¹/ 0.15h⁻¹ was reached and
156 characterized, the accelerostat phase was launched with an acceleration factor of 0.0025 h⁻¹
157 from D 0.12/ 0.15 to 0.25 h⁻¹ (linear increase of dilution rate). Samples were characterized
158 along the steady state and accelerostat phases.

159 For the study regarding its role, cAMP was added directly in the medium feed solution to a
160 final concentration of 25mM.

161 **Biomass characterization**

162 *Biomass concentration*

163 For the 1L bioreactor experiments, the biomass concentration was quantified by
164 spectrophotometric (OD_{620nm}) and dry weight measurements, following the protocol described
165 [14]. For the 1mL microfluidic bioreactor experiments, biomass concentration was quantified
166 spectrophotometrically (OD_{600nm} and OD_{620nm}) using a Nanodrop 1000 spectrophotometer,

167 (ThermoFisher Scientific, Nanodrop Products, Courtaboeuf, France). This particular
168 instrument has the ability to measure a sample of 1 or 2µl and the pathlength was set at 1mm.

169 *Cell viability and morphology*

170 Cell viability was assessed by flow cytometry following the protocol described previously
171 [14]. Cell morphology was assessed by flow cytometry, morphogranulometry and light
172 microscopy as described [14].

173 **Sugar and organic acid analysis by high-performance liquid chromatography (HPLC)** 174 **and ionic chromatography (HPIC)**

175 During batch, and perfused phases, glucose and organic acid (acetate, pyruvate, succinate and
176 citrate) concentrations were determined by HPLC as described [14]. Under continuous mode,
177 quantification of glucose and organic acids (acetate, pyruvate, succinate, malate, fumarate and
178 citrate) present at low concentrations in the broth, was carried out by HPIC. All procedures
179 and details of these apparatus were followed according to previously described methods [14].

180 **Cyclic AMP quantification**

181 During chemostat and accelerostat, intracellular cAMP was quantified using the Cyclic AMP
182 Competitive ELISA Kit (Invitrogen, ThermoFisher, Courtaboeuf, France). The acetylated
183 version of the protocol was followed with regard to the intracellular cAMP concentration
184 range encountered. Cell lysates were obtained from samples containing 10^6 cells mL⁻¹ treated
185 with 0.1 M HCl (VWR Chemicals, France) as described in the kit protocol.

186 **Gas analysis and monitoring**

187 The online analysis of the inlet and outlet gas compositions for the 1L bioreactor cultivations
188 was performed as described [14]. For the 1mL microfluidic bioreactor, gas analysis was not
189 possible (limit of detection of the equipment).

190 **Calculations**

191 All the calculations (off-gas rates, glucose consumption and biomass production rates and the
192 analysis of cell size distribution at the population level) are described in detail in previously
193 published work [14].

194 **Results**

195 Chemostat – Accelerostat cultures in the 1L lab-scale bioreactor

196 In order to assess the role of the residual glucose concentration on the onset of the stress
197 response of *Y. lipolytica*, a coupled chemostat/accelerostat approach was implemented.
198 Chemostat cultures were carried out at a selected dilution rate (0.12 h^{-1}) in order to stabilize
199 and characterize the behavior of the cells placed in a steady state. At steady state, the
200 environment being constant, the entire cell population grew at a constant growth rate and
201 exhibited the same physiological state. Subsequently, the accelerostat approach implemented
202 was to gradually increase the cell growth rate up (via the dilution rate) until reaching the cell
203 wash-out ($D \geq \mu_{\text{max}}$ of the strain), which consequently led to a gradual increase in glucose
204 availability for cultured cells.

205 In previous work [14], it was shown that the dilution rate in the tested range ($0.03, 0.07, 0.10$
206 and 0.20 h^{-1}) had no impact on the pH stress response of *Y. lipolytica* at pH7. Indeed, under
207 well-controlled chemostat culture at pH7, no filamentation was observed whatever the
208 dilution rate tested, indicating that the growth rate of the cells was not the effector of the
209 dimorphic transition observed during the pH7-batch bioreactor or pH7-pulses batch
210 bioreactor. Thus, the current study was carried out at only one dilution rate (0.12 h^{-1}).

211 *Chemostat in steady state as a reference*

212 For the chemostat phase, the steady state was considered to be achieved after a period of at
213 least 5 residence times, and was validated by a constant production of biomass and a stable
214 composition of the exhaust gases. Characterization of the steady state at pH6.5 was carried
215 out by taking up at least 7 samples within a period of 2 to 3 residence times. Evolutions of
216 biomass and residual glucose concentrations, as well as changes in pH and DO during this
217 phase are illustrated in **Figure 2**. Constant production of biomass ($\approx 4.6 \pm 0.2 \text{ g L}^{-1}$) and
218 stable composition of the exhaust gases ($19.44 \pm 0.04 \% \text{ O}_2$, $1.43 \pm 0.01 \% \text{ CO}_2$ / data not
219 shown) were detected which revealed the stability of the steady state. In addition, negligible
220 amounts of residual glucose ($< 5 \text{ mg L}^{-1}$) were quantified in the culture broth, thus confirming
221 the C-limited growth. An O_2 unlimited condition was maintained throughout the chemostat
222 experiments with a DO concentration always $> 40\%$. These results were similar to that
223 previously determined during chemostat cultures of *Y. lipolytica* at pH7 and dilution rate of
224 0.1 h^{-1} [14].

225 Regarding the macroscopic behavior at the global population scale, specific consumption and
226 production rates, biomass yields as well as C and elemental recoveries were calculated from
227 raw data and reported in **Table 1**. Comparing to results obtained in our previous work [13],
228 the same range of magnitude was obtained, the slight difference being due to the 20%
229 increase of the dilution rate. No production of organic acids was observed. The mean residual
230 glucose concentration was lower than 5mg L^{-1} (Figure 2). Respiratory quotients were around
231 1.1, reflecting the conservation of a fully oxidative metabolism.

232 As previously described [13, 14], the steady state was also characterized at the subpopulation
233 level via cytometry, microscopy and morpho-granulometry measurements. This work
234 confirmed that cells in steady state at pH6.5 were perfectly ovoid-shaped with a unimodal size
235 distribution and a mean cell diameter of about $4.23\pm 0.23\ \mu\text{m}$. In addition, the viability
236 assessed either by cFDA/PI or cFDA/Sytox double staining methods was maintained $>97\%$.

237 *Accelerostat*

238 After stabilization and characterization of the steady state, *Y. lipolytica* behavior under
239 glucose-limited chemostat at μ and $D = 0.12\ \text{h}^{-1}$, the accelerostat approach was implemented
240 to progressively increase the dilution rate up to reaching the cell wash out, which
241 consequently would lead to the increase of residual glucose concentration in the bioreactor.

242 In order to understand the dynamics of morphological changes of *Y. lipolytica* in response to
243 increasing residual glucose concentration, profiles of cell size distribution were analysed
244 regularly during the course of the accelerostat. The width signal of the forward scatter light
245 (FSC), measured by flow cytometry, was used to discriminate subpopulations of different
246 sizes within the culture broth. Number size distributions (**Figure 3A**), based on cell length
247 measurements were determined during the time course of fermentation, and data were
248 displayed as box plots (Figure 3B) illustrating the dispersion and size difference between
249 samples.

250 As shown in Figures 2 and 3, up to a critical dilution rate (D_{crit}) of about $0.19\ \text{h}^{-1}$, where a
251 clear dimorphic transition could be observed, both the macroscopic and microscopic
252 behaviors of cells were similar to those described under steady state conditions. As expected,
253 the increase in the dilution rate above the critical value D_{crit} led to a gradual decrease in the
254 biomass concentration from 4.5 to 3g CDW L^{-1} , which consequently led to an increase in
255 residual glucose from 5mg L^{-1} to 2.5g L^{-1} in the culture broth. At D_{crit} , the residual glucose

256 concentration was about 0.35g L⁻¹. No organic acids were detected, and cell viability was
257 always >97%. Similarly, for the chemostat, an unlimited O₂ condition was maintained for the
258 entire course of the accelerostat (DO concentration always >40%).

259

260 Regarding cell morphology, above the dilution rate of 0.19 h⁻¹, a dispersion of the cell length
261 was observed with a gradual increase in the spread of the distribution of the FSC-Width
262 signal. The time of flight across the laser beam of 95% of the cells increased from 75 to 145
263 during the accelerostat, while remaining stable around 75 at steady state chemostat and in the
264 accelerostat at D below 0.18 h⁻¹ (Figure 3B). This result was confirmed by microscopic
265 observations in real time (Figure 3C). While no filamentation was previously observed at
266 pH7 in glucose-limited chemostat at dilution rates between 0.03 and 0.20 h⁻¹ [14], here it has
267 been possible to generate dimorphic transition from yeast to filamentous forms under
268 accelerostat mode at pH7 and under unlimited O₂ condition by increasing the glucose
269 residual concentration.

270 **Chemostat – accelerostat cultures in the 1ml micro-scale bioreactor**

271 A link between cAMP and filamentation has already been described for *Y. lipolytica* [16-19].
272 In order to identify a more complex interaction between residual glucose concentration,
273 cAMP and dimorphic transition under pH stress conditions, the chemostat-accelerostat
274 approach was carried out using a well-controlled microfluidic bioreactor to be able to
275 continuously supplement with cAMP.

276

277 This set of experiments was divided into 4 phases: (i) the steady state chemostat at D = 0.15 h⁻¹
278 ¹, (ii) the accelerostat from 0.15 h⁻¹ to 0.25 h⁻¹ without cAMP, (iii) a second steady state
279 chemostat at D = 0.15 h⁻¹ (data not shown), (iiii) the accelerostat from 0.15 h⁻¹ to 0.25 h⁻¹ with
280 cAMP. Due to the small volume of the bioreactor and in order to not destabilize the system,
281 only 100μL samples were taken at each time point in order to measure residual glucose and
282 analyze biomass (morphology and viability). pH, DO concentration, and biomass were also
283 on-line monitored (**Figure 4**). As with the 1L-lab scale glucose-limited chemostat culture, a
284 steady state was reached with a stable biomass of about 5 gDW L⁻¹, a stable non-limiting DO
285 concentration (>40%), and a stable residual glucose concentration <1 mg L⁻¹ (Figure 4).

286 Viability was always >97 % and yeast-like cells were largely predominant with a mean
287 diameter of $4.44 \pm 0.10 \mu\text{m}$.

288 In order to be able to run the complete experiment with the same reactor cassette and to avoid
289 clogging of withdrawal lines with filamentous cells (in the absence of cAMP), the accelerostat
290 with cAMP was carried out first. As shown in **Figure 5**, in the course of the accelerostat,
291 while biomass concentration was decreasing and residual concentration increasing, the yeast
292 cells retained their yeast-like form with an average diameter of about $4.76 \pm 0.34 \mu\text{m}$ (Figure
293 5A). The time of flight across the laser of 95% of cells was stable at around 80 during the
294 course of the accelerostat (Figure 5B). In contrast, the accelerostat, performed without
295 supplementation of cAMP in the medium, activated dimorphic transition for a residual
296 glucose concentration of about 0.37 g L^{-1} obtained at a D_{crit} of about 0.20 h^{-1} . This D_{crit} value
297 is quite consistent with the results obtained with the 1L bioreactor setup. Without cAMP, a
298 larger size distribution can be observed in the box plot (Figure 4A). To prevent clogging the
299 microfluidic cassette, the filamentation was kept at a lower level (by running shorter time
300 cultivation) than that observed in the lab-scale bioreactor (Figure 5C). In both accelerostat
301 conditions with and without cAMP, the DO concentration was maintained at ~40%, ensuring
302 unlimited O_2 conditions.

303 **cAMP quantification during accelerostat**

304 Quantification of cAMP was carried out in order to evaluate the intracellular level and to
305 compare it between the chemostats and two accelerostat conditions (with or without
306 supplementation of exogenous cAMP in the medium). The results show that under chemostat
307 (steady-state) and accelerostat conditions without cAMP, the intracellular cAMP
308 concentration was below the level of detection of the competitive immunoassay ELISA kit
309 (**Figure 6**). In contrast, it tended to increase progressively during the accelerostat indicating
310 that cAMP was able to enter the cells and consequently may have played a crucial role in
311 inhibiting the dimorphic transition in response to pH stress under glucose excess condition.

312 **Discussion**

313 The objective of this study was to further elucidate the hypothesis of a potential relationship
314 between the level of residual glucose and the dimorphic transition regulation. In order to
315 modulate the residual glucose concentration under stress conditions, well-controlled
316 accelerostat approaches using both classical lab-scale reactor and microfluidic reactor were

317 implemented. In addition, the role of cAMP was evaluated based on the quantification of
318 intracellular cAMP and the continuous feeding of cAMP during the accelerostat cultures.
319 Dynamic behavior of *Y. lipolytica* based on quantitative physiological and morphological
320 characterizations under accelerostat condition with or without cAMP allowed support for this
321 hypothesis.

322 In a previous study [14], it was shown that *Y. lipolytica*, considered a model yeast strain for
323 dimorphic transition studies, was able to trigger or not filamentation in response to pH stress
324 depending on the mode of cultivation implemented. Specifically, in batch bioreactors where
325 cells proliferated at their maximum growth rate, mycelia were mainly formed (up to 93%
326 (v/v) at pH7; whereas, in continuous cultures, at controlled growth rates (from 0.03 to 0.20 h⁻¹
327 ¹) even close to the maximum growth rate of the strain (0.24 h⁻¹), only ovoid cell forms were
328 observed. In order to determine whether this behavior was the same under a different stressor
329 (different level of DO concentration), similar experiments have been reported [13]. This set of
330 experiments confirmed that morphological responses of *Y. lipolytica* to various DO levels
331 were also different between batch and chemostat [13]. More specifically, it was suggested that
332 the level of residual glucose in the culture broth might have an impact on the signaling
333 pathways regulating dimorphic transition in *Y. lipolytica*, as the same phenomenon with both
334 stressors pH and DO was observed [13, 14].

335 The mechanism of regulation of dimorphic transition of *Y. lipolytica* has been investigated by
336 others [16-18,22,23,41-48]. Those investigations have been mainly carried out in non-
337 controlled batch mode using test-tubes and Erlenmeyer flask cultures except in [47], where
338 chemostat cultures were implemented. Regulation of the dimorphic transition in *Y. lipolytica*
339 was identified as based on the operation of the MAPK and PKA signaling pathways as for
340 other fungi such as *S. cerevisiae*, *C. albicans*, *K. Marxianus*, *U. maydis* [49, 50]. However, for
341 *Y. lipolytica*, these pathways were shown to operate in opposition during the yeast-to-
342 mycelium transition [45]. The MAPK cascade is involved in mycelial growth whereas an
343 activated PKA pathway is required for growth in the yeast-like form. When inactive, PKA is
344 composed of a heterotetramer of two catalytic subunits (cPKA) attached to a dimer of
345 regulatory PKA subunit (rPKA). When the concentration of intracellular cAMP increases,
346 two molecules of cAMP bind to each rPKa subunit, releasing the catalytic subunit (cPKA)
347 that is then able to phosphorylate target proteins on serine or threonine residues. The
348 intracellular increase in cAMP can be caused either by adenylate cyclase activation or by
349 entry of the exogenous nucleotides into the cell [16-18].

350 Several genes and proteins have been identified implementing an easier target approach such
351 as specific gene deletion and insertion [16,20,22,45] or global approaches such as proteomic
352 [23] and transcriptomic [45,47] approaches. Based on forward genetic screen and whole-
353 genome sequencing, genes involved in MAPK signaling pathway such as transcription factor
354 *Ylmsn2*, the histidine kinase *Ylchk1* and *Ylnik1* as well as the MAP kinase of the of the GOG
355 (high-osmolarity glycerol response) (*Ylssk2*, *Ylpbs2*, and *Ylhog1*) were identified [47].
356 Furthermore, they have shown that overexpression of either *Ylmbp1* or *Ylswi6* decreased
357 hyphal growth and deletion of *Ylmbp1* or *Ylswi6* promoted hyphal growth. Nevertheless,
358 despite those molecular studies, the mechanism and effectors of regulation remains unraveled
359 in *Y. lipolytica* unlike for the other yeasts [49].

360 In addition, the link between glucose sensing pathway and signaling pathway involved in the
361 dimorphic transition have been clearly identified for *S. cerevisiae* [26], *C. albicans* [27, 51,
362 52] and *K. marxianus*. Indeed, different glucose signal pathways are involved depending on
363 the level of glucose in the medium. A simplified scheme has been proposed depicting the
364 sensing and signaling components involved in the induction of the pseudo-hyphal growth by
365 hexose [26]. Two pathways were proposed with glucose as substrate: activation of cAMP-
366 PKA pathway leading to filamentation, and activation of a Glucose/Repression-Induction
367 signaling pathway that would also trigger filamentation via a specific regulator Snf1.
368 Nevertheless, there is no information concerning the level of residual glucose needed for the
369 induction of either pathway. Earlier, it was concluded from another study, that dimorphic
370 transition in *C. albicans* was not regulated by the pH and that glucose or its metabolites may
371 play an important role [51]. Such a result has also been confirmed by [52], showing that
372 glucose starvation led to filamentation under whatever pH condition (neutral or acidic),
373 whereas for unstarved culture (glucose in excess), filamentation was observed only at pH6.7
374 [52].

375 No such link between glucose signaling pathway and dimorphic transition signaling pathway
376 has been described in literature for *Y. lipolytica*. Others noticed that a *Y. lipolytica* Δ tpk1
377 mutant, *tpk1* coding for the PKA catalytic subunit, showed growth problems when galactose
378 was used as carbon source suggesting a role for PKA in galactose metabolism, although the
379 level of action of PKA remains unknown [16]. Furthermore, there is no description in the
380 literature of the glucose signaling pathway in *Y. lipolytica*. In a recent article, concerning
381 metabolism of alternative substrate metabolism in *Y. lipolytica*, the sugar transporters and
382 mechanism of regulation were discussed based on genome analysis [37] and Blast search, and

383 it was concluded that the mechanism of regulation is more divergent from those seen in *S.*
384 *cerevisiae*, but without further explanation [53]. Until now, the mechanism of regulation of
385 glucose sensing has remained unclear in *Y. lipolytica* and thereby the link between the glucose
386 sensing and the filamentation. Here it is demonstrated that there is a link between the level of
387 residual glucose and the response to pH stress. As long as the residual glucose was under a
388 certain threshold, only ovoid-yeast cells were present in the culture medium at $\text{pH} \geq 6.5$. As
389 soon as the concentration increased, filamentous cells appeared. Based on those results, it can
390 be concluded that also in *Y. lipolytica*, the level of residual glucose is strongly involved in the
391 signaling response. Such a conclusion has not been reported earlier, probably because of the
392 experimental approaches, based on batch cultures, that have been implemented. Furthermore,
393 it was confirmed that the addition of cAMP could prevent the dimorphic transition even in the
394 presence of a residual glucose concentration. Such effect of cAMP on dimorphic transition
395 has been highlighted earlier [19]. Thanks to the microfluidic bioreactor, it has been possible to
396 carry out a well-controlled accelerostat with a continuous feeding of cAMP at a lower cost,
397 and to observe the absence of dimorphic transition even when the level of residual glucose
398 exceeded the threshold value. Further studies are needed to decipher the mechanism,
399 nevertheless this work has clearly demonstrated that the dimorphic transition in *Y. lipolytica* is
400 much more controlled by a sugar signaling pathway, most probably via cAMP-PKA-type
401 signaling pathway, than by the pH or by DO responses. The responses to both stressors (pH
402 and DO) were indeed clearly different depending on the residual glucose concentration in the
403 medium.

404 Filamentation greatly impacts the rheological behavior of the fermentation broth, and transfer
405 phenomena inside bioreactors and consequently bioprocess performance. Being able to
406 control dimorphic transition via a fine control of the residual glucose level in the bioreactor
407 based on well-controlled feeding strategy could be a relevant lever for bioprocess
408 development.

409 **Formatting of funding sources**

410 This research did not receive any specific grant from funding agencies in the public,
411 commercial, or not-for-profit sectors.

412

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588

589

590 **Figure captions**

591 **Fig. 1:** Picture of the two experimental designs. **A.** Conventional 1L-lab-scale bioreactor, **B.**
592 1mL-microfluidic bioreactor.

593 **Fig. 2:** Dynamic evolution of pH, DO, dilution rate, biomass and residual glucose
594 concentration during the steady state and accelerostat phases of the 1-L bioreactor culture: (×)
595 pH, (Δ) DO level, black line (—) dilution rate, (o) Biomass, (●) Residual glucose
596 concentration.

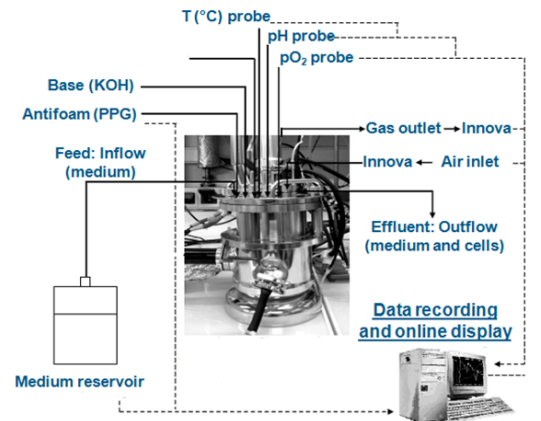
597 **Fig. 3:** Effect of residual glucose concentration on the physiological states of *Y. lipolytica*
598 populations cultivated under accelerostat mode in 1L-lab-scale bioreactor culture. **A.** Dynamic
599 evolution of the filamentous subpopulation, in volume (o), and residual glucose concentration
600 (●) during the accelerostat. **B.** Box plots comparing the time-evolution of length distribution
601 measurements for cells under chemostat and accelerostat modes (data quantified by
602 flow cytometry). The lower boundary of the box indicates the 25th percentile, a black line
603 marks the median, a red line marks the mean and the upper boundary of the box indicates the
604 75th percentile. Whiskers above and below the box indicate the 90th and 10th percentiles. The
605 black dots indicate the 95th and 5th percentiles. **C.** Light micrographs showing morphological
606 changes of *Y. lipolytica* W29 in response to the increase of dilution rate under accelerostat
607 modes. As growth progressed, observations were performed using a light microscope, without
608 oil fixation, and at magnifications of 40 x.

609 **Fig. 4:** Dynamic evolutions of pH, DO, dilution rate, biomass and residual glucose
610 concentration during the steady state and accelerostat phases of the 1mL-microfluidic
611 bioreactor cultures: (×) pH, (Δ) DO level, black line (—) dilution rate, (o) biomass, (●)
612 residual glucose concentration.

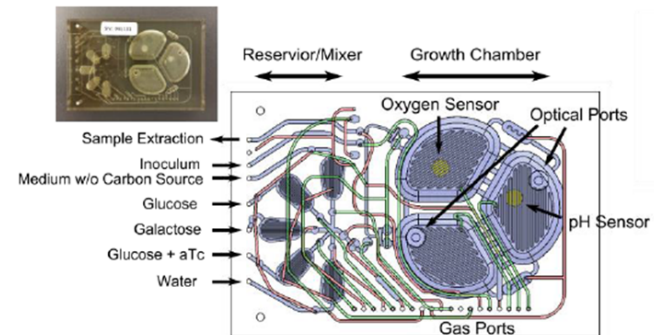
613 **Fig. 5:** Effect of residual glucose concentration on the physiological states of *Y. lipolytica*
614 populations cultivated under accelerostat mode in 1mL-microfluidic bioreactor. **A.** Dynamic
615 evolution of the filamentous subpopulation, in volume (o), and Residual glucose
616 concentration (●) during the accelerostat without cAMP supplementation. **B.** Box plots
617 comparing the time-evolution of length distribution measurements for cells under
618 accelerostat modes with and without cAMP supplementation (data quantified by flow
619 cytometry). The lower boundary of the box indicates the 25th percentile, a black line marks

620 the median, a red line marks the mean and the upper boundary of the box indicates the 75th
621 percentile. Whiskers above and below the box indicate the 90th and 10th percentiles. The black
622 dots indicate the 95th and 5th percentiles. **C.** Light micrographs showing morphological
623 changes of *Y. lipolytica* W29 in response the increase of residual glucose during accelerostat
624 without cAMP. As growth progressed, observations were performed using a light microscope,
625 without oil fixation, and at magnifications of 40 x.

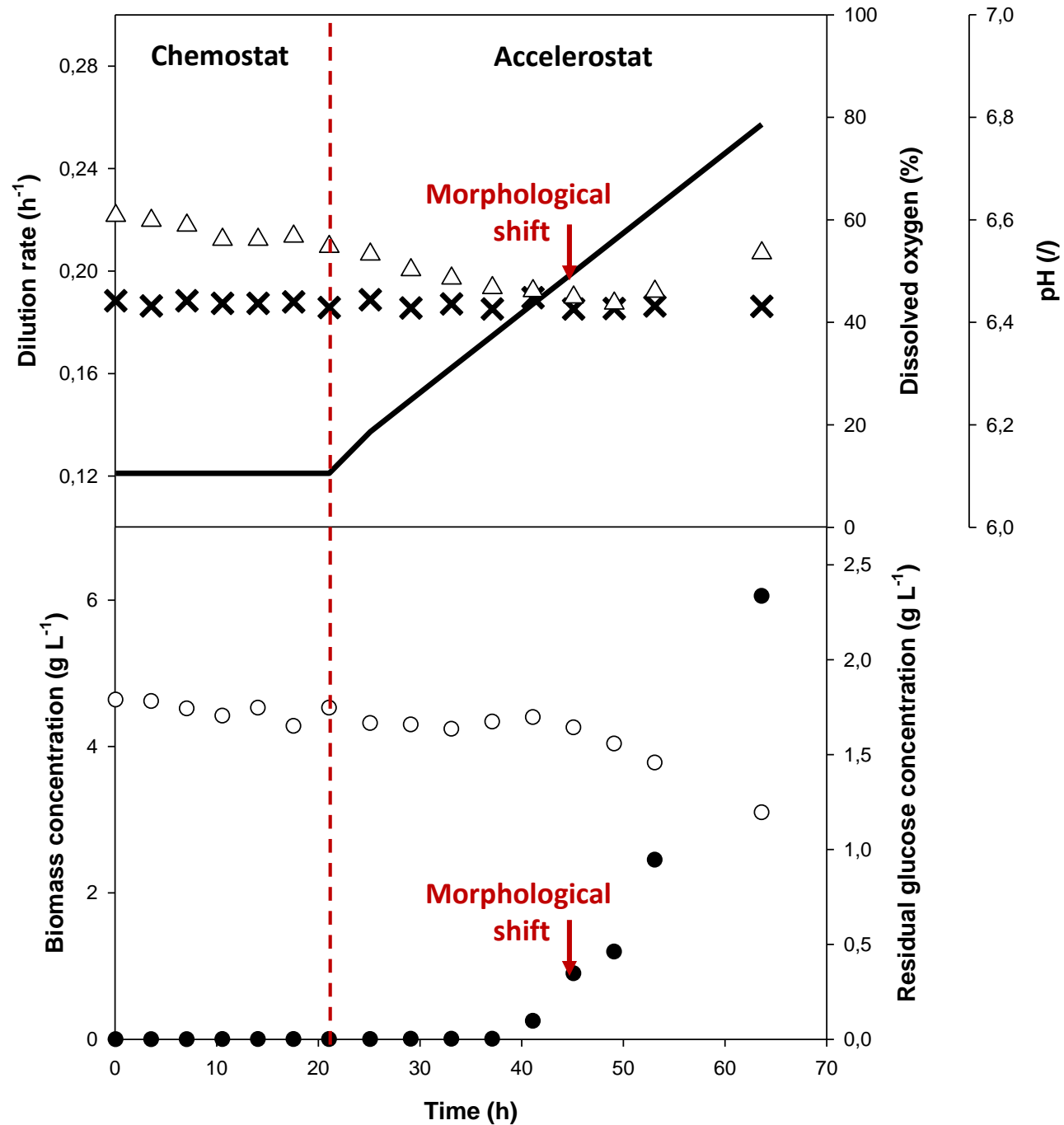
626 **Fig. 6:** Dynamic evolutions of cAMP intracellular concentration during the steady state and
627 accelerostat phases with and without cAMP of the 1mL-microfluidic and 1L- bioreactor.

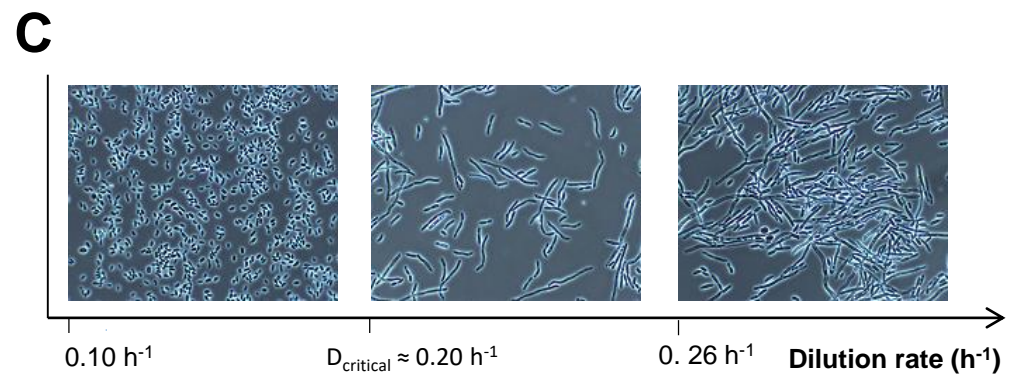
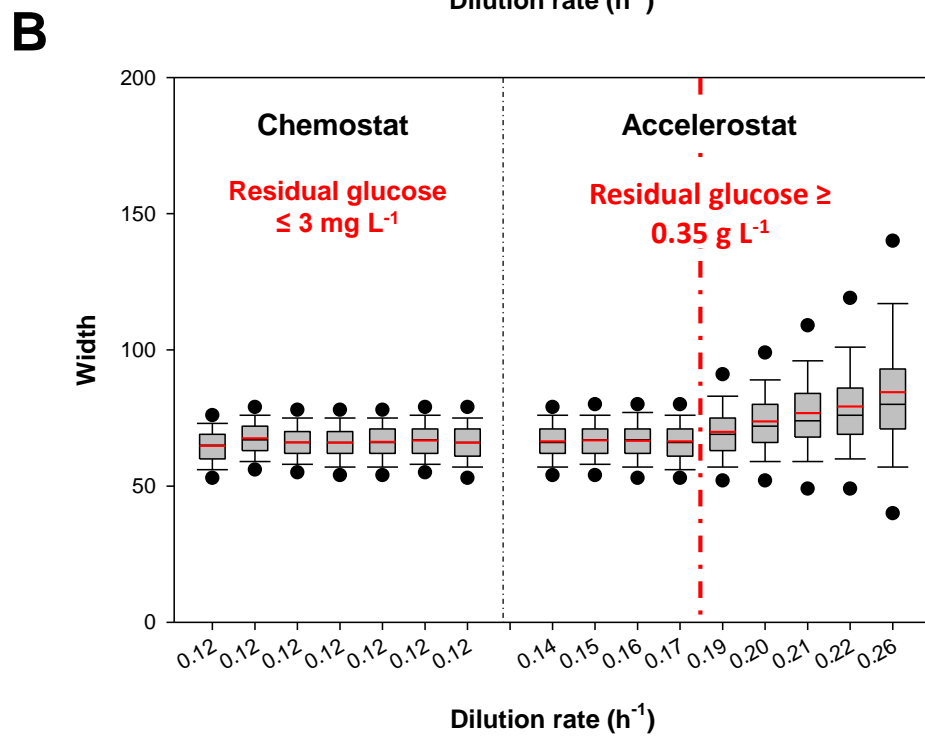
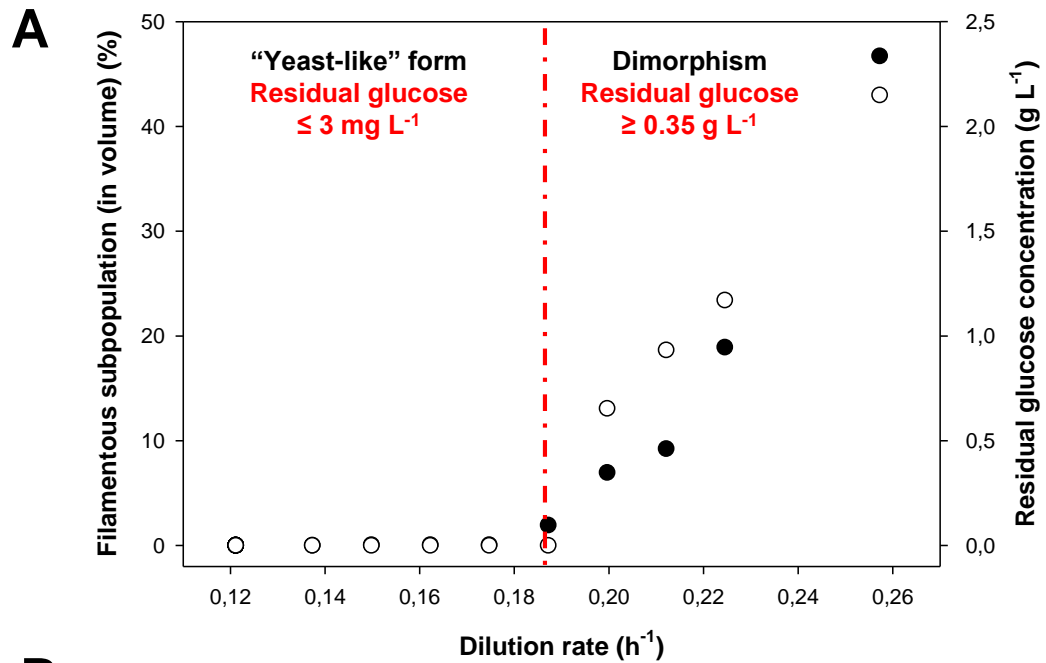
A

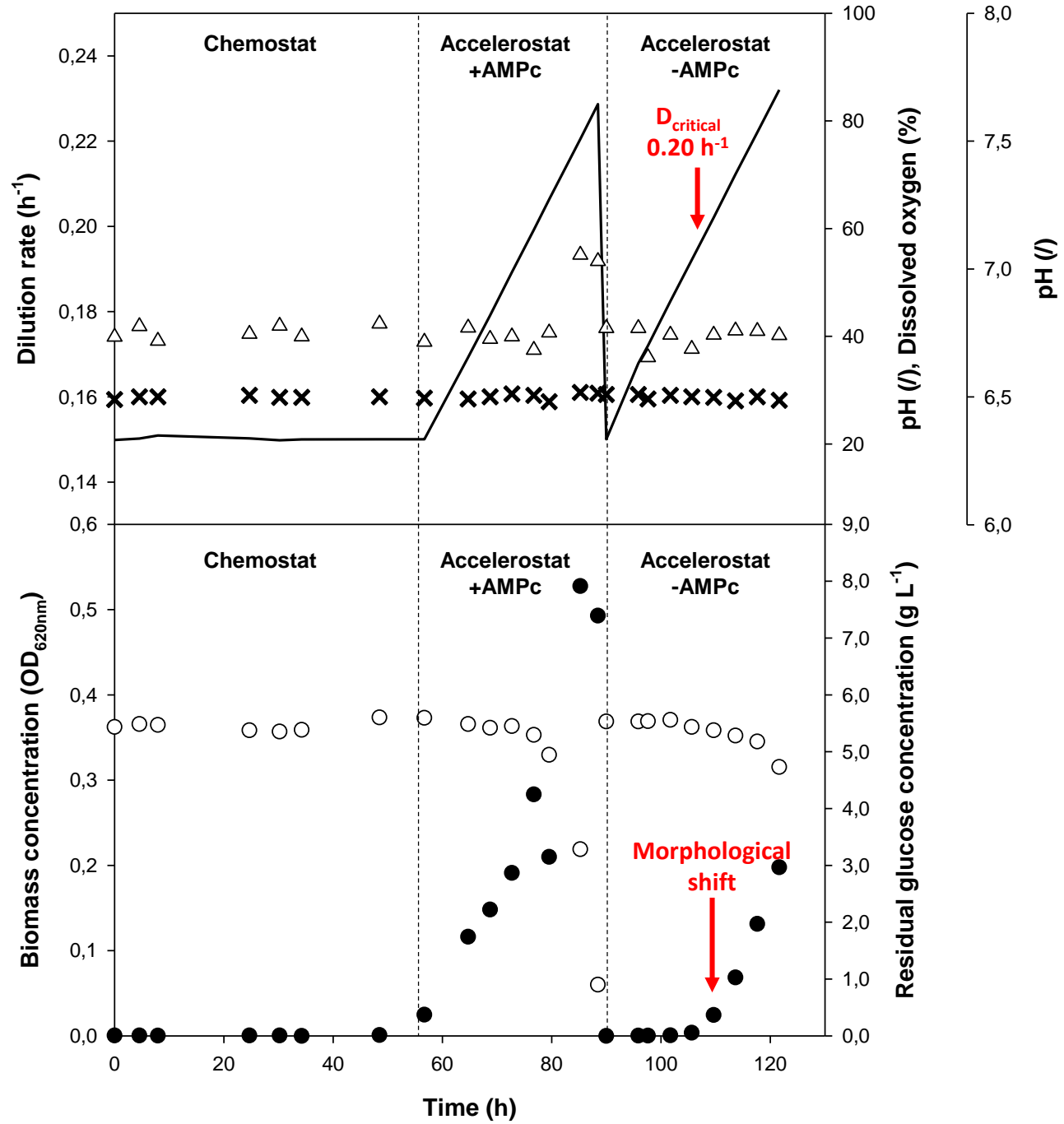
- **1L - lab-scale bioreactor**
- Accelerostat mode (ACC): $D= 0.12$ to 0.24 h^{-1}
- Culture without cAMP
- pH stress: Chemostat vs ACC at pH5.6

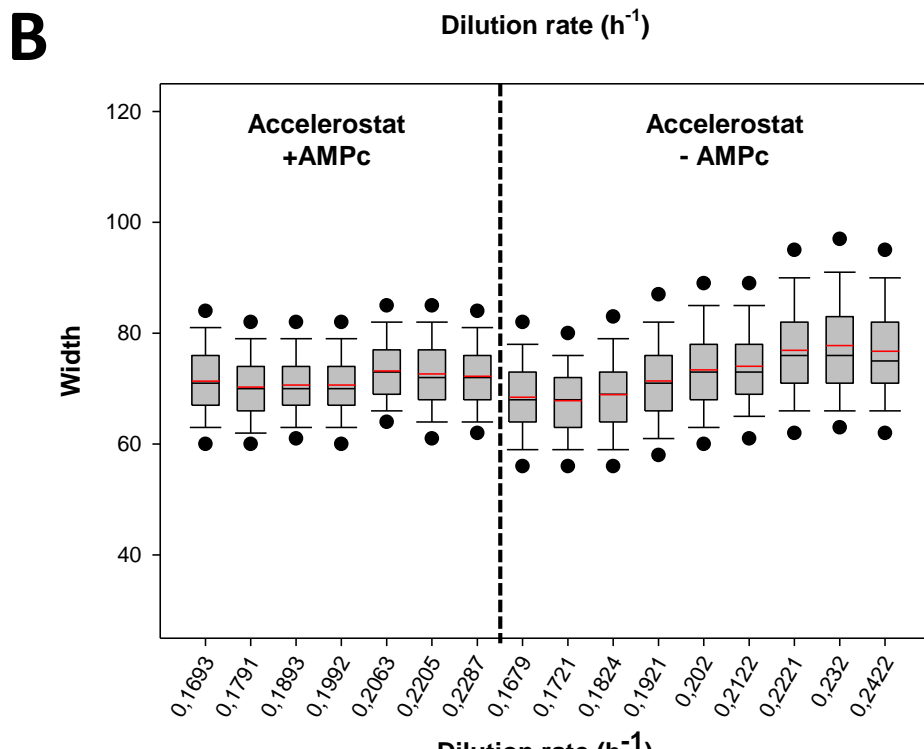
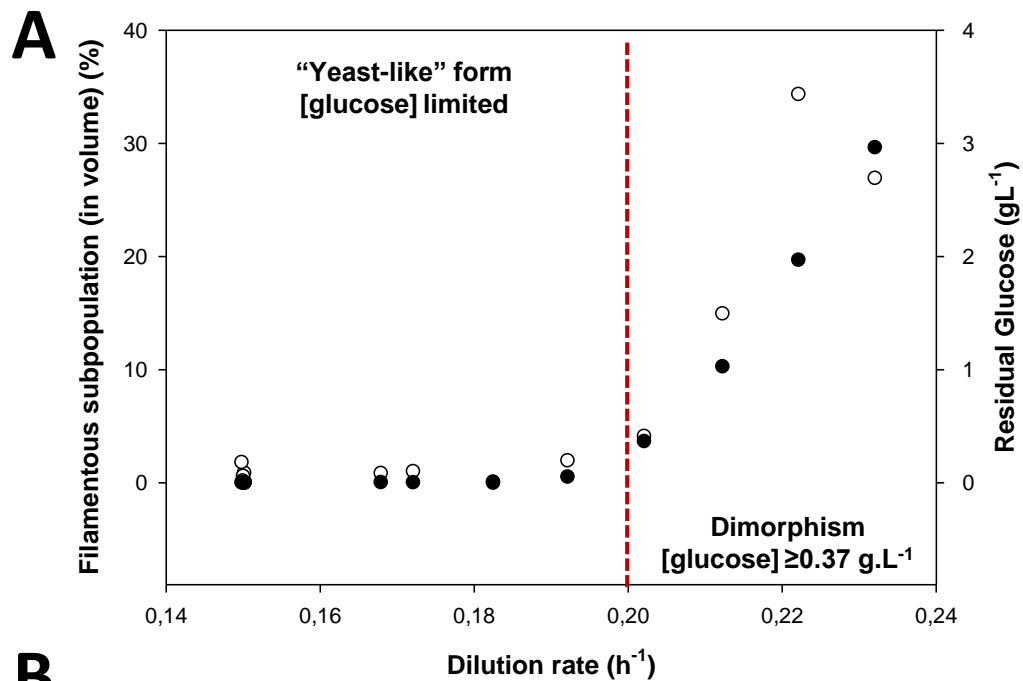
B

- **Single-use 1mL - microreactor**
- Mode Accelerostat: $D=0.15$ to 0.24 h^{-1}
- Culture with and without cAMP
- pH stress: Chemostat vs ACC at pH5.6

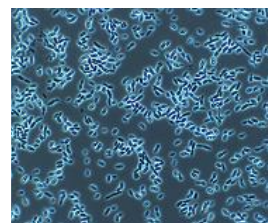




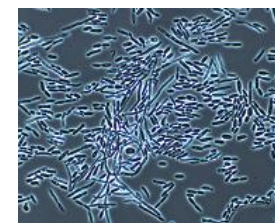




C



“Yeast-like” form
 ∇ D and
[Glucose] limited



Dimorphism
[Glucose] $\geq 0.37 \text{ g.L}^{-1}$
 $D_{\text{critical}} \approx 0.20 \text{ h}^{-1}$

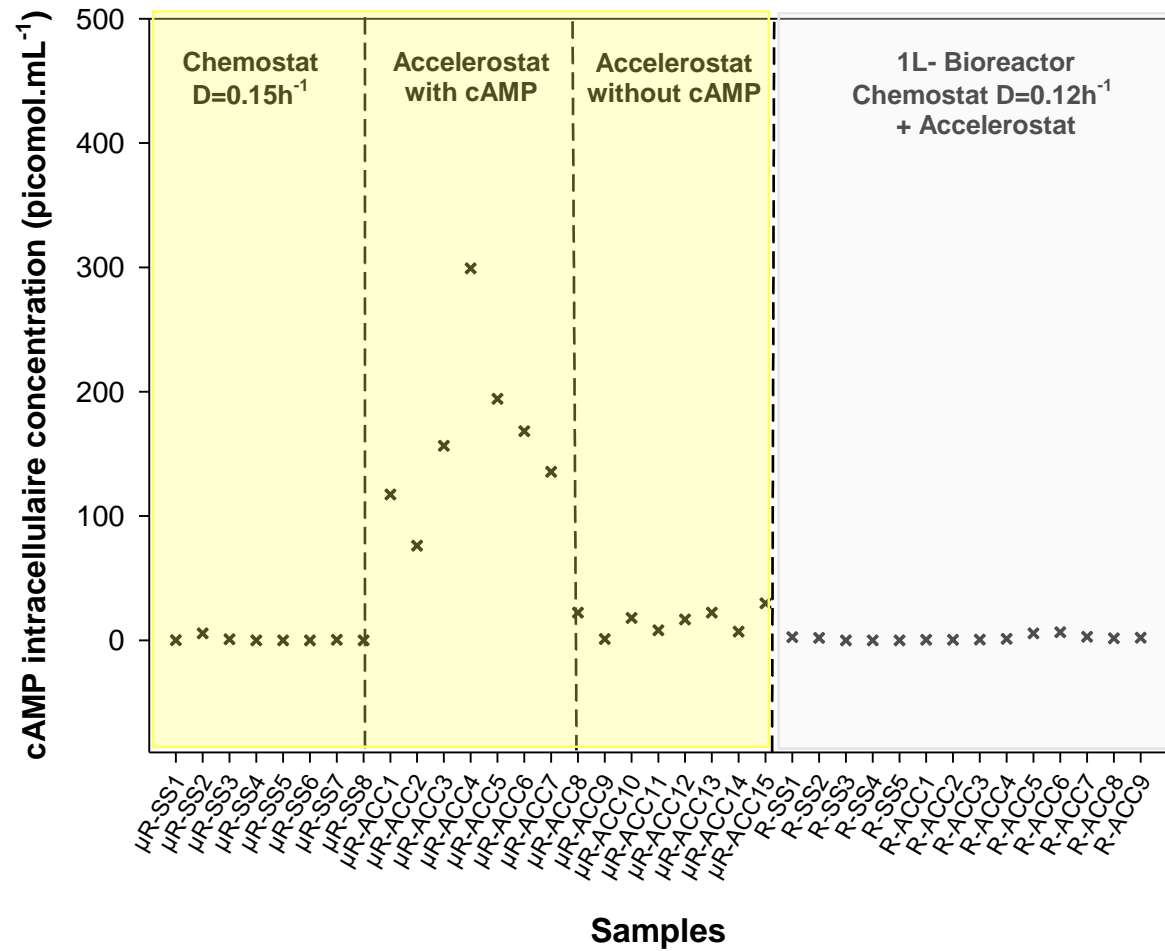


Table 1. Kinetic parameters of the continuous cultures during the steady-state phase: Average values of specific rates, yields, respiratory quotients, carbon and redox recoveries were expressed with their associated standard deviations

| Dilution rate | pH | -qS | qCO ₂ | -qO ₂ | Y _{x/s} | RQ _{mean} | Carbon recovery | Redox recovery | Reference |
|--------------------|--------|---|------------------|------------------|----------------------------|--------------------|-----------------|----------------|-----------|
| (h ⁻¹) | | (Cmol CmolX ⁻¹ h ⁻¹) | | | (Cmol Cmol ⁻¹) | (/) | (%) | | |
| 0.10 | pH 7 | 0.160±0.001 | 0.053±0.010 | 0.044±0.003 | 0.65±0.01 | 1.10±0.05 | 97.4±1.3 | 100.6±0.8 | [14] |
| 0.12 | pH 6.5 | 0.194±0.002 | 0.081±0.006 | 0.073±0.005 | 0.61±0.01 | 1.11±0.01 | 103±4 | 105±6 | This work |