



**HAL**  
open science

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

Julie Lesage, Asma Timoumi, Stéphanie Cenard, Eric Lombard, Harry L.T. Lee, Stéphane Guillouet, Nathalie Gorret

### ► To cite this version:

Julie Lesage, Asma Timoumi, Stéphanie Cenard, Eric Lombard, Harry L.T. Lee, et al.. Accelerostat study in conventional and microfluidic bioreactors to assess the key role of residual glucose in the dimorphic transition of *Yarrowia lipolytica* in response to environmental stimuli. *New Biotechnology*, 2021, 64, pp.37-45. 10.1016/j.nbt.2021.05.004 . hal-03628080

**HAL Id: hal-03628080**

**<https://hal.inrae.fr/hal-03628080>**

Submitted on 13 Jun 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

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

5 Julie Lesage<sup>1§</sup>, Asma Timoumi<sup>1§</sup>, Stéphanie Cenard<sup>1</sup>, Eric Lombard<sup>1</sup>, Harry L. T. Lee<sup>2</sup>,  
6 Stéphane E. Guillouet<sup>1</sup> and Nathalie Gorret<sup>1\*</sup>

7

8 <sup>1</sup>Toulouse Biotechnology Institute (TBI), Université de Toulouse, CNRS, INRA, INSA, 135  
9 Avenue de Rangueil. 35077 Toulouse Cedex, FRANCE

10 <sup>2</sup>Erbi Bio, Inc, 325 New Boston Stress, Unit 6, Woburn, MA 01801, USA

11 <sup>§</sup>These authors contributed equally to this work.

12 \* Corresponding author. E-mail: [ngorret@insa-toulouse.fr](mailto:ngorret@insa-toulouse.fr)

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<sup>-1</sup>.  
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<sup>-1</sup>); 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<sup>-1</sup> to 0.20 h<sup>-1</sup>), 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<sup>-1</sup> DO concentration), did not engender  
58 mycelial transition. However, filamentation was observed under conditions where limiting O<sub>2</sub>  
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 O<sub>2</sub> -  
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 O<sub>2</sub> 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<sup>-1</sup>) was evaluated under chemostat at 0.032h<sup>-1</sup> 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 ( $\mu_{\max}$ ).  
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<sup>-1</sup>) 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  $\gamma$ -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  $\mu$ L sections, of which only  
133 two were full at any time to ensure both the 1000 $\mu$ 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 NaHCO<sub>3</sub> (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<sup>-1</sup> and 0.15 h<sup>-1</sup> 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<sup>-1</sup>/0.15 h<sup>-1</sup>, 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<sup>-1</sup>/ 0.15h<sup>-1</sup> was reached and  
156 characterized, the accelerostat phase was launched with an acceleration factor of 0.0025 h<sup>-1</sup>  
157 from D 0.12/ 0.15 to 0.25 h<sup>-1</sup> (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<sub>620nm</sub>) and dry weight measurements, following the protocol described  
165 [14]. For the 1mL microfluidic bioreactor experiments, biomass concentration was quantified  
166 spectrophotometrically (OD<sub>600nm</sub> and OD<sub>620nm</sub>) 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<sup>-1</sup> 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 ( $0.12 \text{ h}^{-1}$ ) 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  $0.20 \text{ h}^{-1}$ ) 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 ( $0.12 \text{ h}^{-1}$ ).

### 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  $\text{O}_2$  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  $0.1 \text{ h}^{-1}$  [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  $5\text{mg L}^{-1}$  (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  $\text{et}$  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 ( $D_{\text{crit}}$ ) 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  $D_{\text{crit}}$  led to a gradual decrease in the  
254 biomass concentration from  $4.5$  to  $3\text{g CDW L}^{-1}$ , which consequently led to an increase in  
255 residual glucose from  $5\text{mg L}^{-1}$  to  $2.5\text{g L}^{-1}$  in the culture broth. At  $D_{\text{crit}}$ , the residual glucose

256 concentration was about 0.35g L<sup>-1</sup>. No organic acids were detected, and cell viability was  
257 always >97%. Similarly, for the chemostat, an unlimited O<sub>2</sub> 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<sup>-1</sup>, 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<sup>-1</sup> (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<sup>-1</sup> [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 O<sub>2</sub> 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<sup>-1</sup>  
278 <sup>1</sup>, (ii) the accelerostat from 0.15 h<sup>-1</sup> to 0.25 h<sup>-1</sup> without cAMP, (iii) a second steady state  
279 chemostat at D = 0.15 h<sup>-1</sup> (data not shown), (iiii) the accelerostat from 0.15 h<sup>-1</sup> to 0.25 h<sup>-1</sup> 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<sup>-1</sup>, a stable non-limiting DO  
285 concentration (>40%), and a stable residual glucose concentration <1 mg L<sup>-1</sup> (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  $0.37 \text{ g L}^{-1}$  obtained at a  $D_{\text{crit}}$  of about  $0.20 \text{ h}^{-1}$ . This  $D_{\text{crit}}$  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  $\text{O}_2$  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<sup>-1</sup>  
327 <sup>1</sup>) even close to the maximum growth rate of the strain (0.24 h<sup>-1</sup>), 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*  $\Delta$ 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

### 413 **References**

- 414 [1] Fickers P, Benetti PH, Wache Y, Marty A, Mauersberger S, Smit MS, et al. Hydrophobic  
415 substrate utilisation by the yeast *Yarrowia lipolytica*, and its potential applications.  
416 Fems Yeast Res 2005;5:527-543. doi: 10.1016/j.femsyr.2004.09.004
- 417 [2] Finogenova TV, Kamzolova SV, Dedyukhina EG, Shishkanova NV, Il'chenko AP,  
418 Morgunov IG, et al. Biosynthesis of citric and isocitric acids from ethanol by mutant  
419 *Yarrowia lipolytica* N 1 under continuous cultivation. Appl Microbiol Biot  
420 2002;59:493-500. doi: 10.1007/s00253-002-1022-8
- 421 [3] Papanikolaou S, Galiotou-Panayotou M, Chevalot I, Komaitis M, Marc I, Aggelis G.  
422 Influence of glucose and saturated free-fatty acid mixtures on citric acid and lipid  
423 production by *Yarrowia lipolytica*. Curr Microbiol 2006;52:134-142.  
424 doi: 10.1007/s00284-005-0223-7
- 425 [4] Makri A, Fakas S, Aggelis G. Metabolic activities of biotechnological interest in *Yarrowia*  
426 *lipolytica* grown on glycerol in repeated batch cultures. Bioresource Technol  
427 2010;101:2351-2358. doi: 10.1016/j.biortech.2009.11.024
- 428 [5] Sarris D, Galiotou-Panayotou M, Koutinas AA, Komaitis M, Papanikolaou S. Citric acid,  
429 biomass and cellular lipid production by *Yarrowia lipolytica* strains cultivated on olive  
430 mill wastewater-based media. J Chem Technol Biot 2011;86:1439-1448.  
431 doi: 10.1002/jctb.2658
- 432 [6] Coelho MAZ, Amaral PFF, Belo I. *Yarrowia lipolytica*: an industrial workhorse Current  
433 Research, Technology and Education Topics in Applied Microbiology and Microbial  
434 Biotechnology Advances 2010:930-940
- 435 [7] Bussamara R, Fuentefria AM, de Oliveira ES, Broetto L, Simcikova M, Valente P, et al.  
436 Isolation of a lipase-secreting yeast for enzyme production in a pilot-plant scale batch  
437 fermentation. Bioresource Technol 2010;101:268-275.  
438 doi: 10.1016/j.biortech.2008.10.063
- 439 [8] Madzak C, Gaillardin C, Beckerich JM. Heterologous protein expression and secretion in  
440 the non-conventional yeast *Yarrowia lipolytica*: a review. J Biotechnol 2004;109:63-  
441 81. doi: 10.1016/j.jbiotec.2003.10.027
- 442 [9] Papanikolaou S, Chevalot I, Galiotou-Panayotou M, Komaitis M, Marc I, Aggelis G.  
443 Industrial derivative of tallow: a promising renewable substrate for microbial lipid,  
444 single-cell protein and lipase production by *Yarrowia lipolytica*. Electron J Biotechnol  
445 2007;10:425-435. doi: 10.2225/vol10-issue3-fulltext-8
- 446 [10] Papanikolaou S, Chatzifragkou A, Fakas S, Galiotou-Panayotou M, Komaitis M, Nicaud  
447 J-M, et al. Biosynthesis of lipids and organic acids by *Yarrowia lipolytica* strains  
448 cultivated on glucose. Eur J Lipid Sci Tech 2009;111:1221-1232.  
449 doi: 10.1002/ejlt.200900055
- 450 [11] Parfene G, Horincar V, Tyagi AK, Malik A, Bahrim G. Production of medium chain  
451 saturated fatty acids with enhanced antimicrobial activity from crude coconut fat by  
452 solid state cultivation of *Yarrowia lipolytica*. Food Chem 2013;136:1345-1349.  
453 doi: 10.1016/j.foodchem.2012.09.057
- 454 [12] Timoumi A, Guillouet SE, Molina-Jouve C, Fillaudeau L, Gorret N. Impacts of  
455 environmental conditions on product formation and morphology of *Yarrowia*  
456 *lipolytica*. Appl Microbiol Biotechnol 2018;102:3831-3848. doi: 10.1007/s00253-  
457 018-8870-3
- 458 [13] Timoumi A, Bideaux C, Guillouet SE, Allouche Y, Molina-Jouve C, Fillaudeau L, et al.  
459 Influence of oxygen availability on the metabolism and morphology of *Yarrowia*  
460 *lipolytica*: insights into the impact of glucose levels on dimorphism. Appl Microbiol  
461 Biotechnol 2017; 101:7317-7333. doi: 10.1007/s00253-017-8446-7
- 462 [14] Timoumi A, Cleret M, Bideaux C, Guillouet SE, Allouche Y, Molina-Jouve C, et al.  
463 Dynamic behavior of *Yarrowia lipolytica* in response to pH perturbations: dependence



- 464 of the stress response on the culture mode. *Appl Microbiol Biotechnol* 2017;101:351-  
 465 366. doi: 10.1007/s00253-016-7856-2
- 466 [15] Bellou S, Makri A, Triantaphyllidou IE, Papanikolaou S, Aggelis G. Morphological and  
 467 metabolic shifts of *Yarrowia lipolytica* induced by alteration of the dissolved oxygen  
 468 concentration in the growth environment. *Microbiol-SGM* 2014;160:807-817.  
 469 doi: 10.1099/mic.0.074302-0
- 470 [16] Cervantes-Chavez JA, Kronberg F, Passeron S, Ruiz-Herrera J. Regulatory role of the  
 471 PKA pathway in dimorphism and mating in *Yarrowia lipolytica*. *Fungal Genet Biol*  
 472 2009;46:390-399. doi: 10.1016/j.fgb.2009.02.005
- 473 [17] Cervantes-Chavez JA, Ruiz-Herrera J. STE11 disruption reveals the central role of a  
 474 MAPK pathway in dimorphism and mating in *Yarrowia lipolytica*. *Fems Yeast Res*  
 475 2006;6:801-815. doi: 10.1111/j.1567-1364.2006.00084.x
- 476 [18] Cervantes-Chavez JA, Ruiz-Herrera J. The regulatory subunit of protein kinase A  
 477 promotes hyphal growth and plays an essential role in *Yarrowia lipolytica*. *Fems*  
 478 *Yeast Res* 2007;7:929-940. doi: 10.1111/j.1567-1364.2007.00265.x
- 479 [19] Ruiz-Herrera J, Sentandreu R. Different effectors of dimorphism in *Yarrowia lipolytica*.  
 480 *Arch Microbiol* 2002;178:477-483. doi: 10.1007/s00203-002-0478-3
- 481 [20] Hurtado CAR, Beckerich JM, Gaillardin C, Rachubinski RA. A Rac homolog is required  
 482 for induction of hyphal growth in the dimorphic yeast *Yarrowia lipolytica*. *J Bacteriol*  
 483 2000;182:2376-2386. doi: 10.1128/jb.182.9.2376-2386.2000
- 484 [21] Topiltin Morales-Vargas A, Dominguez A, Ruiz-Herrera J. Identification of dimorphism-  
 485 involved genes of *Yarrowia lipolytica* by means of microarray analysis. *Res Microbiol*  
 486 2012;163:378-387. doi: 10.1016/j.resmic.2012.03.002
- 487 [22] Martinez-Vazquez A, Gonzalez-Hernandez A, Dominguez A, Rachubinski R, Riquelme  
 488 M, Cuellar-Mata P, et al. Identification of the Transcription Factor Znc1p, which  
 489 Regulates the Yeast-to-Hypha Transition in the Dimorphic Yeast *Yarrowia lipolytica*.  
 490 *Plos One* 2013;8. doi: 10.1371/journal.pone.0066790
- 491 [23] Morin M, Monteoliva L, Insenser M, Gil C, Dominguez A. Proteomic analysis reveals  
 492 metabolic changes during yeast to hypha transition in *Yarrowia lipolytica*. *J Mass*  
 493 *Spectrom* 2007;42:1453-1462. doi: 10.1002/jms.1284
- 494 [24] Buu L-M, Chen Y-C. Impact of glucose levels on expression of hypha-associated  
 495 secreted aspartyl proteinases in *Candida albicans*. *J Biomed Sci* 2014;21:22-22.  
 496 doi: 10.1186/1423-0127-21-22
- 497 [25] Laurian R, Dementhon K, Doumèche B, Soulard A, Noel T, Lemaire M, et al.  
 498 Hexokinase and Glucokinases Are Essential for Fitness and Virulence in the  
 499 Pathogenic Yeast *Candida albicans*. *Front Microbiol* 2019;10. doi:  
 500 10.3389/fmicb.2019.00327
- 501 [26] Van de Velde S, Thevelein JM. Cyclic AMP-protein kinase A and Snf1 signaling  
 502 mechanisms underlie the superior potency of sucrose for induction of filamentation in  
 503 *Saccharomyces cerevisiae*. *Eukaryot Cell* 2008;7:286-293. doi: 10.1128/EC.00276-07
- 504 [27] Johnson C, Kweon HK, Sheidy D, Shively CA, Mellacheruvu D, Nesvizhskii AI, et al.  
 505 The yeast Sks1p kinase signaling network regulates pseudohyphal growth and glucose  
 506 response. *PLoS Genet* 2014;10:e1004183-e1004183.  
 507 doi: 10.1371/journal.pgen.1004183
- 508 [28] Rødkær SV, Færgeman NJ. Glucose- and nitrogen sensing and regulatory mechanisms in  
 509 *Saccharomyces cerevisiae*. *FEMS Yeast Res* 2014;14:683-696.  
 510 doi: 10.1111/1567-1364.12157
- 511 [29] Rolland F, Winderickx J, Thevelein JM. Glucose-sensing and -signalling mechanisms in  
 512 yeast. *FEMS Yeast Res* 2002;2:183-201. doi: 10.1111/j.1567-1364.2002.tb00084.x

- 513 [30] Santangelo GM. Glucose signaling in *Saccharomyces cerevisiae*. *Microbiol Mol Biol*  
514 *Rev* 2006;70:253-282. doi: 10.1128/MMBR.70.1.253-282.2006
- 515 [31] Sabina J, Brown V. Glucose sensing network in *Candida albicans*: a sweet spot for  
516 fungal morphogenesis. *Eukaryot Cell* 2009;8:1314-1320. doi: 10.1128/EC.00138-09
- 517 [32] Zaman S, Lippman SI, Schnepfer L, Slonim N, Broach JR. Glucose regulates  
518 transcription in yeast through a network of signaling pathways. *Mol Syst Biol*  
519 2009;5:245-245.  
520 doi: 10.1038/msb.2009.2
- 521 [33] Kutykrishnan S, Sabina J, Langton LL, Johnston M, Brent MR. A quantitative model of  
522 glucose signaling in yeast reveals an incoherent feed forward loop leading to a  
523 specific, transient pulse of transcription. *P Natl Acad Sci USA* 2010;107:16743-  
524 16748.  
525 doi: 10.1073/pnas.0912483107
- 526 [34] Kim J-H, Roy A, Jouandot D, Cho KH. The glucose signaling network in yeast.  
527 2013;1830:5204-5210. <https://doi.org/10.1016/j.bbagen.2013.07.025>
- 528 [35] Roy A, Kim YB, Cho KH, Kim JH. Glucose starvation-induced turnover of the yeast  
529 glucose transporter Hxt1. *Biochim Biophys Acta* 2014;1840:2878-2885.  
530 doi: 10.1016/j.bbagen.2014.05.004
- 531 [36] Cairey-Remonnay A, Deffaud J, Wésolowski-Louvel M, Lemaire M, Soulard A.  
532 Glycolysis controls plasma membrane glucose sensors to promote glucose signaling in  
533 yeasts. *Mol Cell Biol* 2015;35:747-757. doi: 10.1128/MCB.00515-14
- 534 [37] Lazar Z, Neuvéglise C, Rossignol T, Devillers H, Morin N, Robak M, et al.  
535 Characterization of hexose transporters in *Yarrowia lipolytica* reveals new groups of  
536 Sugar Porters involved in yeast growth. *Fungal Genet Biol* 2017;100:1-12.  
537 <https://doi.org/10.1016/j.fgb.2017.01.001>
- 538 [38] Lee KS, Ram RJ. Plastic-PDMS bonding for high pressure hydrolytically stable active  
539 microfluidics. *Lab Chip* 2009;9:1618-1624. doi: 10.1039/B820924C
- 540 [39] Lee KS, Boccazzi P, Sinskey AJ, Ram RJ. Microfluidic chemostat and turbidostat with  
541 flow rate, oxygen, and temperature control for dynamic continuous culture. *Lab Chip*  
542 2011;11:1730-1739. doi: 10.1039/C1LC20019D
- 543 [40] Bower DM, Lee KS, Ram RJ, Prather KLJ. Fed-batch microbioreactor platform for scale  
544 down and analysis of a plasmid DNA production process. *Biotechnol Bioeng*  
545 2012;109:1976-1986. doi: 10.1002/bit.24498
- 546 [41] Torres-Guzmán JC, Domínguez A. HOY1, a homeo gene required for hyphal formation  
547 in *Yarrowia lipolytica*. *Mol Cell Biol* 1997;17:6283-6293. doi:  
548 10.1128/mcb.17.11.6283
- 549 [42] Hurtado CA, Rachubinski RA. MHY1 encodes a C2H2-type zinc finger protein that  
550 promotes dimorphic transition in the yeast *Yarrowia lipolytica*. *J Bacteriol*  
551 1999;181:3051-3057. doi: 10.1128/JB.181.10.3051-3057.1999
- 552 [43] Gonzalez-Lopez CI, Ortiz-Castellanos L, Ruiz-Herrera J. The ambient pH response Rim  
553 pathway in *Yarrowia lipolytica*: Identification of YIRIM9 and characterization of its  
554 role in dimorphism. *Curr Microbiol* 2006;53:8-12. doi: 10.1007/s00284-005-0070-6
- 555 [44] Kronberg F, Giacometti R, Ruiz-Herrera J, Passeron S. Characterization of the regulatory  
556 subunit of *Yarrowia lipolytica* cAMP-dependent protein kinase. Evidences of a  
557 monomeric protein. *Arch Biochem Biophys* 2011;509:66-75.  
558 doi: 10.1016/j.abb.2011.03.001
- 559 [45] Morales-Vargas AT, Dominguez A, Ruiz-Herrera J. Identification of dimorphism-  
560 involved genes of *Yarrowia lipolytica* by means of microarray analysis. *Res Microbiol*  
561 2012;163:378-387. doi: 10.1016/j.resmic.2012.03.002

- 562 [46] Liang SH, Wu H, Wang RR, Wang Q, Shu T, Gao XD. The TORC1-Sch9-Rim15  
563 signaling pathway represses yeast-to-hypha transition in response to glycerol  
564 availability in the oleaginous yeast *Yarrowia lipolytica*. *Mol Microbiol* 2017;104:553-  
565 567. doi: 10.1111/mmi.13645
- 567 [47] Pomraning KR, Bredeweg EL, Kerkhoven EJ, Barry K, Haridas S, Hundley H, et al.  
568 Regulation of Yeast-to-Hyphae Transition in *Yarrowia lipolytica*. *mSphere*  
569 2018;3:e00541-00518. doi: 10.1128/mSphere.00541-18
- 570 [48] Li M, Li YQ, Zhao XF, Gao XD. Roles of the three Ras proteins in the regulation of  
571 dimorphic transition in the yeast *Yarrowia lipolytica*. *FEMS Yeast Res* 2014;14:451-  
572 463. doi: 10.1111/1567-1364.12129
- 573 [49] Lengeler KB, Davidson RC, D'Souza C, Harashima T, Shen WC, Wang P, et al. Signal  
574 transduction cascades regulating fungal development and virulence. *Microbiol Mol*  
575 *Biol Rev* 2000;64:746-785. doi: 10.1128/membr.64.4.746-785.2000
- 576 [50] Borges-Walmsley MI, Walmsley AR. cAMP signalling in pathogenic fungi: control of  
577 dimorphic switching and pathogenicity. *Trends Microbiol* 2000;8:133-  
578 141. [https://doi.org/10.1016/S0966-842X\(00\)01698-X](https://doi.org/10.1016/S0966-842X(00)01698-X)
- 579 [51] Pollack JH, Hashimoto T. The Role of Glucose in the pH Regulation of Germ-tube  
580 Formation in *Candida albicans*. *Microbiology* 1987;133:415-  
581 424. <https://doi.org/10.1099/00221287-133-2-415>
- 582 [52] Bruatto M, Gremmi M, Nardacchione A, Amerio M. Effect of glucose starvation on  
583 germ-tube production by *Candida albicans*. *Mycopathologia* 1993;123:105-110.  
584 doi: 10.1007/bf01365088
- 585 [53] Spagnuolo M, Shabbir Hussain M, Gambill L, Blenner M. Alternative Substrate  
586 Metabolism in *Yarrowia lipolytica*. *Front Microbiol* 2018;9:1077. doi:  
587 10.3389/fmicb.2018.01077

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.

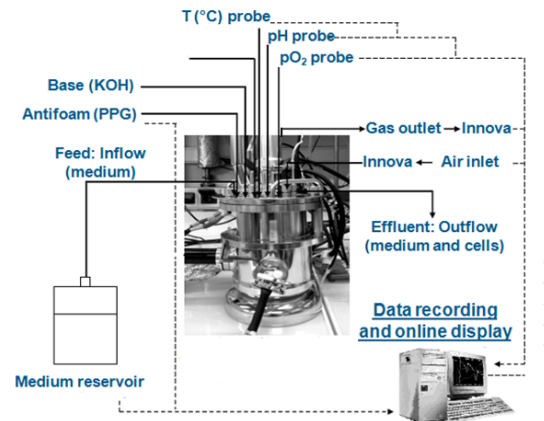
597 **Fig. 3:** Effect of residual glucose concentration on the physiological states of *Y. lipolytica*  
598 populations cultivated under accelerostat mode in 1L-lab-scale bioreactor culture. **A.** Dynamic  
599 evolution of the filamentous subpopulation, in volume (o), and residual glucose concentration  
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 25<sup>th</sup> percentile, a black line  
603 marks the median, a red line marks the mean and the upper boundary of the box indicates the  
604 75<sup>th</sup> percentile. Whiskers above and below the box indicate the 90<sup>th</sup> and 10<sup>th</sup> percentiles. The  
605 black dots indicate the 95<sup>th</sup> and 5<sup>th</sup> 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.

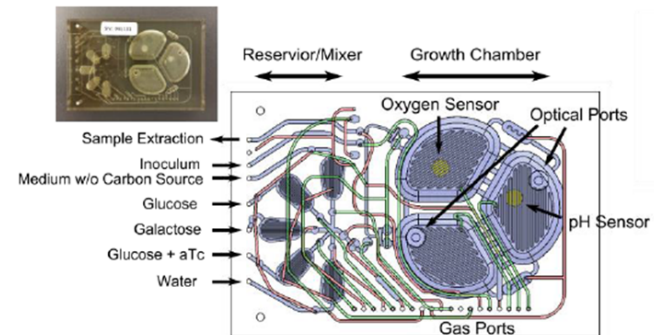
613 **Fig. 5:** Effect of residual glucose concentration on the physiological states of *Y. lipolytica*  
614 populations cultivated under accelerostat mode in 1mL-microfluidic bioreactor. **A.** Dynamic  
615 evolution of the filamentous subpopulation, in volume (o), and Residual glucose  
616 concentration (●) 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 25<sup>th</sup> percentile, a black line marks

620 the median, a red line marks the mean and the upper boundary of the box indicates the 75<sup>th</sup>  
621 percentile. Whiskers above and below the box indicate the 90<sup>th</sup> and 10<sup>th</sup> percentiles. The black  
622 dots indicate the 95<sup>th</sup> and 5<sup>th</sup> 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.

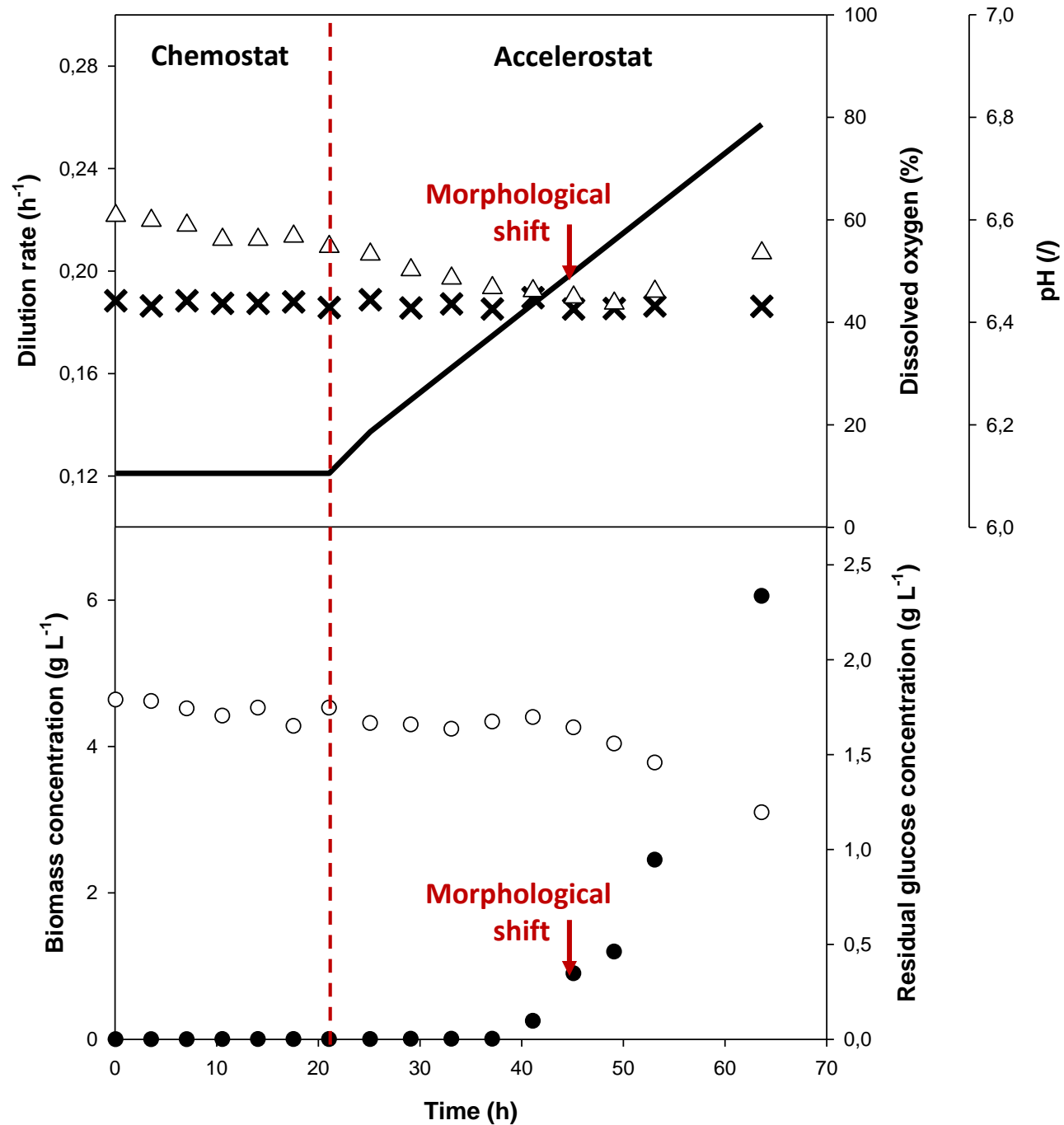
626 **Fig. 6:** Dynamic evolutions of cAMP intracellular concentration during the steady state and  
627 accelerostat phases with and without cAMP of the 1mL-microfluidic and 1L- bioreactor.

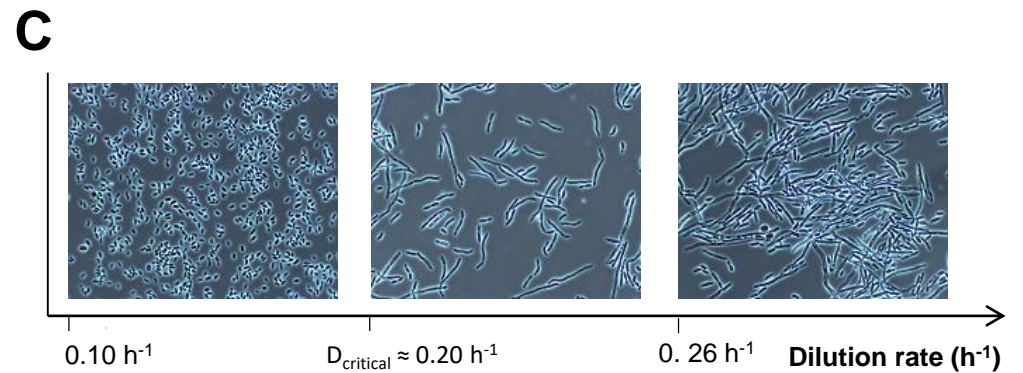
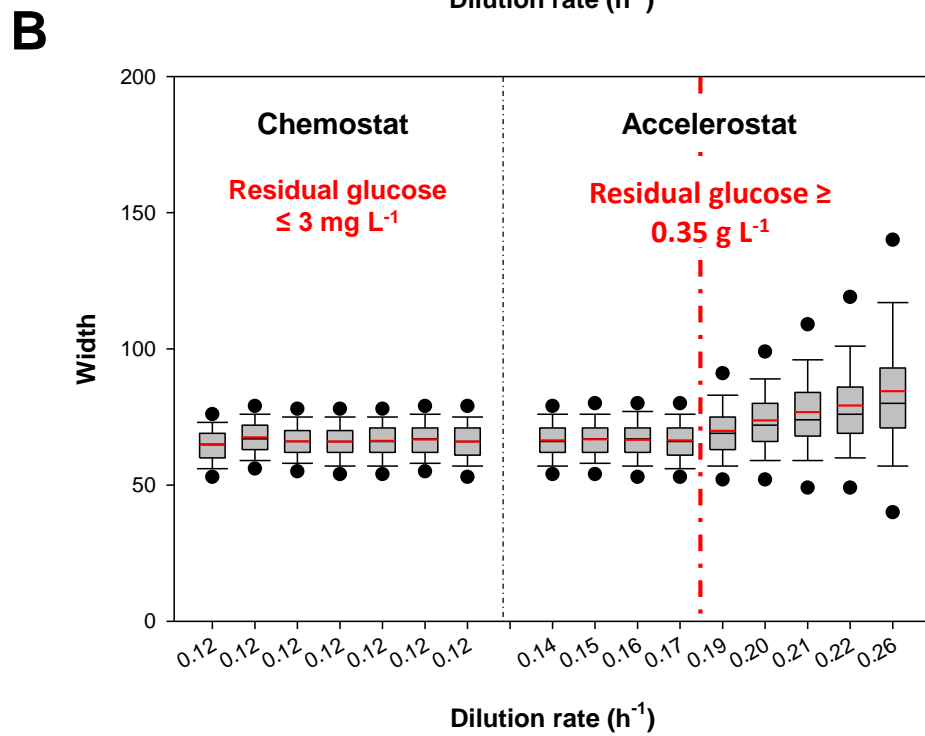
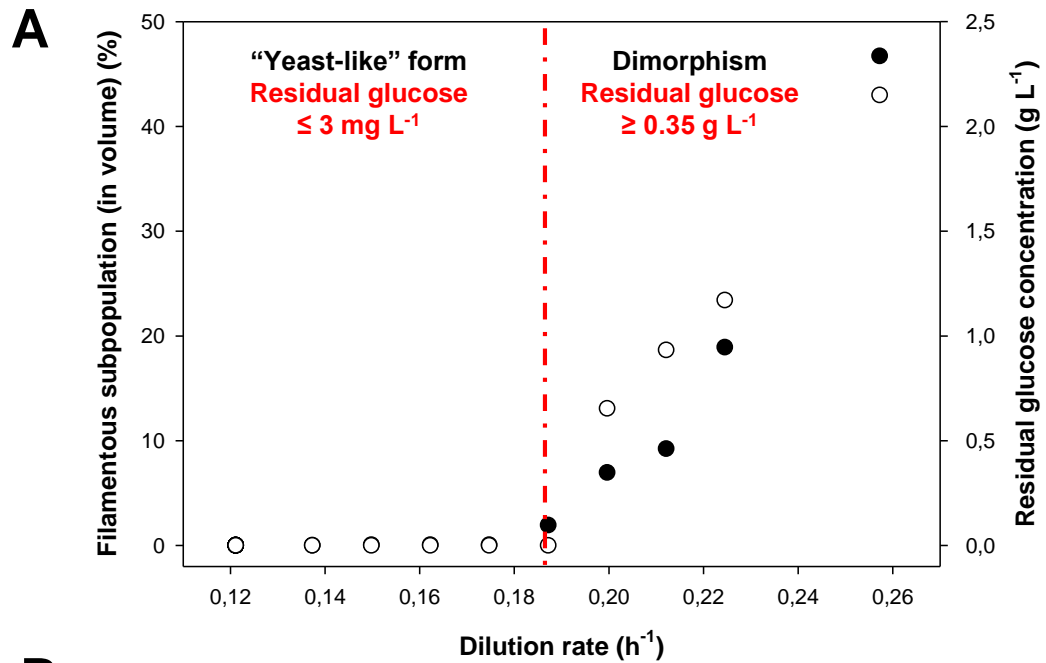
**A**

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

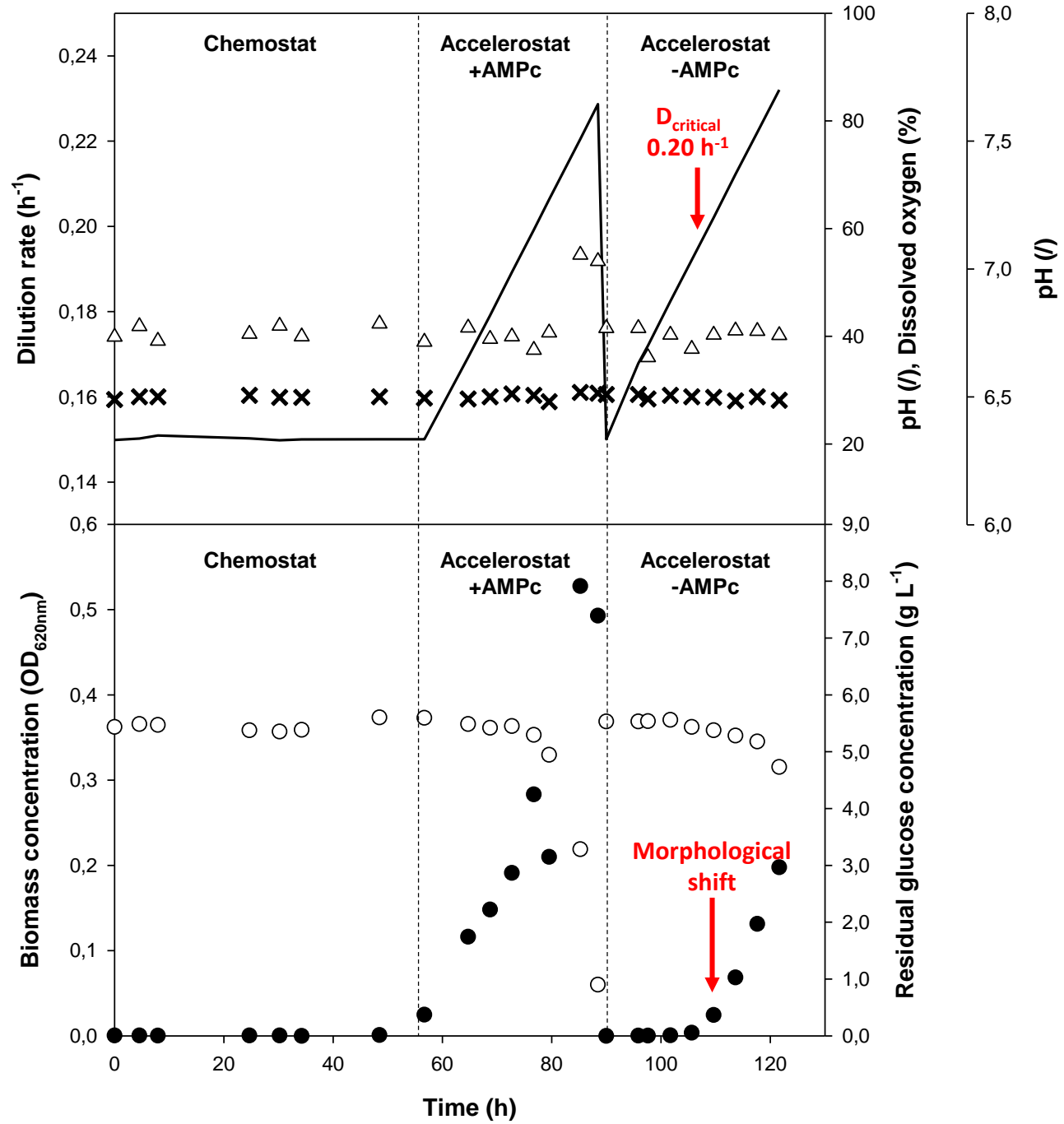
**B**

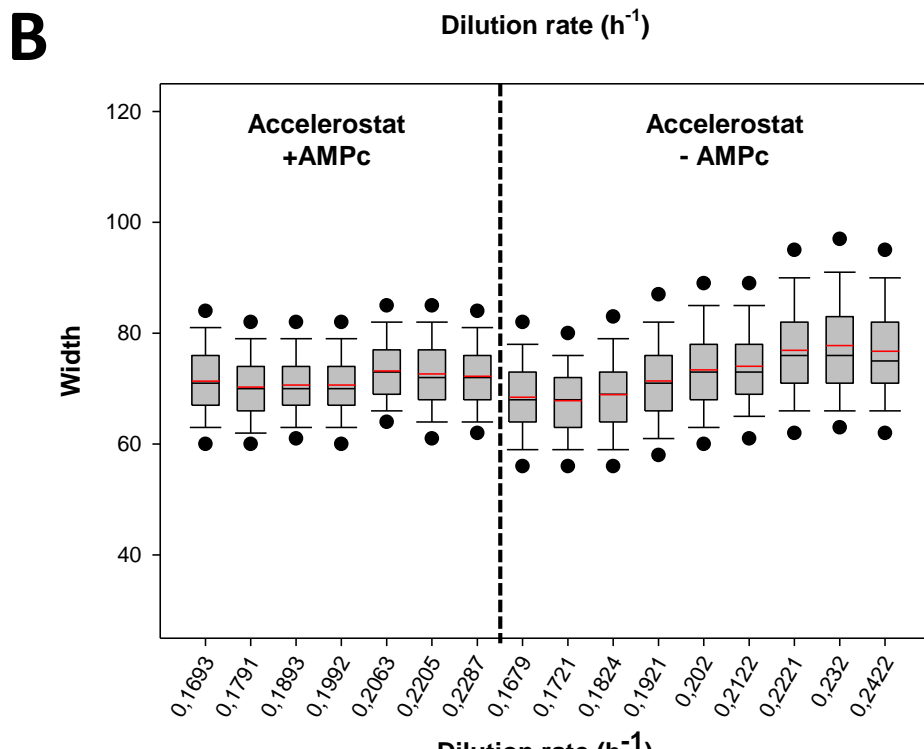
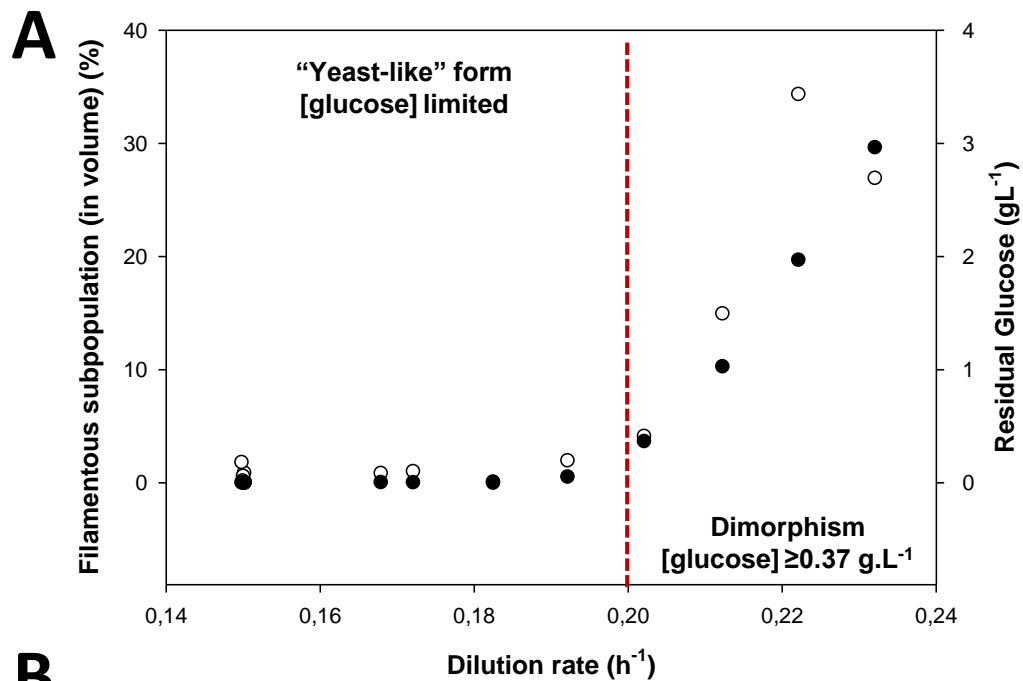
- **Single-use 1mL - microreactor**
- Mode Accelerostat:  $D=0.15$  to  $0.24 \text{ h}^{-1}$
- Culture with and without cAMP
- pH stress: Chemostat vs ACC at pH5.6



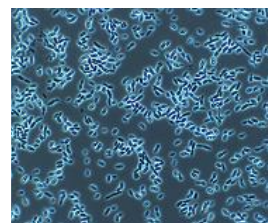




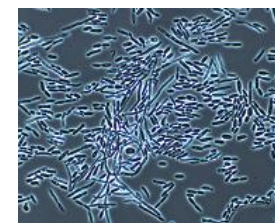




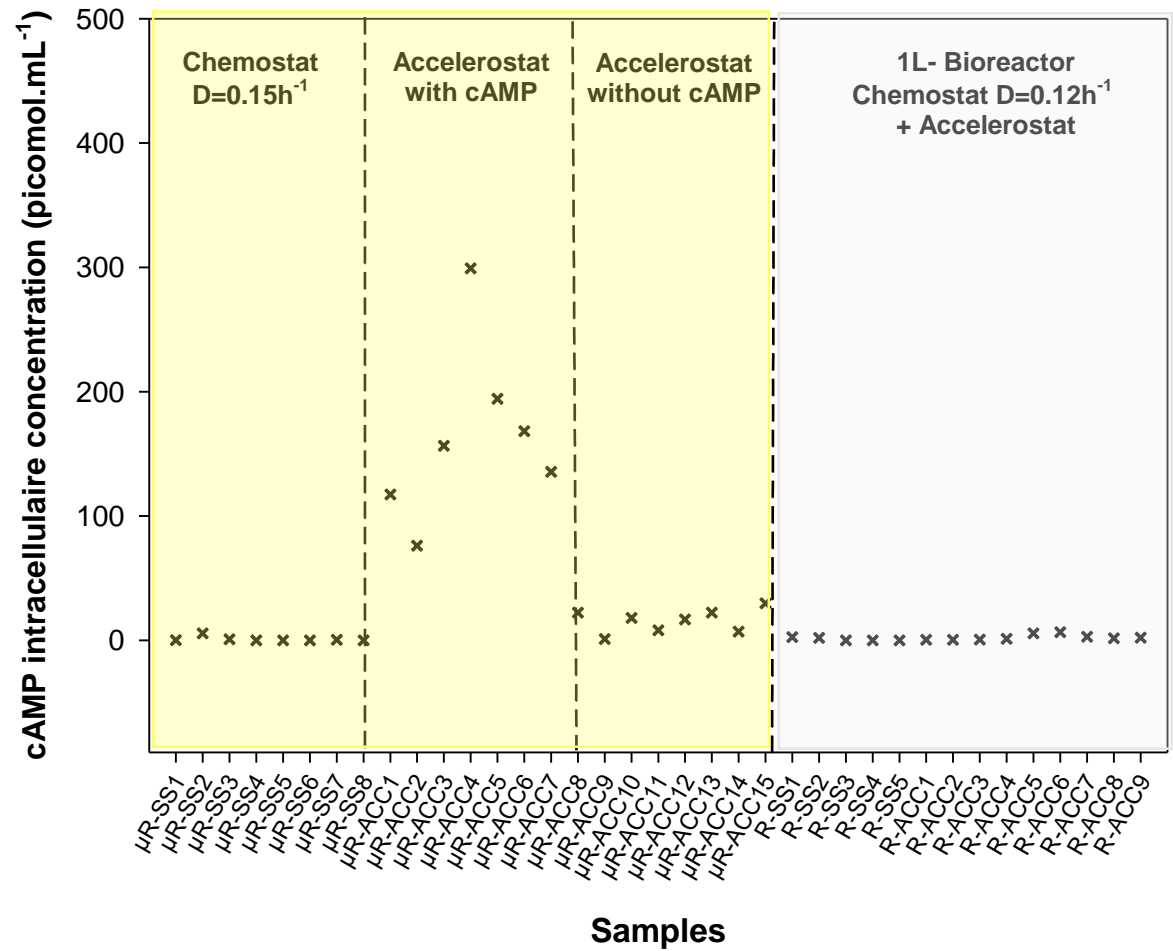
**C**



“Yeast-like” form  
 $\nabla$  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 <sub>2</sub>	-qO <sub>2</sub>	Y <sub>x/s</sub>	RQ <sub>mean</sub>	Carbon recovery	Redox recovery	Reference
(h <sup>-1</sup> )		(Cmol CmolX <sup>-1</sup> h <sup>-1</sup> )			(Cmol Cmol <sup>-1</sup> )	(/)	(%)		
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