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HAL Authorization

1 **Process for symbiotic culture of *Saccharomyces cerevisiae* and *Chlorella vulgaris* for *in situ* CO<sub>2</sub>**  
2 **mitigation**

3  
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11  
12 **Abstract**

13 Industrial biotechnology relies heavily on fermentation processes that release considerable amounts of CO<sub>2</sub>.  
14 Apart from the fact that this CO<sub>2</sub> represents a considerable part of the organic substrate, it has a negative  
15 impact on the environment. Microalgae cultures have been suggested as potential means of capturing the CO<sub>2</sub>  
16 with further applications in high value compounds production or directly for feed applications. We developed  
17 a sustainable process based on a mixed co-dominant culture of *Saccharomyces cerevisiae* and *Chlorella*  
18 *vulgaris* where the CO<sub>2</sub> production and utilization controlled the microbial ecology of the culture. By mixing  
19 yeast and microalga in the same culture the CO<sub>2</sub> is produced in dissolved form and is available to the  
20 microalga avoiding degassing and dissolution phenomena. With this process, the CO<sub>2</sub> production and  
21 utilization rates were balanced and a mutual symbiosis between the yeast and the microalga was set-up in the  
22 culture. In this study the reutilization of CO<sub>2</sub> and growth of *C. vulgaris* was demonstrated. The two organism  
23 populations were balanced at approximately 20×10<sup>6</sup> cells ml<sup>-1</sup> and almost all the CO<sub>2</sub> produces by yeast was  
24 reutilized by microalga within 168 h of culture. The *C. vulgaris* inoculum preparation played a key role in  
25 establishing co-dominance of the two organisms. Other key factors in establishing symbiosis were the  
26 inoculum ratio of the two organisms and the growth medium design. A new method allowed the independent  
27 enumeration of each organism in mixed culture. This study could provide a basis for the development of  
28 green processes of low environmental impact.

29  
30 **Keywords**

31 Photo-bioreactor; Flow cytometer; Medium design; Autotrophy; Heterotrophy; Co-dominant culture

32

33 **Introduction**

34 Industrial biotechnology such as bioethanol production, alcoholic beverage production and liquid effluent  
35 treatments form a considerable part of human biological activity. These industries, involving fermentation  
36 technology, release large quantities of CO<sub>2</sub>. At the same time, the current trend is towards more sustainable  
37 industrial processes. Waste recycling is increasingly seen not only as an obligation but an opportunity in  
38 industry.

39

40 Commercially, the loss of a considerable part of the substrate in the form of CO<sub>2</sub> is an inefficient practice that  
41 cannot be avoided with microbial cultures. CO<sub>2</sub> mitigation from exhausts gases could provide an opportunity  
42 where the substrate would be entirely used at the same time rendering the process sustainable. To this end,  
43 photosynthesis is the best candidate to be associated to the normal production process. This is present in  
44 nature, often based on symbiotic relationships between organisms, and in some industrial sectors such as  
45 sewage treatment.

46

47 Biotransformation can be achieved through the use of GMO organisms. Alternatively, biotransformation can  
48 be performed by the use of specific consortia to create the desired microbial ecology. The ability to control  
49 mixed cultures is key to the use of consortia for biotransformation.

50

51 The term “symbiosis” is credited to Heinrich Anton de Bary who first used and described it as “the living  
52 together of unlike named organisms” in 1879 (Oulhen et al. 2016). One of the most studied natural composite  
53 organisms, considered as the model of symbiosis, is the lichen. Lichen arises from a symbiotic relationship  
54 between a fungi and algae or cyanobacteria (Gargas et al. 1995) and the metabolites obtained from lichens  
55 have application in industries such as pharmaceutical industry (Müller 2001).

56

57 Co-culture systems based on symbiosis between microbial species have been attempted for biotechnological  
58 applications in bioprocess and environmental protection (Santos and Reis 2014; Magdouli et al. 2016). The  
59 choice of microbial species (microalgae, bacteria or yeast) depends on the final aims of co-culture: harvesting  
60 by bioflocculation (Subashchandrabose et al. 2011; Rai et al. 2012), wastewater treatment (Arumugam et al.  
61 2014), production of extracellular polymeric substances (Haggstrom and Dostalek 1981) or growth promotion  
62 and lipid production (Milledge and Heaven 2013; Pragma et al. 2013).

63

64 Reports of studies on symbiotic co-cultures of microalgae and yeast have been increasingly appearing in the  
65 scientific literature, with the aim of improving biomass and target molecule productivity. These co-cultures  
66 fall into two categories: studies with bioreactors in series where the exhaust gases from the heterotrophic  
67 culture are fed into the autotrophic culture, and studies where both yeast and microalgae are concomitantly in

68 the same culture. We have decided to refer to the former as coupled cultures and the latter as mixed cultures  
69 (Fig. 1).

70

71 Coupled cultures consists of an upstream heterotrophic yeast-culture connected to an autotrophic culture of  
72 microalgae in photo-bioreactor through the exhaust gases from yeast culture (Puangbut and Leesing 2012;  
73 Santos et al. 2013; Dillschneider et al. 2014; Chagas et al. 2015). Studies on coupled cultures have suggested  
74 an increase in the final microalgae biomass and lipid production that is achieved by effectively enriching the  
75 air supply to the microalgae cultures with CO<sub>2</sub> from the heterotrophic culture. In a coupled-culture system, the  
76 autotrophic organism benefits from the heterotrophic organism with no positive or negative impact on the  
77 latter, therefore the symbiosis is commensal.

78

79 The mixed culture system of microalgae and yeast focuses on the symbiotic potential of associating both  
80 organisms in the same culture. This system has an advantage over coupled-cultures in that it provides an  
81 opportunity for direct gaseous exchange in dissolved form bypassing the dissolution and degassing rates of  
82 the gas supply. Usually, any gas supplied to a bioreactor has to pass from a gaseous phase into a liquid phase  
83 (dissolution) and the gases produced by the culture have to pass from the liquid phase into the gaseous phase  
84 (degassing). These transfers are subject to specific surface limitations as well as mixing phenomena that can  
85 limit CO<sub>2</sub> supply for the autotroph and O<sub>2</sub> supply for the heterotroph in a coupled culture. In a mixed culture  
86 of microalgae and yeast, each organism would use the gas produced by the other organism *in situ* and without  
87 passing through a gaseous phase, the organisms would benefit from each other, so the symbiosis based on  
88 these gas exchanges would be mutual.

89

90 From a CO<sub>2</sub> mitigation viewpoint, as the heterotrophic CO<sub>2</sub> production rate is usually largely superior to it  
91 autotrophic consumption, the two populations must be balanced in such way so that the photosynthetic  
92 population can cope with the rate of CO<sub>2</sub> production. Hence the heterotrophic activity must be in step with the  
93 CO<sub>2</sub> removal rate. This could be achieved though co-dominance of the populations allowing synergy between  
94 the two organisms based on gaseous exchange. So far, no scientific studies have been published with the  
95 stated aim of developing co-dominant symbiotic mixed cultures.

96

97 One of the main challenges for a mixed culture of yeast and microalgae appears to be the dominance of one  
98 organism over the other by the end of incubation period. The dominance seems to be due to the use of a  
99 culture medium that preferentially promotes either the growth of the yeast or that of microalgae (Dong and  
100 Zhao 2004; Cai et al. 2007; Xue et al. 2010; Cheirsilp et al. 2011; Shu et al. 2013; Zhang et al. 2014). In  
101 Zhang et al. 2014, a mixed culture of yeast *Rhodotorula glutinis* and microalga *Chlorella vulgaris* showed a  
102 yeast dominance of 88 % of the total population after 2.5 days of growth (18 g l<sup>-1</sup> of yeast and 2.4 g l<sup>-1</sup> of  
103 microalgae). Additionally, the maximum biomass concentration reached by the yeast in monoculture was 8.5

104 times higher than of the microalga in monoculture (14.5 and 1.7 g l<sup>-1</sup> respectively), this suggests that the  
105 medium designed for mixed culture was more suitable for yeast than microalgae. Inversely in Cai et al.  
106 (2007), the microalga *Isochrysis galbana* was dominant in the mixed culture at the end of the experiment (97  
107 % of the total population). In this study, the medium seems to have been more adapted to the microalgae  
108 rather than yeast *Ambrosiozyma cicatricose*, leading to microalgae dominance. In these studies, on mixed  
109 cultures, no mention of the enumeration method in the mixed culture is made.

110

111 The present study was conducted to develop a co-dominant and symbiotic mixed culture of two model  
112 organisms: the yeast *Saccharomyces cerevisiae* and the microalga *Chlorella vulgaris*. In order to promote co-  
113 dominance between the two organisms, a new growth medium was specifically designed, and the inoculum  
114 ratio was adjusted. The two species were grown in the same medium and in a non-aerated photo-bioreactor  
115 fitted with a fermentation lock to prevent gas exchange with the outside atmosphere. The absence of external  
116 air supply is intended to force the mutual symbiosis through synergetic effects of *in situ* gas exchange. To  
117 monitor the proportion of populations, a flow cytometric method was used to determine the cell concentration  
118 of each population in the mixed-culture. Dissolved O<sub>2</sub> and CO<sub>2</sub> were continuously measured in-line to  
119 evaluate the *in situ* gas exchange between the two species and to proof the mutual symbiosis.

120

121 Through this study, we propose a general methodology for the design of a co-dominant symbiotic mixed  
122 culture of a heterotroph and an autotroph in general and assess the success and the challenges of such strategy.  
123 The work presented here was performed on well-known model organisms but can provide the basis for more  
124 applied studies. The potential advantage of this work is that a symbiotic mixed culture would self-regulate the  
125 speed of the bioconversion hence the CO<sub>2</sub>-production and -utilization rates; it could potentially eliminate the  
126 need for gas supply and can lead to full utilization of the substrate. The potential savings would be those of  
127 recovering the cost of the portion of the substrate that is normally lost as CO<sub>2</sub>, making considerable savings in  
128 terms of gas supply avoidance and reducing environmental CO<sub>2</sub> emissions. In an economical assessment, all  
129 these savings would have to be weighed against the losses incurred by moderating the bioconversion speed in  
130 step with the photosynthetic rate.

131

## 132 **Materials and methods**

133

### 134 **Strategy of this study**

135 A diagram (Fig. 2) demonstrates the strategy used to establish co-dominant culture of *S. cerevisiae* and *C.*  
136 *vulgaris*.

137

### 138 **Microbial strains and their maintenance**

139 *S. cerevisiae* strain ID YLR249W was supplied by Life Technologies-University of California San Francisco.  
140 This clone expresses a cytoplasm fusion protein coupled to a green fluorescent protein (GFP). The protein of  
141 interest is the translation elongation factor 3 encoded by the gene YEF3 (Qin et al. 1987). The strain was  
142 maintained on YPG agar stock plates incubated at 25°C for 3 days and subsequently stored at 4°C for 3  
143 months before subculture. The YPG agar medium was composed of (g l<sup>-1</sup>): yeast extract (10), peptone (20)  
144 glucose (10) and agar (15) and the stock plates were renewed every three months.

145 *C. vulgaris* SAG 211-12 was obtained from the Culture Collection of Algae (SAG), University of Göttingen,  
146 Germany. The strain was maintained in liquid culture (50 ml in 250 ml flask) through weekly subculture into  
147 fresh medium, incubated at 25°C on an orbital shaker (120 rpm) with continuous lighting at 20 μmol m<sup>-2</sup> s<sup>-1</sup> at  
148 the surface of the culture and in air enriched with 1.5% (v/v) CO<sub>2</sub>. The liquid inorganic medium used was  
149 MBM (modified 3N-Bristol medium) (Clément-Larosière et al. 2014), with the following composition (mg l<sup>-1</sup>):  
150 NaNO<sub>3</sub> (750); CaCl<sub>2</sub>.2H<sub>2</sub>O (25); MgSO<sub>4</sub>.7H<sub>2</sub>O (75); FeEDTA (20); K<sub>2</sub>HPO<sub>4</sub> (75); KH<sub>2</sub>PO<sub>4</sub> (175); NaCl  
151 (20); H<sub>3</sub>BO<sub>3</sub> (2.86); MnCl<sub>2</sub>.4H<sub>2</sub>O (1.81); ZnSO<sub>4</sub>.7H<sub>2</sub>O (0.22); CuSO<sub>4</sub>.7H<sub>2</sub>O (0.08); MoO<sub>3</sub> 85% (0.036);  
152 CoSO<sub>4</sub>.7H<sub>2</sub>O, (0.09).

### 154 **Design of a specific medium for mixed culture**

155 Monocultures of *S. cerevisiae* and *C. vulgaris* were grown in three different media in order to define a  
156 medium suitable for co-dominance of the organisms in mixed culture. The media were based on different  
157 combinations of the microalgae growth medium (MBM) (described above) and components from the  
158 commonly used yeast growth YPG (yeast extract, peptone and glucose) medium (g l<sup>-1</sup>):

- 159 - MBM-G: MBM medium with glucose (10)
- 160 - MBM-GY: MBM medium with glucose (10) and yeast extract (10)
- 161 - MBM-GP: MBM medium with glucose (10) and peptone (20)

162 Erlenmeyer flasks (50 ml working volume; 250 ml total volume) were used for the monoculture of *C. vulgaris*  
163 and *S. cerevisiae* in the above media and the inoculation ratio was 1% (v/v) from a fully-grown culture. The  
164 flasks were incubated at 25°C on an orbital shaker (120 rpm) with continuous lighting at 80 μmol m<sup>-2</sup> s<sup>-1</sup>  
165 (LI250A Light Meter; LI-COR, USA) at the surface of the cultures. Yeast monocultures were conducted for 3  
166 days and microalgae monocultures for 5 days.

167 The medium finally selected and specifically designed for the mixed culture was named MBM-GP and was  
168 composed of (mg l<sup>-1</sup>): NaNO<sub>3</sub> (1,500); CaCl<sub>2</sub>.2H<sub>2</sub>O (50); MgSO<sub>4</sub>.7H<sub>2</sub>O (150); FeEDTA (40); K<sub>2</sub>HPO<sub>4</sub> (75);  
169 KH<sub>2</sub>PO<sub>4</sub> (175); NaCl (20); H<sub>3</sub>BO<sub>3</sub> (2.86); MnCl<sub>2</sub>.4H<sub>2</sub>O (1.81); ZnSO<sub>4</sub>.7H<sub>2</sub>O (0.220); CuSO<sub>4</sub>.7H<sub>2</sub>O (0.08);  
170 MoO<sub>3</sub> 85% (0.036); CoSO<sub>4</sub>.7H<sub>2</sub>O, (0.09), glucose (10,000); peptone (20,000).

171

### **Cultures in photo-bioreactors**

All experiments in photo-bioreactor (PBR) were conducted in a stirred bioreactor (5-liter working volume) (BIOSTAT Bplus – 5 L CC; Sartorius Stedim biotech, Göttingen, Germany). The PBR was lit with six LED lamps (Ledare 130 lumen, 2700 Kelvin, 27° dispersion angle, IKEA, Leiden, Netherlands). The light intensity at the inner surface of the bioreactor for each lamp was measured at  $1,600 \mu\text{mol m}^{-2} \text{s}^{-1}$  (LI250A Light Meter; LI-COR, USA). The stirring speed was 750 rpm with a 3-blades pitch-blade impeller (UniVessel 51, Germany), each inclined at 45° from the horizontal axis. The planar diameter of the impellers was 65 mm. The temperature was maintained at 25°C and the pH was controlled at 6.5 with automatic base KOH (1 mol l<sup>-1</sup>) or acid H<sub>3</sub>PO<sub>4</sub> (1 mol l<sup>-1</sup>) solutions addition based on the continuous measurements made by an internal pH probe (EasyFerm PLUS K8 325, Hamilton, Bonaduz, Switzerland). Dissolved oxygen (pO<sub>2</sub>) in cultures was measured with an internal probe (VisiFerm DO H2, Hamilton, Bonaduz, Switzerland). The pO<sub>2</sub> was expressed in terms of % of O<sub>2</sub> partial pressure in the liquid phase of the culture. The *S. cerevisiae* and *C. vulgaris* specific growth rates ( $\mu$ ) were calculated as the slope of the linear part of the logarithm of cell concentration plotted versus time.

### **Mixed cultures in PBR**

Two non-aerated mixed cultures in PBR were grown using MBM-GP medium. The experimental set up (Fig. 3) involved hermetically isolating the bioreactor to limit the exchange of gases with the atmosphere at the exterior of the bioreactor. Dissolved CO<sub>2</sub> (pCO<sub>2</sub>) was measured only in the mixed culture No. 1 with an external minisensor integrated in a flow cell (CO<sub>2</sub> Flow-Through Cell FTC-CD1, PreSens, Regensburg, Germany). The culture was circulated (90 ml min<sup>-1</sup>) through the flow cell with the aid of a peristaltic pump (520S/R, Watson Marlow) and back into the bioreactor. The flow-through cell was placed as close to the outlet from the bioreactor as possible. The passage of the culture over the sensor in the flow cell allowed the continuous measurement of pCO<sub>2</sub> via an optical fiber. As with the pO<sub>2</sub>, the pCO<sub>2</sub> was expressed in % of CO<sub>2</sub> partial pressure in the liquid phase of the culture. *S. cerevisiae* inoculum preparation was the same for both mixed cultures; *S. cerevisiae* was grown on MBM-GP medium, at 25°C, for 2 days. The preparation of the *C. vulgaris* inocula for the two mixed cultures differed; for the mixed culture No. 1, the *C. vulgaris* inoculum was grown on autotrophic MBM medium under continuous illumination, for 15 days, at 25°C and for mixed culture No. 2 the *C. vulgaris* inoculum was grown on heterotrophic MBM-GP medium under continuous lighting, for 15 days, at 25°C.

### **Monoculture of *S. cerevisiae* in PBR**

The monoculture of *S. cerevisiae* was grown in a non-aerated PBR in MBM-GP medium, with culture parameters as described above and the photo-bioreactor configuration was the same as for mixed culture (Fig.

207 3), there was no aeration and gas outlet was closed as described with a fermentation lock. The culture was lit  
208 as for mixed culture. The *S. cerevisiae* inoculum was grown in MBM-GP medium, at 25°C, for 2 days.

209

#### 210 ***Monocultures of C. vulgaris in PBR***

211 Two monocultures of *C. vulgaris* in PBR were grown, one in heterotrophic MBM-GP medium and the other  
212 in autotrophic MBM medium. For the first one the inoculum was prepared in MBM-GP medium, the second  
213 one using MBM medium and both under continuous light at 25°C for 15 days. Both culture conditions were  
214 set up as described above and the photo-bioreactor was continuously aerated with sterile air (Midisart 2000  
215 0.2 µm PTFE, Sartorius, Göttingen, Germany) at 500 ml min<sup>-1</sup> (0.1 vvm) (1 atm, 25°C).

216

#### 217 **The impact of ethanol on *C. vulgaris* growth**

218 *C. vulgaris* was grown on MBM medium in Erlenmeyer flasks (50 ml working volume; 250 ml total volume)  
219 and the flasks were incubated at 25°C on an orbital shaker (120 rpm) with continuous lighting at 20 µmol m<sup>-2</sup>  
220 s<sup>-1</sup> and in air enriched with CO<sub>2</sub> 1.5% (v/v). Four ethanol concentrations were tested (0, 2, 4 and 6 g l<sup>-1</sup>)  
221 (ethanol 96 %).

222

#### 223 **Analytical methods**

224

#### 225 ***Simultaneous enumeration of C. vulgaris and S. cerevisiae by flow cytometry***

226 A flow cytometer (Guava easyCyte™, EMD Millipore, Burlington, United States) was used to simultaneously  
227 determine the cell concentrations of *S. cerevisiae* and *C. vulgaris* in mixed culture. The excitation wavelength  
228 of the blue laser was 488 nm and detectors separately captured the Forward-scattered light (FSC) that is  
229 proportional to cell-surface area (size), the Side-scattered light (SSC) that indicated particles granularity and  
230 the fluorescence emitted by the cell (auto-fluorescence). Samples were diluted so that the cell enumeration  
231 was always performed at cell concentrations between 1×10<sup>5</sup> and 1×10<sup>6</sup> cells ml<sup>-1</sup>. The method for cell  
232 enumeration by flow cytometer suspensions containing only one of the microorganisms was previously  
233 validated against a Thoma counting chamber as the referent method (data not shown). Cell viability of *C.*  
234 *vulgaris* was also determined by flow cytometry using the Guava ViaCount Reagent (EMD Millipore,  
235 Burlington, United States).

236

#### 237 ***Glucose and ethanol measurements***

238 Culture supernatants were prepared by sample centrifugation (10 min, 3500 g), filtration (PTFE Syringe Filter  
239 0.2 µm, Fisherbrand, Waltham, United States) and High Pressure Liquid Chromatography (HPLC) (Ultimate  
240 3000, Thermo Scientific, United States). A cationic column (Aminex HPX-87H, Bio-Rad, United States) was  
241 used with 2 mM sulfuric acid as the mobile phase with a flow rate of 0.5 ml min<sup>-1</sup>, an injection volume of 10

242  $\mu\text{l}$ , a temperature of 45°C and a pressure of 60 bar. Detection was by means of a refractive index (RI) detector  
243 (RI 101, Shodex, Japan).

244

#### 245 ***Dry weight***

246 The dry weight was determined by sampling and centrifuging 10 ml of culture (10 min and 1,800 g). The  
247 pellet was washed with an equal volume of deionized water, and was centrifuged again (10 min, 1800 g) and  
248 the final pellet was transferred into a dry pre-weight ceramic cup (24 h, 105 °C). The pellet was dried  
249 overnight at 105 °C and cooled in a desiccator containing dry silica gel prior to weighing. A correlation  
250 between the dry weight and the cell concentration was established for *S. cerevisiae* and *C. vulgaris*:  $DW_{\text{yeast}} =$   
251  $3.25 \times 10^{-8} N_{\text{yeast}}$  (9 data points and  $R^2=0.91$ ) and  $DW_{\text{algae}} = 1.5 \times 10^{-8} N_{\text{algae}}$  (13 data points and  $R^2=0.96$ ) with  
252 DW the dry weight ( $\text{g l}^{-1}$ ) and N the cell concentration ( $\text{cells ml}^{-1}$ ). The experimental data points for the yeast  
253 were obtained from a monoculture in PBR using the MBM-GP medium and for the microalgae from a  
254 monoculture in PBR using the autotrophic medium MBM.

255

#### 256 ***CO<sub>2</sub> production and consumption***

257 CO<sub>2</sub> produced by yeast was assumed to be the main cause of culture acidification, resulting in the automatic  
258 addition of base (KOH) under the experimental conditions designed to keep the pH at 6.5. Consequently, the  
259 quantity of KOH solution is directly proportional to the CO<sub>2</sub> produced and was used to estimate the amount of  
260 CO<sub>2</sub> produced by yeast. The difference in the KOH added into the yeast monoculture and the mixed culture  
261 indicated the amount of CO<sub>2</sub> used by *C. vulgaris* and was used to perform a carbon mass balance.

262

### 263 **Results**

264

#### 265 **Validation of simultaneous enumeration of *C. vulgaris* and *S. cerevisiae* by flow cytometry**

266 A method for separately-enumerating *C. vulgaris* and *S. cerevisiae* populations in a mixed suspension was  
267 developed. The two species were distinguished on the basis of their specific auto-fluorescence detected by  
268 flow cytometry. *C. vulgaris* cells were distinguished through chlorophyll fluorescence (emission wavelength  
269 of 650 nm) and *S. cerevisiae* with the fluorescence of the constitutively expressed GFP protein (emission  
270 wavelength of 525 nm) (Fig. S1).

271 To validate the method, eleven mixed suspensions were prepared over a range of precise microalgae:yeast  
272 ratios (reference ratios) and the two populations in the mixed suspensions were measured with flow cytometry  
273 (experimental ratios). By plotting the experimental *C. vulgaris* ratio as a function of the referent microalgae  
274 ratio (Fig. 4), a linear relationship was obtained with a slope of 1.048 (correlation coefficient of 0.997; 11 data  
275 points). A linear relationship was also found for *S. cerevisiae* with a slope of 0.996 and a correlation  
276 coefficient of 0.998, validating the method for enumerating microalgae and yeast simultaneously in mixed  
277 suspensions.

278

279 **Strategy for a co-dominance of *C. vulgaris* and *S. cerevisiae* in mixed culture**

280

281 ***Design of a specific medium for mixed culture***

282 A growth medium that allowed the growth of both organisms was necessary. According to Fig. 5, MBM-G  
283 medium allowed only microalgae growth and *S. cerevisiae* growth was barely detectable. Monocultures of *C.*  
284 *vulgaris* and *S. cerevisiae* in MBM-GY medium showed the opposite results from those in MBM-G medium:  
285 MBM-GY allowed good growth of *S. cerevisiae* but not of *C. vulgaris*.

286 In MBM-GP medium, both *C. vulgaris* and *S. cerevisiae* could grow: the maximum *C. vulgaris* population  
287 was  $2 \times 10^8$  cells ml<sup>-1</sup> and the maximum yeast population was 10 times lower ( $2 \times 10^7$  cells ml<sup>-1</sup>).

288

289 ***Definition of parameters for mixed culture in PBR***

290 The temperature and pH in PBR were chosen to favor *C. vulgaris* growth. According to Kumar et al. (2010),  
291 temperatures of 15-26°C and neutral pH is optimal for microalgae growth. The form of the dissolved CO<sub>2</sub>  
292 concentration and the pH of the culture are directly linked so we chose to control the pH at 6.5 to achieve a  
293 good compromise between having a neutral pH and the dissolved CO<sub>2</sub> and bicarbonate species proportioned  
294 at around 0.5 at 25°C (Edwards et al. 1978).

295 The inoculum ratio was set up in way to minimize dominance of yeast and favor microalgae growth:

296

$$X_{0 \text{ } C. \text{ vulgaris}} = \frac{X_{0 \text{ } S. \text{ cerevisiae}} e^{\mu_{S. \text{ cerevisiae}} t}}{e^{\mu_{C. \text{ vulgaris}} t}} \tag{1}$$

297

298 with:

299  $X_{0 \text{ } C. \text{ vulgaris}}$ : initial *C. vulgaris* population

300  $X_{0 \text{ } S. \text{ cerevisiae}}$ : initial *S. cerevisiae* population

301  $\mu_{S. \text{ cerevisiae}}$ : *S. cerevisiae* specific growth rate

302  $\mu_{C. \text{ vulgaris}}$ : *C. vulgaris* specific growth rate

303 t: duration of the *S. cerevisiae* exponential phase.

304

305 **Monocultures in PBR**

306 Yeast and microalgae monocultures in PBR served as reference cultures for mixed culture. In the case of *C.*  
307 *vulgaris*, two reference conditions were tested: heterotrophic growth in the presence of glucose and,  
308 autotrophic growth in the absence of glucose. The behavior of the individual species in mixed cultures would  
309 then be compared to the latter reference conditions.

310

### 311 ***Monoculture of S. cerevisiae in closed and non-aerated PBR***

312 *S. cerevisiae* was grown on MBM-GP medium in PBR without aeration, exactly under the same conditions as  
313 for the subsequent mixed culture.

314 The yeast exponential growth phase ( $\mu=0.27\text{ h}^{-1}$ ) occurred within the first 24 h of incubation (15 h of  
315 exponential phase) and was accompanied with glucose and O<sub>2</sub> consumption. *S. cerevisiae* used all glucose  
316 within the first 31 h of incubation reaching a maximum population of  $2.2\times 10^7$  cells ml<sup>-1</sup> (Fig. 6). Within the  
317 first 31 h, *S. cerevisiae* also produced ethanol to a peak concentration of 4 g l<sup>-1</sup>.

### 319 ***Monocultures of C. vulgaris in aerated PBR in mixotrophic conditions***

320 *C. vulgaris* was grown on MBM-GP medium in PBR in the same way as *S. cerevisiae* in monoculture and as  
321 mixed cultures except that the *C. vulgaris* monocultures were continuously aerated. Aeration was mandatory  
322 for CO<sub>2</sub> provision to *C. vulgaris* for photosynthesis. The pO<sub>2</sub> in the culture was expected to be stable at 20.9  
323 % in the absence of net production or consumption of O<sub>2</sub> by *C. vulgaris*.

324 During the first 48 h of *C. vulgaris* growth in MBM-GP (Fig. 7a), the glucose and O<sub>2</sub> concentrations did not  
325 decrease while the population increased slightly from  $1\times 10^6$  to  $1.8\times 10^6$  cells ml<sup>-1</sup>. From 48 to 116 h of  
326 incubation, glucose decreased to complete depletion while the microalgae population increased from  $1.8\times 10^6$   
327 to  $4\times 10^8$  cells ml<sup>-1</sup>. During this period, the presumed heterotrophic microalgae growth was exponential with  
328  $\mu=0.09\text{ h}^{-1}$ .

### 330 ***Monocultures of C. vulgaris in aerated PBR in photo-autotrophic conditions***

331 *C. vulgaris* was grown in autotrophic monoculture using MBM medium in the absence of glucose (photo-  
332 autotrophically) and with continuous aeration (Fig. 7b) to supply atmospheric CO<sub>2</sub> as carbon source. The  
333 microalgae firstly grew exponentially ( $\mu=0.02\text{ h}^{-1}$ ), increasing the population from  $9\times 10^5$  to  $2\times 10^7$  cells ml<sup>-1</sup>  
334 and producing O<sub>2</sub> via photosynthesis. Starting from a value of 21%, the pO<sub>2</sub> reached 22% at the end of the  
335 exponential growth phase (100 h) then it continued to increase up to 22.3% and remained constant at the same  
336 level. This is a significant level of O<sub>2</sub> production considering the continuous flow of the air through the photo-  
337 bioreactor and the concentration of cells in the culture.

### 339 **Impact of ethanol on *C. vulgaris* growth**

340 The impact of the ethanol produced by *S. cerevisiae* when grown in MBM-GP medium in photo-bioreactor  
341 culture was assessed on *C. vulgaris* growth. Ethanol was added to *C. vulgaris* shake-flask cultures when the  
342 population reached  $7\times 10^6$  cells ml<sup>-1</sup> (corresponding to the initial *C. vulgaris* population in the mixed culture).  
343 Four ethanol concentrations (2, 4, 6 g l<sup>-1</sup> and 0 g l<sup>-1</sup>) were chosen according to the range of ethanol  
344 concentrations that could be produced by *S. cerevisiae* in monoculture and mixed culture (Fig. S2). The *C.*  
345 *vulgaris* growth profile was the same in all cultures (with or without the addition of ethanol). Moreover, cell  
346 viability of the four cultures remained at approximately 98 %.

347

348 **Mixed cultures of *S. cerevisiae* and *C. vulgaris* in closed and non-aerated PBR**

349

350 ***Mixed culture No. 1***

351 In the first mixed culture, the yeast inoculum was prepared in the newly designed MBM-GP medium while  
352 the microalgae inoculum was prepared in autotrophic MBM medium.

353 The *S. cerevisiae* behavior was similar in both mixed culture No. 1 (Fig. 8) and in the reference yeast  
354 monoculture (Fig. 6) (same maximum population, same specific growth rate, and same ethanol productivity).

355 On the other hand, the *C. vulgaris* growth in mixed culture No. 1 was weak compared with the reference  
356 photoautotrophic mixed culture. The microalgae population only slightly increased from  $7 \times 10^6$  to  $9 \times 10^6$  cells  
357  $\text{ml}^{-1}$  within the first 13 h and remained mainly constant until the end of incubation (168 h) but the dissolved  
358  $\text{CO}_2$  concentration gradually decreased from 16 % to 0 % from 48 h to 168 h at the end of the experiment.

359

360 ***Mixed culture No. 2***

361 For this second mixed culture, both the yeast and the microalgae inocula were prepared in the MBM-GP  
362 medium. This was in contrast to the mixed culture No. 1 where the microalgae inoculum was prepared in  
363 autotrophic MBM medium. The mixed cultures No. 2 and No. 1 only differed in the microalgae inoculum  
364 preparation. All other conditions including the microalgae:yeast inoculum ratio were identical.

365 The *S. cerevisiae* behavior was similar in both mixed cultures (Fig. 9) and in the reference yeast monoculture  
366 (Fig. 6) in terms of maximum population, specific growth rate, and ethanol productivity.

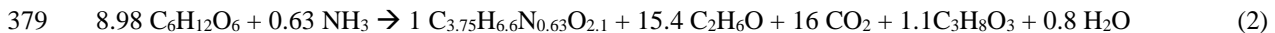
367 *C. vulgaris* started to grow from the beginning of incubation period, and without a lag phase, until 24 h and  
368 reached a maximum population of  $2.4 \times 10^7$  cells  $\text{ml}^{-1}$ , then its population remained stable until the end of the  
369 experiment.

370

371  **$\text{CO}_2$  production by *S. cerevisiae* in monoculture**

372 In monoculture of *S. cerevisiae* using MBM-GP medium, yeast biomass, ethanol and  $\text{CO}_2$  were produced  
373 during growth, the latter resulting in the acidification of the culture medium. Since a stable pH was specified  
374 for the fermentation, the acidification of the culture resulted in the automatic addition of KOH in step with  
375 yeast growth during the first 41 h of the culture. Ethanol ( $3.95 \text{ g l}^{-1}$ ) was produced (Fig. 6) and  $\text{CO}_2$  ( $3.91 \text{ g l}^{-1}$ )  
376 was released. The  $\text{CO}_2$  concentration was calculated by adopting the stoichiometric fermentation equation 2  
377 (Verduyn et al. 1990) using the ethanol yield ( $3.95 \text{ g l}^{-1}$ ) of the yeast monoculture in MBM-GP medium:

378



380

381 The  $\text{CO}_2$  released into the culture medium reacts with water to form carbonic acid  $\text{H}_2\text{CO}_3$  and then dissociates  
382 into  $\text{H}^+$  and  $\text{HCO}_3^-$  (Peña et al. 2015) acidifying the culture medium. Under the pH-control regime, the KOH

383 solution is added to maintain the pH at 6.5. The stoichiometry of the reaction between CO<sub>2</sub> and KOH is 1:1. A  
 384 total KOH volume of 337 ml was added during the yeast growth phase, which corresponded to 0.337 mole of  
 385 KOH added to the 5-liter culture medium. For ease of the mass balance calculation, the amount of KOH  
 386 added was expressed as a concentration ( $6.74 \times 10^{-2} \text{ mol l}^{-1}$ ):  
 387

$$[\text{KOH}] = \frac{V_{\text{KOH}} \times C_{\text{KOH}}}{V} \quad (3)$$

388

389 with:

390 [KOH]: base KOH concentration in the culture medium ( $\text{mol l}^{-1}$ )

391  $V_{\text{KOH}}$ : volume of KOH added to the culture medium (l)

392  $C_{\text{KOH}}$ : concentration of the KOH solution added to the photo-bioreactor ( $\text{mol l}^{-1}$ )

393 V: working volume (5 l)

394

395 Assuming that the KOH reacted exclusively with the H<sup>+</sup> from the hydration of the CO<sub>2</sub> produced,  $6.74 \times 10^{-2}$   
 396  $\text{mol l}^{-1}$  of KOH was used for pH adjustment:

397



398

399 The CO<sub>2</sub> concentration produced by yeast and neutralized by the KOH was  $2.97 \text{ g l}^{-1}$  and was calculated as:

400

$$[\text{CO}_2]_{\text{KOH}} = [\text{KOH}] \times M_{\text{CO}_2} \quad (5)$$

401

402 with:

403  $[\text{CO}_2]_{\text{KOH}}$ : concentration of CO<sub>2</sub> produced by yeast and reacted with KOH ( $\text{g l}^{-1}$ )

404 [KOH]: base KOH concentration in the culture medium ( $\text{mol l}^{-1}$ )

405  $M_{\text{CO}_2}$ : molar mass of CO<sub>2</sub> ( $44 \text{ g mol}^{-1}$ )

406

407 From the above calculation,  $3.91 \text{ g l}^{-1}$  of CO<sub>2</sub> would have been produced during yeast monoculture but only  
 408  $2.97 \text{ g l}^{-1}$  of CO<sub>2</sub> was measured based on the KOH used. This means that  $0.94 \text{ g l}^{-1}$  of CO<sub>2</sub> remained in  
 409 solution and/or passed into gaseous phase (Fig. 10b).

410

### 411 **CO<sub>2</sub> mass balance for *S. cerevisiae* and *C. vulgaris* in mixed culture No. 2**

412 The CO<sub>2</sub> production and biofixation was studied only in mixed culture No. 2 since the dominance between  
 413 microalgae and yeast was reached in this mixed culture and not in mixed culture No. 1.

414

415 In mixed culture No. 2, the KOH solution was added during the first 39 h of culture corresponding to yeast  
 416 growth. As explained above, the *S. cerevisiae* behavior was similar in both mixed culture No. 2 and in the

417 reference yeast monoculture (Fig. 10a); again the assumption was made that the KOH solution was mainly  
418 added to the mixed culture No. 2 to compensate for the medium acidification by the CO<sub>2</sub> release by the yeast.  
419 KOH (283 ml) was added during the growth phase of the yeast corresponding to  $5.7 \times 10^{-2}$  mol l<sup>-1</sup> of CO<sub>2</sub>  
420 equivalent to 2.49 g l<sup>-1</sup> of CO<sub>2</sub> (equation 3 and 5).

421

422 In the yeast reference monoculture 2.97 g l<sup>-1</sup> of CO<sub>2</sub> reacted with KOH whereas in mixed culture No. 2 only  
423 2.49 g l<sup>-1</sup>, of CO<sub>2</sub> reacted with KOH. The difference in CO<sub>2</sub> concentration most likely corresponds to the  
424 amount of CO<sub>2</sub> assimilated by microalgae in the mixed culture: 0.48 g l<sup>-1</sup> of CO<sub>2</sub> i.e. 0.13 g l<sup>-1</sup> of carbon. This  
425 concentration of carbon is coherent with the concentration of carbon required for the *C. vulgaris* biomass  
426 measured in mixed culture No. 2;  $1.5 \times 10^7$  cells ml<sup>-1</sup> of *C. vulgaris* was produced corresponding to a dry  
427 weight of 0.23 g l<sup>-1</sup> or  $8.8 \times 10^{-3}$  mol l<sup>-1</sup> (the microalgae composition is C<sub>1</sub>H<sub>1.78</sub>N<sub>0.165</sub>O<sub>0.495</sub> according to  
428 Scherholz and Curtis, 2013), and consequently 0.11 g l<sup>-1</sup> of carbon was required for the microalgae biomass  
429 production. Hence, the amount of carbon fixed by microalgae was determined by two different methods; the  
430 carbon fixation by *C. vulgaris* calculated from the microalgae biomass concentration corresponded to 85% of  
431 that calculated from the KOH consumption.

432

### 433 **Discussion**

434 The aim of this work was to establish a symbiotic relationship between a heterotroph organism and an  
435 autotroph organism based on gaseous (CO<sub>2</sub>) exchange. For this relationship to be useful in terms of in situ  
436 CO<sub>2</sub> mitigation, the rates for CO<sub>2</sub> production and consumption must be equal and in order to achieve this  
437 neither organisms must dominate, hence a co-dominant culture is necessary.

438 One of the main challenges in developing a mixed culture of a heterotroph and an autotroph is the selection of  
439 medium that would allow the co-dominance of the two species. As the  $\mu$  of *C. vulgaris* is considerably  
440 smaller (slower) than that of *S. cerevisiae*, the growth medium was designed to favor *C. vulgaris* development  
441 and to limit *S. cerevisiae* growth. The MBM-GP medium was a good compromise for a co-dominant culture  
442 of *C. vulgaris* and *S. cerevisiae* (Fig. 5). Both organisms were able to grow in this medium based on the  
443 available nitrogen and carbon for both *C. vulgaris* and *S. cerevisiae*: *C. vulgaris* could obtain nitrogen from  
444 nitrate, both organisms could access the short peptides and amino acids in the peptone and *S. cerevisiae* could  
445 additionally use the NH<sub>3</sub> supplied by the peptone. Glucose as carbon source would be available to both  
446 organisms but CO<sub>2</sub> would be additionally available to *C. vulgaris*. *S. cerevisiae* growth was limited by the  
447 availability of assimilable nitrogen to this organism. *S. cerevisiae* did not grow in the absence of peptone, as  
448 was the case with MBM-G medium. The addition of yeast extract to MBM-G medium to give MBM-GY  
449 medium provided a nitrogen source as well as other nutrients that could be used by the yeast for growth,  
450 however, the addition of yeast extract to the growth medium proved toxic to *C. vulgaris*. Finally, the MBM-  
451 GP medium allowed growth of both yeast and microalgae and also compensated for the higher  $\mu$  of yeast by  
452 limiting the maximum yeast population at 10% of the microalgae population.

453

454 Having designed a medium suitable for the growth of both organisms, three reference cultures were grown in  
455 photo-bioreactor: a fermentative culture of *S. cerevisiae* without aeration (MBM-GP medium), a culture of *C.*  
456 *vulgaris* under mixotrophic conditions with continuous aeration (MBM-GP medium) and an autotrophic  
457 culture of *C. vulgaris* with continuous aeration and in the absence of glucose (MBM medium). Two mixed  
458 cultures were also grown without aeration in MBM-GP medium.

459

460 The glucose was mainly fermented by *S. cerevisiae* in monoculture (Fig. 6) although the possibility of some  
461 respiration cannot be ruled out. *S. cerevisiae* mixes respiration and fermentation in the presence of O<sub>2</sub> and  
462 when external glucose concentration exceeds 0.8 mmol l<sup>-1</sup> (0.1 g l<sup>-1</sup>) (Verduyn et al. 1984; Otterstedt et al.  
463 2004). This phenomenon is called the “Crabtree effect” (Verduyn et al. 1984).

464

465 Under mixotrophic conditions *C. vulgaris* in monoculture (Fig. 7a), grew without using glucose and O<sub>2</sub> at the  
466 beginning of the culture, which, indicated photoautotrophic growth of the organism also reported by Ben  
467 Amor-Ben Ayed et al. (2017). After 48 of incubation, *C. vulgaris* started to grow heterotrophically using  
468 glucose and O<sub>2</sub>. *C. vulgaris* seems to “privilege” autotrophy as long as the microalgae population is small  
469 enough to allow satisfactory light penetration into the PBR. After that, *C. vulgaris* seems to have, at least,  
470 partly switched to heterotrophic metabolism. Microbial growth leads to an increase in light absorption and  
471 auto-shading by the microorganisms (Pfaffinger et al. 2016). The population in the shaded volume (central  
472 section of the PBR) may have used glucose and O<sub>2</sub> for growth through respiration, while the population in the  
473 lit volume (at the edge of the PBR and closed to the light source) could have grown photoautotrophically. In a  
474 well-mixed culture, as employed in this study, this means that as the average amount of light available to each  
475 cell decreases, *C. vulgaris* increasingly progresses towards a more heterotrophic metabolism.

476

477 In mixed culture, *S. cerevisiae* with its higher  $\mu$  could be expected to rapidly consume all glucose before *C.*  
478 *vulgaris* would have time to grow heterotrophically. This means that the latter would grow fully  
479 photoautotrophically in mixed culture. For this reason, a reference culture of *C. vulgaris* was grown under  
480 photoautotrophic conditions in the usual growth medium used for this purpose; MBM medium (Fig. 7b).

481

482 For the *C. vulgaris* photoautotrophic monoculture the exponential growth phase was followed by a longer  
483 linear growth phase from the point where the population density increased beyond 2×10<sup>7</sup> cells ml<sup>-1</sup>. This is  
484 most likely due to light limitation; once the culture reaches a certain population density that would result in  
485 considerable autoshadowing and restricted light penetration into the core of the culture. The growth would  
486 then be directly related to the light arrival rate, which is constant resulting in linear growth.

487

488 In summary, the *S. cerevisiae* monoculture (Fig. 6) and the microalgae *C. vulgaris* autotrophic monoculture  
489 (Fig. 7b) were used as reference cultures to compare with the mixed cultures. The respective  $\mu$  were used to  
490 adjust the microalgae:yeast inoculation ratio to 30:1 (equation 1) in an attempt to minimize the possibility of  
491 yeast domination in the mixed culture. Two mixed cultures were grown without aeration where an attempt  
492 was made to coordinate the growth of the two organisms by adjusting their respective inoculation rates as  
493 described above.

494

495 In the first mixed culture (No. 1) (Fig. 8), *S. cerevisiae* consumed the glucose within the first 48 h as had been  
496 observed in the reference yeast monoculture (Fig. 6). The *S. cerevisiae* biomass and ethanol production were  
497 the same for both the mixed culture and the yeast reference culture. Since no glucose was available for the  
498 microalgae, *C. vulgaris* probably grew fully photosynthetically in the mixed culture. This was additionally  
499 supported by the observation that in the *C. vulgaris* monoculture in the presence of glucose, the glucose was  
500 not consumed during the first 48 h (Fig. 7a). On the other hand, *C. vulgaris* growth in mixed culture was weak  
501 with the maximal microalgae population 2.5 times lower than the reference monoculture ( $9 \times 10^6$  and  $2 \times 10^7$   
502 cells ml<sup>-1</sup> respectively) (Fig. 7b). Ethanol or CO<sub>2</sub> toxicity can both be excluded as reasons for this low  
503 microalgal biomass production, as discussed below. Although the *C. vulgaris* population was weak, the  
504 microalgal cells remained active during the entire experiment (168 h). During the latter phases of the  
505 experiment, there were instances where the sun shone directly on the PBR; intermittent negative pCO<sub>2</sub>  
506 troughs and concomitant positive pO<sub>2</sub> peaks were observed during these transient periods. This can be taken  
507 as a strong indicator that both organisms in the mixed culture were metabolically active and that synergy  
508 effects between yeast and microalgae occurred. The final pCO<sub>2</sub> concentration reached almost its initial level  
509 indicating that in principle, *in situ* CO<sub>2</sub> mitigation in mixed culture is feasible, although the efficiency of the  
510 process remains to be improved.

511

512 A second mixed culture (No. 2) was grown to increase the microalga population in mixed culture (Fig. 9). The  
513 *C. vulgaris* inoculum was prepared in the same medium as used for the mixed culture (MBM-GP) in order to  
514 pre-adapt the organism to this medium and promote immediate growth of *C. vulgaris* straight after inoculation  
515 into the photo-bioreactor. *C. vulgaris* grew straight away from the start reaching a maximum population 2.7  
516 times higher than that of the first mixed culture. By modifying the preparation of the inoculum, it was  
517 possible to achieve the same population concentration for both organisms ( $2 \times 10^7$  cells ml<sup>-1</sup>). Presumably the  
518 pre-adaptation allowed *C. vulgaris* to have the enzymes necessary for the utilization of the amino acids and  
519 the small peptides present in the peptone in the photo-bioreactor medium. Like in the first mixed culture, *C.*  
520 *vulgaris* grew on the CO<sub>2</sub> produced by *S. cerevisiae* as there was no other source of CO<sub>2</sub>. Of the CO<sub>2</sub>  
521 produced by *S. cerevisiae* in mixed culture No. 2, 12% was consumed directly by *C. vulgaris*, and the 64% of  
522 CO<sub>2</sub> captured by the KOH was in the HCO<sub>3</sub><sup>-</sup> form and still available to the microalgae for utilization (Fig.  
523 10c).

524

525 Ethanol is known to exhibit antimicrobial activity by attacking cell membranes (Patra et al. 2006) and it was  
526 important to assess the potential toxicity of the ethanol produced by *S. cerevisiae* in mixed culture on *C.*  
527 *vulgaris*. Firstly, the growth of the microalgae in the mixed culture No.2 provided the first indication that at 4  
528 g l<sup>-1</sup>, ethanol was not toxic to *C. vulgaris*. This observation was further confirmed with a shake-flask  
529 experiment where exogenous ethanol (2, 4 and 6 g l<sup>-1</sup>) was added to growing autotrophic cultures of *C.*  
530 *vulgaris*. The same growth profiles were observed for both control cultures and cultures containing ethanol,  
531 even at concentrations higher than those measured in mixed cultures. The cell viabilities of these  
532 monocultures were high (approx. 100%) even at the end of the incubation period (411 h).

533

534 In conclusion, in order to encourage mutual symbiosis, we developed a mixed culture of *C. vulgaris* and *S.*  
535 *cerevisiae* in PBR in a way that neither organism dominated the other in terms of population concentration.  
536 The method developed for simultaneous cell enumeration with flow cytometry permitted to rigorously  
537 monitor the two populations in the mixed culture. The dissolved O<sub>2</sub> and CO<sub>2</sub> probes brought relevant  
538 measurements that allowed us to follow gas evolution. The results indicated that the medium design, the  
539 culture conditions, the inoculum ratio and the *C. vulgaris* inoculum preparation all contributed for co-  
540 dominance of the two species. By comparing the physiological behavior of microalgae and yeast in  
541 monoculture and mixed culture, co-dominance and a mutual symbiosis based on *in situ* gas exchange were  
542 demonstrated. This work opens the perspective for *in situ* CO<sub>2</sub> mitigation, full utilization of the organic  
543 substrate and a reduction in aeration costs of biotransformation processes.

544

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548

#### 549 **Compliance with ethical standards**

550

- 551 • Conflict of Interest Statement

552 The authors declare no conflict of interest.

553

- 554 • Statement of Informed Consent, Human/Animal Rights

555 This article does not contain any studies with human participants or animals performed by any of the authors.

556

- 557 • Declaration of authors agreement

558 The authors declare their agreement to authorship and submission of the manuscript for peer review.

559

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647

648 **Figure captions**

649

650 **Fig. 1** A coupled culture (a) and a mixed culture (b) of heterotrophic and autotrophic organisms; (a) gases  
651 pass from the liquid phase of the heterotrophic culture into a gaseous phase (blue dashed arrows) and they  
652 then pass from the gaseous phase into the liquid phase of the photo-bioreactor (red solid arrows); (b) Diagram  
653 of a mixed culture of heterotrophic and autotrophic organisms with CO<sub>2</sub> produced by heterotrophic  
654 metabolism (the gases are generated and reused in situ)  
655

656 **Fig. 2** Flow chart for the development of a co-dominant mixed culture between *S. cerevisiae* and *C. vulgaris*  
657

658 **Fig. 3** Diagram showing the closed PBR configuration used for the mixed culture of microalgae and yeast.  
659 The culture was not aerated, and the bioreactor was fitted with a fermentation lock, by using a U-tube  
660 manometer filled with 200 mm of H<sub>2</sub>O. The pO<sub>2</sub> was monitored continuously using the immersed optic pO<sub>2</sub>  
661 probe and the pCO<sub>2</sub> sensors was placed in a loop that passed culture over it continuously by means of a  
662 peristaltic pump  
663

664 **Fig. 4** Validation of simultaneous enumeration of *C. vulgaris* and *S. cerevisiae* by flow cytometer;  
665 experimental *versus* reference ratios of *C. vulgaris* in eleven mixed cells suspensions (black circles) at  
666 different microalgae:yeast ratios  
667

668 **Fig. 5** Design of a specific medium for mixed culture; maximum population of *S. cerevisiae* GFP (light grey)  
669 or *C. vulgaris* (dark grey) in monoculture using three candidate media for mixed culture. Each monoculture of  
670 yeast or microalgae was performed in shake-flask and in duplicate  
671

672 **Fig. 6** *S. cerevisiae* monoculture in non-aerated PBR using heterotrophic MBM-GP medium. The yeast  
673 population is represented by orange circles. Glucose (filled triangles) and ethanol (empty triangles) are  
674 represented by symbols connected by dashed lines. Dissolved O<sub>2</sub> is represented by blue solid line. Error bars  
675 represent standard deviations of duplicate analyses of population concentration  
676

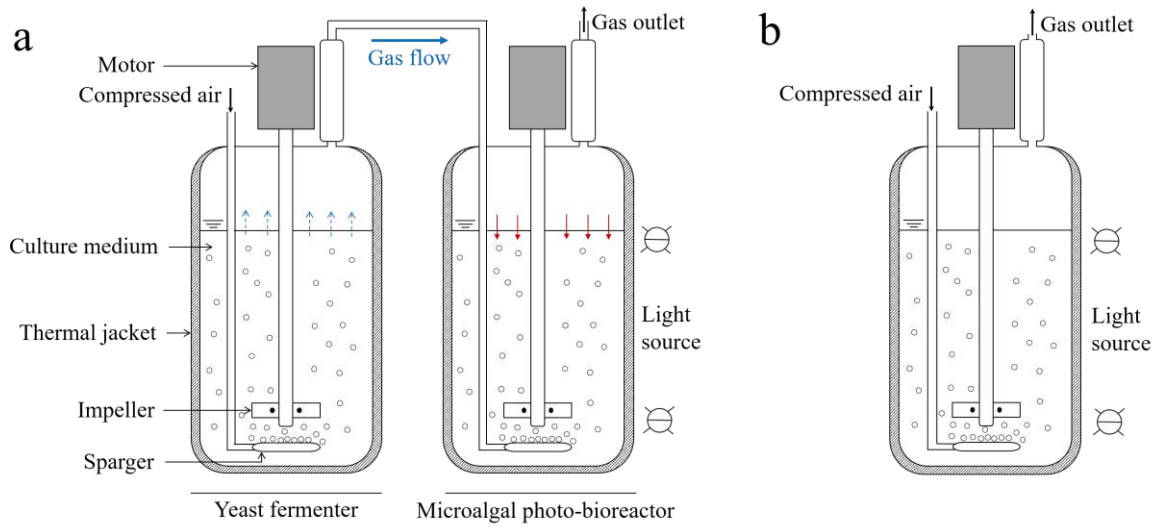
677 **Fig. 7** *C. vulgaris* monoculture in aerated PBR using heterotrophic MBM-GP medium (a) and *C. vulgaris*  
678 monoculture in aerated PBR using autotrophic MBM medium (without glucose and peptone) (b). The  
679 microalgae population is represented by green squares. Dissolved O<sub>2</sub> is represented by blue solid line and  
680 glucose (filled triangles) by symbols connected by dashed lines. Error bars represent standard deviations of  
681 duplicate analyses of population concentration  
682

683 **Fig. 8** Mixed culture No. 1 of *C. vulgaris* and *S. cerevisiae* in closed and non-aerated PBR using MBM-GP  
684 medium. The yeast inoculum was prepared using heterotrophic MBM-GP medium while the microalgae  
685 inoculum was prepared in autotrophic MBM medium. The yeast population is represented by orange circles  
686 and the microalgae population by green squares. Glucose (filled triangles) and ethanol (empty triangles) are  
687 represented by symbols connected by dashed lines. Dissolved O<sub>2</sub> and CO<sub>2</sub> are represented by blue dashed line  
688 and red solid line respectively. Error bars represent standard deviations of duplicate analyses  
689

690 **Fig. 9** Mixed culture No.2 of *C. vulgaris* and *S. cerevisiae* in closed and non-aerated PBR using MBM-GP  
691 medium. The yeast and microalgae inocula were both prepared in heterotrophic MBM-GP medium. The yeast  
692 population is represented by orange circles and the microalgae population by green squares. Glucose (filled  
693 triangles) and ethanol (empty triangles) are represented by symbols connected by dashed lines. Dissolved O<sub>2</sub>  
694 is represented by blue solid line. Error bars represent standard deviations of duplicate analyses  
695

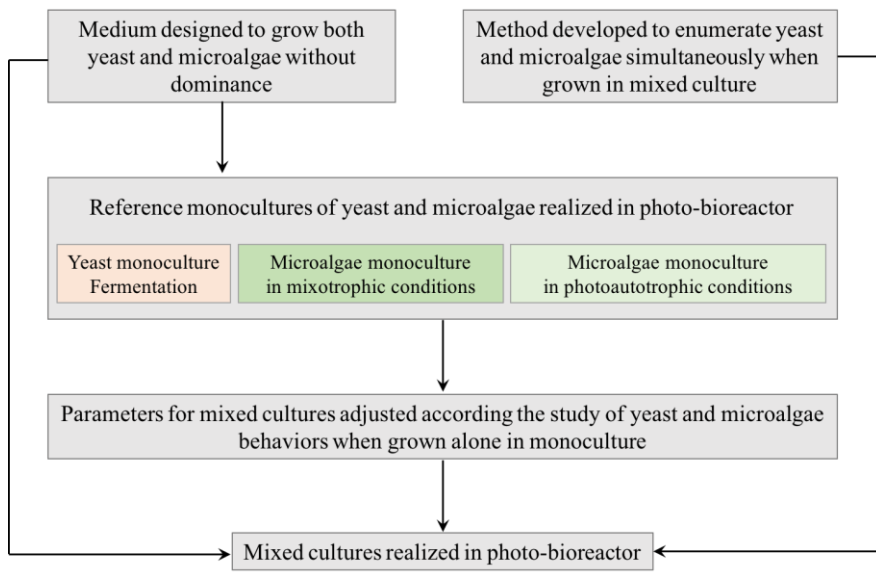
696 **Fig. 10** Automatic addition of base KOH solution in *S. cerevisiae* monoculture and in mixed culture No. 2 for  
697 pH adjustment at 6.5 (a). Repartition of CO<sub>2</sub> produced by *S. cerevisiae* in *S. cerevisiae* monoculture (b) and in  
698 mixed culture No. 2 (c). Error bars represent standard deviations of duplicate analyses of yeast population  
699 concentration  
700

701 **Figures**



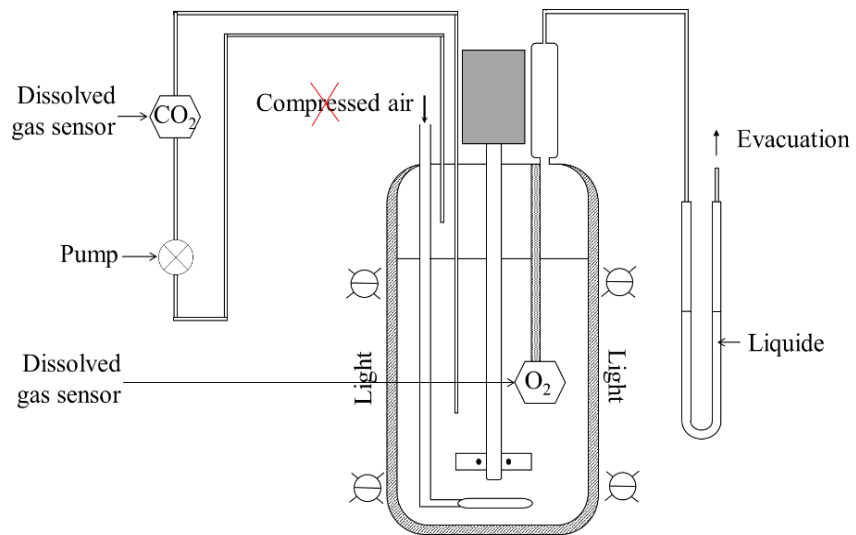
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703 **Fig. 1** program: PowerPoint



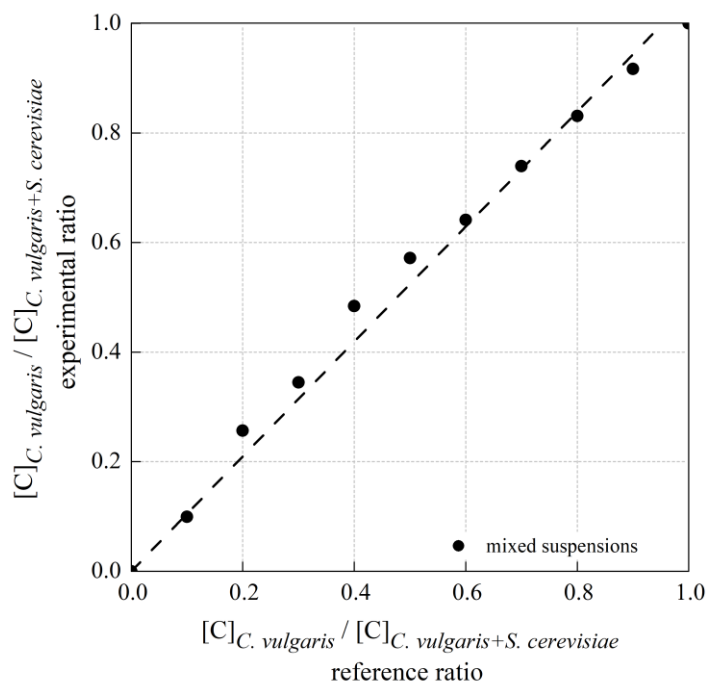
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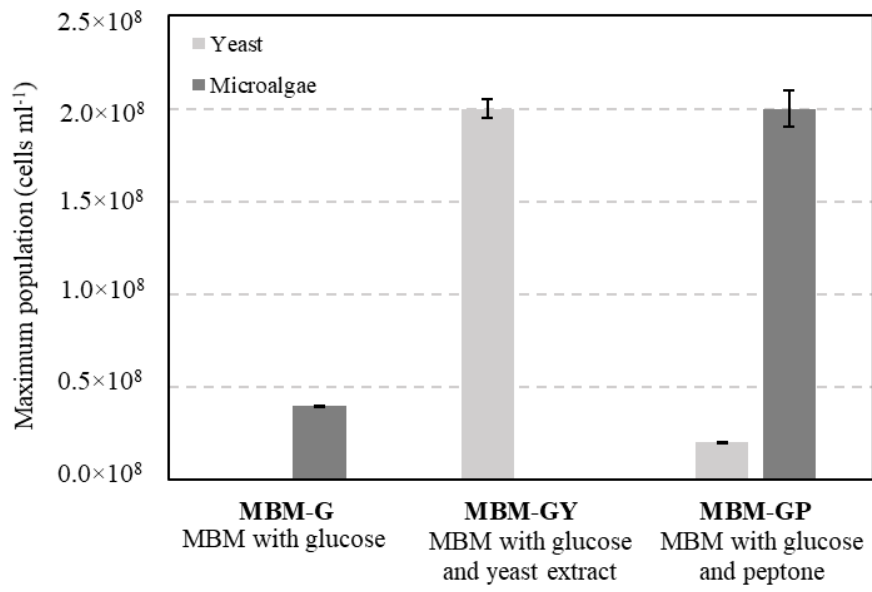
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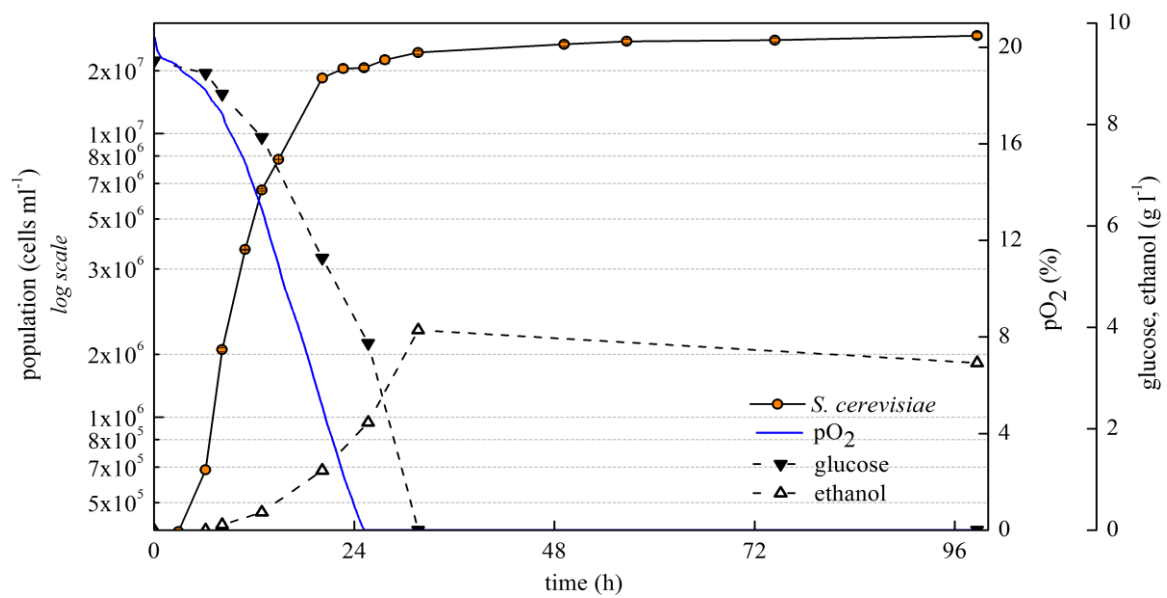
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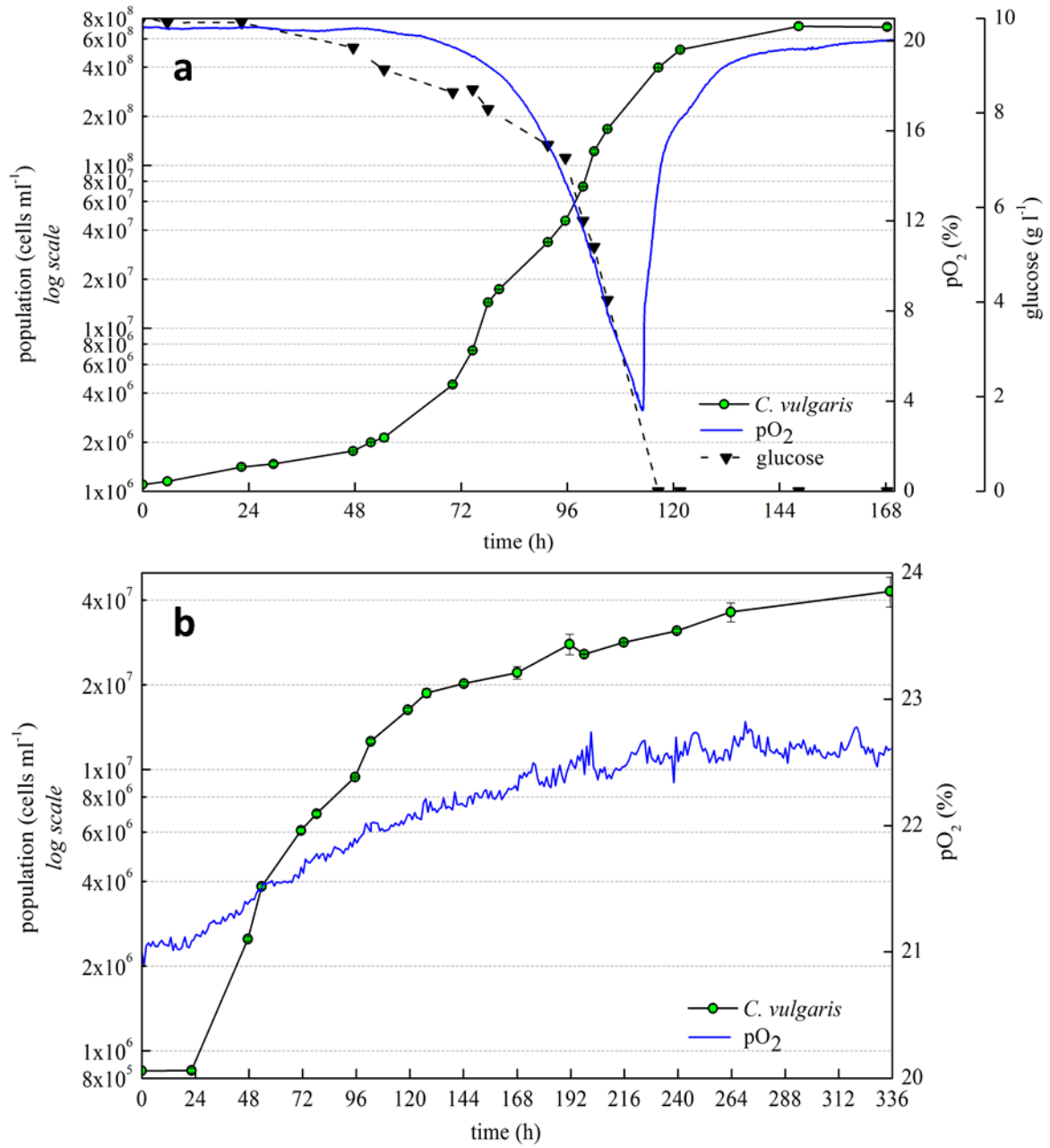
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711 **Fig. 5** program: OriginPro



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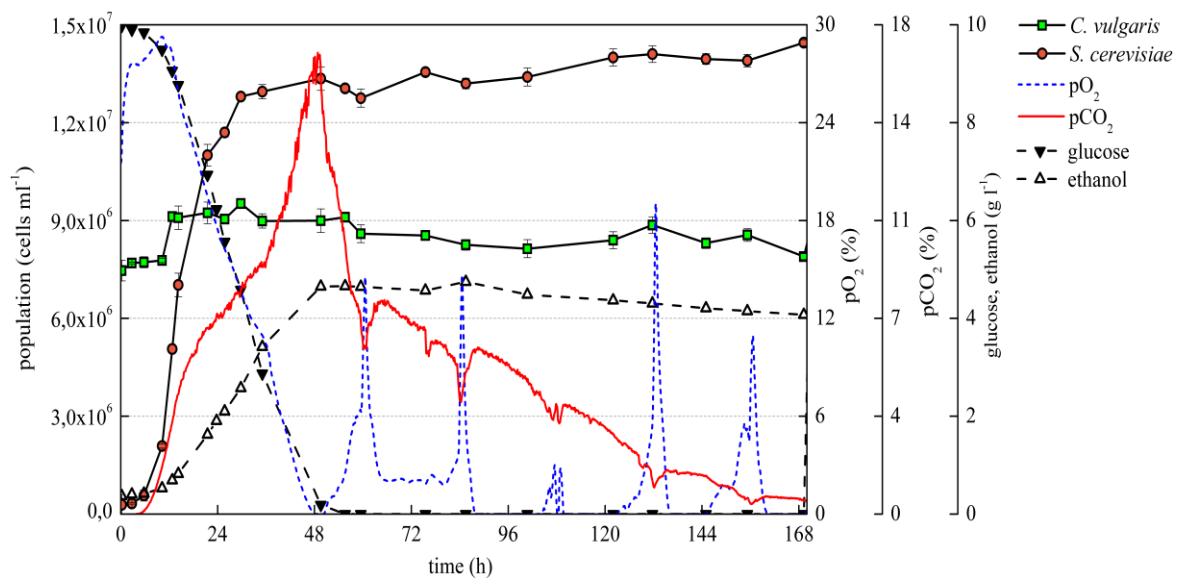
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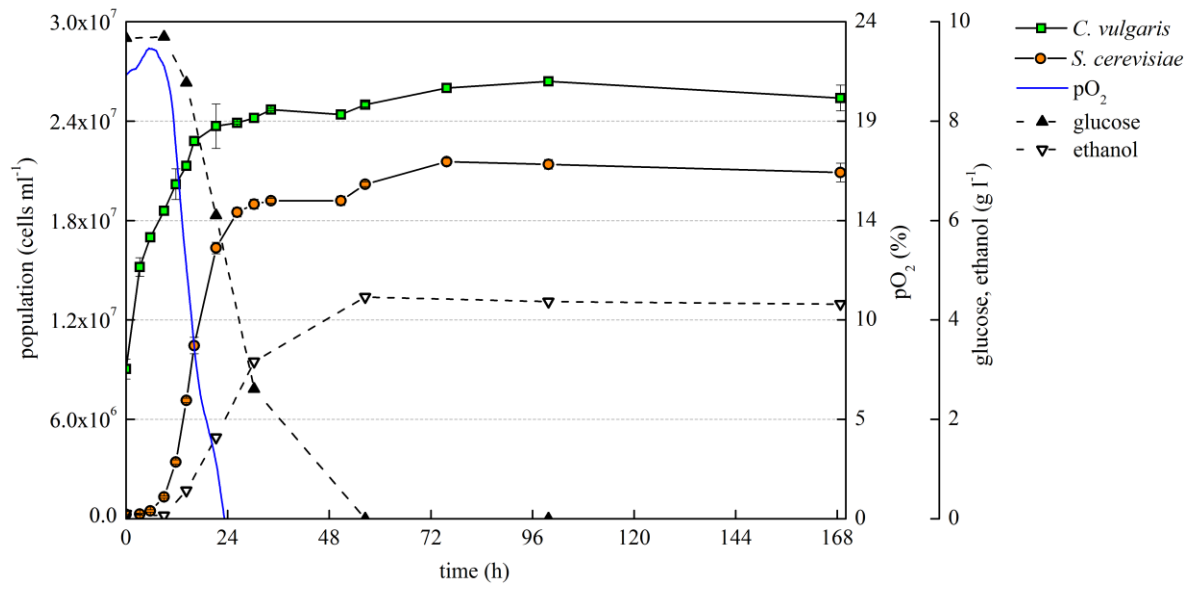
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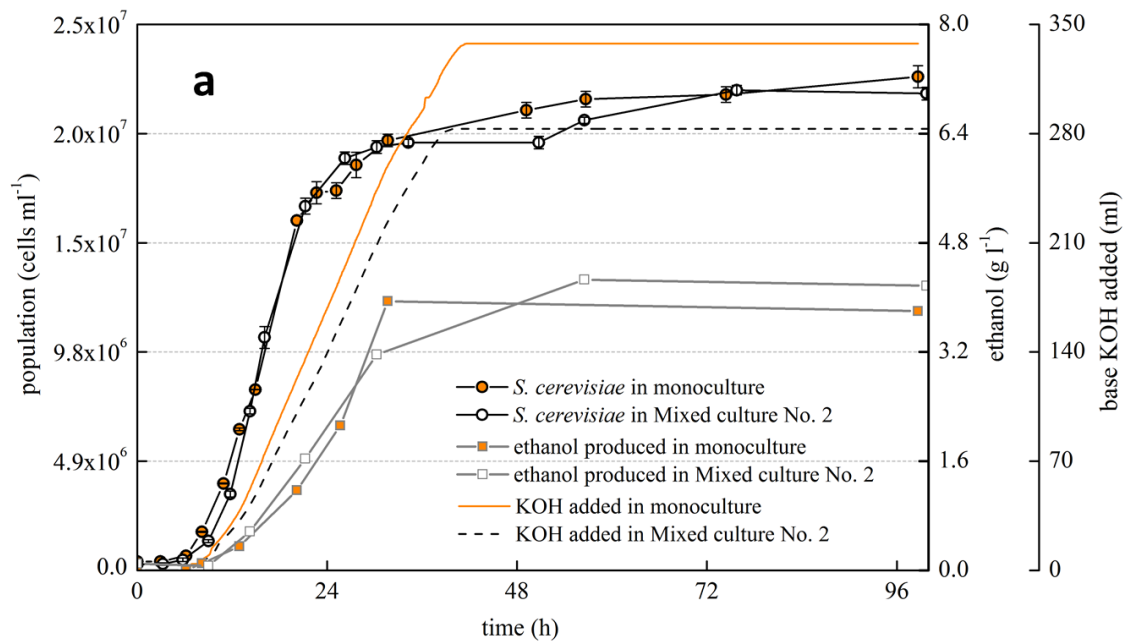
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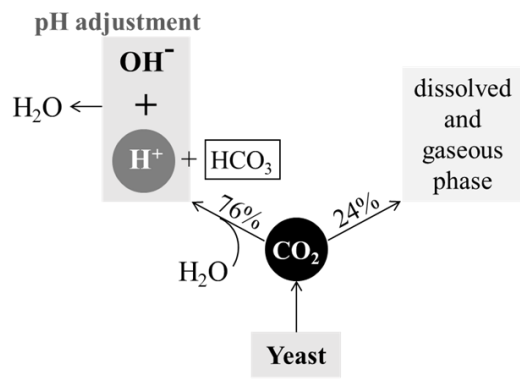
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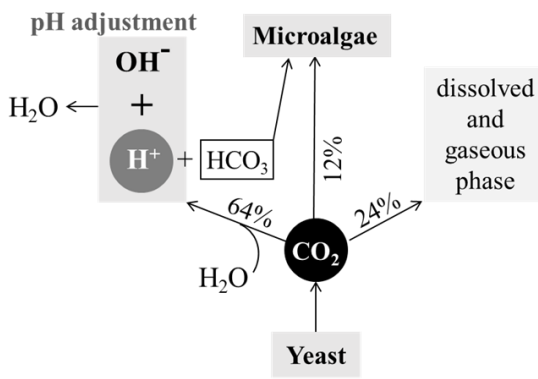
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**b** *S. cerevisiae* monoculture in PBR



**c** Mixed culture No. 2 in PBR



722  
723  
724

**Fig. 10** program: OriginPro (a) and PowerPoint (b and c)