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1 **Use of fermentative metabolites for heterotrophic microalgae**
2 **growth: yields and kinetics**

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10 **Abstract**

11 The growth of two lipid-producing *Chlorella* species on fermentative end-products
12 acetate, butyrate and lactate, was investigated using a kinetic modeling approach.
13 *Chlorella sorokiniana* and *Auxenochlorella protothecoides* were grown on synthetic
14 media with various (acetate: butyrate: lactate) ratios. Both species assimilated
15 efficiently acetate and butyrate with yields between 0.4 and 0.5 g carbon of biomass/ g
16 carbon of substrate, but did not use lactate. The highest growth rate on acetate, 2.23 d⁻¹,
17 was observed for *Chlorella sorokiniana*, and on butyrate, 0.22 d⁻¹, for *Auxenochlorella*
18 *protothecoides*. Butyrate removal started after complete acetate exhaustion (diauxic
19 effect). However, butyrate consumption may be favored by the increase of biomass
20 concentration induced by the initial use of acetate. A model combining Monod and
21 Haldane functions was then built and fitted the experimental data well for both species.
22 Butyrate concentration and (acetate: butyrate) ratios were identified as key parameters
23 for heterotrophic growth of microalgae on fermentative metabolites.

24 **Keywords:** Heterotrophy, Volatile Fatty Acids, *Chlorella*, Kinetic model, Diauxic
25 effect

26 **1 Introduction**

27 Production of microalgae has gained a lot of interest in the past decades due to their
28 ability to synthesize biomolecules having potential industrial applications such as
29 energy generation. The use of microalgae to produce biofuels offers the main advantage
30 of improving biomass and lipid yields and land use when compared to oil crops.

31 Georgianna and Mayfield (2012) assessed that microalgae could produce the same
32 amount of oil than palm oil using six times less area. Due to higher growth rates,
33 expressed in days instead of months or years, and lipid contents up to 80 % of dry
34 biomass, microalgae are very promising candidates to produce massively and constantly
35 biofuels (Wu et al., 2012).

36 Growing microalgae in darkness and on organic compounds presents several advantages
37 compared to autotrophic cultivation systems: (1) a higher biomass density can be
38 achieved, leading to reduce the costs of microalgae harvesting compared with low
39 density systems operated in autotrophy (Doucha and Lívanský, 2011). (2) Higher
40 growth rates are observed in heterotrophy, reducing the time of cultivation (Kim et al.,
41 2013). (3) Higher lipid yields can be achieved in heterotrophic cultures, improving
42 economic competitiveness of microalgae biofuels (Wan et al., 2012).

43 Industrial production of heterotrophic microalgae is hampered by the high economic
44 and environmental costs of glucose, commonly used as main carbon source. Therefore,
45 glycerol, acetate or wastewaters containing glucose, such as whey permeate, are
46 considered as the most promising alternatives of low cost carbon substrates (Espinosa-

47 Gonzalez et al., 2014). Glycerol is a by-product of biodiesel and can be used to sustain
48 heterotrophic algal growth and reduces the overall process cost (Heredia-Arroyo et al.,
49 2010). Acetate is a by-product of anaerobic digestion and often accumulates in dark
50 fermentation processes. Interestingly, microalgae can easily convert acetate into acetyl-
51 CoA which is the main precursor for lipid synthesis (Ramanan et al., 2013).

52 *Auxenochlorella protothecoides* was successfully grown on glycerol and acetate, with
53 maximal biomass concentrations of 3.97 and 3.62 g L⁻¹ and maximal lipids contents of
54 20.33 and 52.38 % of dry biomass, respectively (Heredia-Arroyo et al., 2010). These
55 results were very similar to the values reported with glucose, i.e. 4.25 g L⁻¹ of biomass
56 and a lipid content of 25.25 % (Heredia-Arroyo et al., 2010). The use of other organic
57 substrates, such as sucrose, lactose and ethanol may not support substantial
58 heterotrophic growth of microalgae (Perez-Garcia et al., 2011b).

59 Coupling dark fermentation, producing hydrogen and volatile fatty acids (VFA), such as
60 acetate, butyrate and lactate, with microalgal mixotrophic bioprocesses has been
61 recently investigated with the purpose of lowering the costs of the overall process by
62 finding new sources of substrates. Hu *et al* (2013) showed the feasibility of growing
63 mixotrophically a newly isolated *Chlorella* sp. on acidogenic swine effluents containing
64 a mixture of acetate, propionate and butyrate. The effluent had to be diluted 8 folds in
65 order to promote the microalgae growth and it was assumed that growth inhibition was
66 caused by high concentrations of VFAs. Consistently, Liu et al (2012) pointed out an
67 inhibitory effect on mixotrophic growth of *Chlorella vulgaris* when butyrate
68 concentration was higher than 0.1 g L⁻¹. In contrast, Wen *et al* (2013) found that
69 butyrate was degraded before acetate but after valerate and ethanol by *Chlorella*
70 *protothecoides* in heterotrophic conditions on anaerobically digested sugarcane

71 wastewaters. Venkata Mohan and Prathima Devi (2012) used a mixed culture of
72 microalgae, containing species of *Scenedesmus* and *Chlorella*, in order to convert dark
73 fermentation effluents into microalgal biomass (1.42 g L^{-1}) and microalgal lipids (26.4
74 % of dry weight), under mixotrophic conditions. They reported that acetate was a
75 preferred substrate compared to butyrate and propionate. Ren *et al* (2013) used
76 sterilized dark fermentation effluents, composed of at least 95 % of acetate and ethanol,
77 to sustain heterotrophic growth of *Scenedesmus* sp. In this study, acetate was
78 completely removed but not ethanol. The subsequent biomass production and lipids
79 content reached 1.98 g L^{-1} and 40.9 % of dry weight, respectively. Liu *et al* (2013)
80 pointed out that butyrate removal was higher under heterotrophic than mixotrophic
81 conditions due to the competition between organic and inorganic carbon uptake.
82 Considering all these studies, the heterotrophic growth of microalgae on a mixture of
83 organic substrates is still difficult to estimate mainly because of the lack of a clear
84 behavior when a mixture of substrates is used.

85 *Auxenochlorella protothecoides* and *Chlorella sorokiniana* are two well-known lipid-
86 producing microalgae, their lipids content can be as high as 57 and 61.5% of dry
87 weight, respectively (Ramanna et al., 2014; Wang et al., 2013). The aim of this study
88 was to characterize the growth *Auxenochlorella protothecoides* and *Chlorella*
89 *sorokiniana*, under strict heterotrophic conditions and in presence of three organic acids,
90 i.e. acetate, butyrate and lactate, mainly generated in dark fermentation processes.
91 Lipids content 51.7 and 61.5 % of dry biomass for *Auxenochlorella protothecoides* and
92 *Chlorella sorokiniana* Kinetic parameters were assessed using a global kinetic model
93 fitting biomass growth and organic carbon removal.

94 **2 Materials and methods**

95 **2.1 Microalgae strains and culture conditions**

96 *Chlorella sorokiniana* (CCAP 211/8K) and *Auxenochlorella protothecoides* (CCAP
97 211/8K) were obtained from the CCAP culture collection (United Kingdom). A
98 modified BG11 medium (UTEX, <http://www.utex.org/>) was used to pre-cultivate the
99 inoculum. Sodium bicarbonate (10 mM), chlorure ammonium (5 mM) and dipotassium
100 phosphate (0.31 mM) were used as inorganic carbon (C), nitrogen (N) and phosphorus
101 (P) sources, respectively. Since *A. protothecoides* is auxotrophic for thiamine (vitamin
102 B1) (Huss et al., 1999), the medium was supplemented with 1 mL/L of F/2 medium's
103 vitamins solution (CCAP, <http://www.ccap.ac.uk/>). The pH of the medium was set at
104 6.5 prior to sterilization. Ammonium and vitamins solution were sterilized using a filter
105 with 0.2 µm pores. All other media components were sterilized by autoclaving at 121
106 °C for 20 min. Both species were cultivated in 500 mL Erlenmeyer flasks, containing
107 200 mL of modified BG11 medium. Inoculum was cultivated in autotrophic conditions
108 to avoid a cometabolism effect, i.e. the induction of a preferred organic substrate
109 (Narang and Pilyugin, 2005). The flasks were placed at 25 °C under a light intensity of
110 100 µmol photons.m⁻².s⁻¹. After 5 to 7 days of cultivation, the culture was used to
111 further inoculate (10 % V/V) the different culture media. Axeny was daily checked by
112 phase contrast microscopy and DAPI coloration microscope observations as well as
113 spreading cultures on ATCC5 solid media (ATCC, <http://www.lgcstandards-atcc.org/>).

114 **2.2 Heterotrophic growth on mixtures of organic acids**

115 In order to evaluate the cell viability of the inoculum, a positive control was cultivated
116 under photoautotrophic conditions. A negative control containing inorganic carbon as

117 carbon source (bicarbonate) was placed in the dark to evaluate the ability of the
118 inoculum to grow on its own cellular reserves.

119 For the growth on organic carbon compounds, different initial concentrations of C
120 sources were tested alone and in mixture (Table 1) but the C: N: P molar ratio was set at
121 48: 16: 1. Prior to sterilization with a 0.2 μm pores filter, working solutions of acetate,
122 butyrate and lactate were neutralized at pH 6.5 with NaOH. To maintain a non-
123 inhibitory pH throughout the experiments, pH was buffered with 100 mM of 2-(N-
124 morpholino) ethanesulfonic acid (MES) (Abdelaziz et al., 2014). The initial pH was set
125 between 6 and 6.5(Zheng et al., 2013). Microalgae were cultivated in 125 mL black
126 Erlenmeyer flasks with cotton plugs containing 40 mL of medium. The flasks were
127 placed under dark conditions at 25 °C on a rotary shaker (150 rpm).

128 An acclimation study was also performed during 4 weeks in order to improve butyrate
129 removal. *Auxenochlorella protothecoides* was successively cultivated on a medium
130 containing 0.1 gC L⁻¹ of butyrate. Medium composition was the same as described
131 above.

132 All experiments and controls were done in triplicates.

133 [Table 1]

134 **2.3 Analytical methods**

135 **2.3.1 Biomass measurement**

136 Biomass growth was quantified by measurement of the Optical Density at 800 nm
137 (OD₈₀₀) to minimize pigment interference (Schmidt et al., 2005). Culture samples of
138 300 μL were dispensed in a 96 well BD Falcon® microplate and analyzed using a

139 Spectrophotometer Infinite Nanoquant M200 (Tecan®). Cellular Dry Weight (CDW)
140 was determined after filtering 15 mL of algal samples on pre-weighed GF/F Whatman®
141 filters that were dried overnight at 105°C. CDW was correlated to OD₈₀₀ using a
142 calibration curve. For *Chlorella sorokiniana* and *Auxenochlorella protothecoides* the
143 equations were $CDW (g L^{-1}) = 1.24 * OD_{800}$ ($R^2 = 0.9533$) and $CDW (g L^{-1}) =$
144 $1.38 * OD_{800}$ ($R^2 = 0.9935$), respectively.

145 **2.3.2 Organic acids measurements**

146 Volatile fatty acids (VFAs), i.e. acetate and butyrate, were quantified using a gas
147 chromatograph (GC 3900 Varian) equipped with a flame ionization detector as
148 previously described by Rafrafi *et al* (2013). Lactate was quantified using a high
149 performance liquid chromatography (HPLC) coupled with a refractometric detector.
150 The column used was an Aminex HPX-87H column (Biorad®). The column
151 temperature was maintained at 35 °C and the flow rate at 0.4 mL/min.

152 The errors associated with OD, CDW and organic acids measurements were 2, 6 and 5
153 %.

154 **2.4 Data analysis**

155 **2.4.1 Model design**

156 A model was built to estimate kinetics parameters in batch cultures: (1) μ_{a_max} the
157 maximum growth rate associated with acetate removal (d^{-1}), (2) K_{Sa} the half saturation
158 constant for acetate ($gC L^{-1}$), (3) Y_a the yield coefficient associated with acetate
159 removal ($g gC^{-1}$), (4) μ_{b_max} is the maximum growth rate associated with butyrate
160 removal (d^{-1}), (5) S_{bopt} is the concentration of butyrate when the growth rate is
161 maximum ($gC L^{-1}$), (6) α is the initial slope ($L.d/gC$), (7) Y_b the yield coefficient

162 associated with butyrate removal (g gC^{-1}) and (8) K_D the half inhibitory constant
 163 representing the inhibitory effect of acetate on butyrate removal (gC L^{-1}). Lag phases
 164 were graphically determined by logarithmic plotting of the CDW versus time and were
 165 excluded from data analysis.

166 In its structure, the model considers that organic carbon, provided by acetate, S_a (gC L^{-1}),
 167 is assimilated by microalgae to produce biomass, X (g L^{-1}) at a rate $\mu_a(S_a)$ (d^{-1}) and
 168 with a yield coefficient, Y_a (g gC^{-1}). This carbon assimilation can be described by the
 169 macroscopic reaction which represents the mass flux between substrate and biomass as
 170 following:



171

172 The growth rate $\mu_a(S_a)$, associated with acetate removal, was assumed as following a
 173 Monod function:

$$\mu_a(S_a) = \mu_{a,max} * \left(\frac{S_a}{S_a + K_{S_a}} \right) \quad \text{Equation 2}$$

174 With S_a the concentration of acetate (gC L^{-1}), K_{S_a} the half saturation constant for acetate
 175 (gC L^{-1}) and $\mu_{a,max}$ the maximum growth rate associated with acetate assimilation (d^{-1}).

176 As for growth on acetate, the assimilation of carbon via butyrate removal can be
 177 described by the following macroscopic reaction:



178

179 The growth rate $\mu_b(S_b)$, associated with butyrate removal, was assumed as following a
 180 Haldane function, supplemented with a diauxic term, K_D (gC L⁻¹), representing the
 181 inhibitory effect of acetate on butyrate removal:

$$\mu_b(S_b) = \mu_{b_max} * \frac{K_D}{K_D + S_a} * \frac{S_b}{S_b + \frac{\mu_{b_max}}{\alpha} * \left(\frac{S_b}{S_{b_opt}} - 1\right)^2} \quad \text{Equation 4}$$

182 With S_b the concentration of butyrate (gC L⁻¹), S_{b_opt} is the concentration of butyrate
 183 when $\mu_b(S_b)$ is maximum (gC L⁻¹), α is the initial slope (L.d gC⁻¹), μ_{b_max} is the
 184 maximum growth rate associated with butyrate assimilation (d⁻¹), K_D is the half
 185 inhibitory constant associated with the diauxic growth (gC L⁻¹).

186 Thanks to a mass-balance, the model was described by the following ordinary
 187 differential equations (ODE) system:

$$\frac{dS_a}{dt} = \mu_a(S_a) * \frac{1}{Y_a} * X \quad \text{Equation 5}$$

$$\frac{dS_b}{dt} = \mu_b(S_b) * \frac{1}{Y_b} * X \quad \text{Equation 6}$$

$$\frac{dX}{dt} = \mu_a(S_a) * X + \mu_b(S_b) * X \quad \text{Equation 7}$$

188

189 **2.4.2 Parameters estimation**

190 Kinetics parameters were estimated using six sets of experimental data: 0.1 and 1 gC L⁻¹
 191 of acetate, 0.1 and 1 gC L⁻¹ of butyrate, 0.25 gC L⁻¹ of acetate and butyrate and 0.9 gC
 192 L⁻¹, 0.1 gC L⁻¹ of acetate, butyrate, respectively. The seven complementary data sets
 193 were used to validate the estimation (Table 1). To minimize the effect of inoculation
 194 and substrate addition differences between each flask of triplicates, each flask was

195 analyzed individually. To estimate the kinetic parameters, squared-error between
196 simulation and experimental data was minimized using the following formula:

| | |
|--|-----|
| $error = \frac{\sum_x \sum_t (x_{measured}(t) - x_{simulated}(t))^2}{n}$ where $x \in \{S_a; S_b; X\}$ and n is the total number of experimental data | (8) |
|--|-----|

197

198 To minimize this error, the Nelder-Mead algorithm (function *fminsearch* under Scilab
199 (<http://www.scilab.org>) was used. To reduce the risk of finding a local minimum,
200 several optimization sets were performed with random initial parameters.

201 **2.4.3 Sensitivity analysis of the parameters**

202 A range of 0-200% of the estimated value for each parameter was tested, the other
203 parameters remaining constant. The results were analyzed by calculating the relative
204 error of the model related to the variation of individual parameters. A parameter is
205 considered as sensitive and accurately estimated when a small variation of its value
206 induces a strong increase in the model error. The parameters were considered to be
207 accurately estimated when a variation higher than 5 % induced more than a 5% increase
208 in the error between the simulation and the experimental data.

209 **3 Results and discussion**

210 **3.1 High cell growth using acetate**

211 Both *Chlorella sorokiniana* and *Auxenochlorella protothecoides* growth was efficient
212 and rapid for the four different concentrations of acetate (0.1^{-1} gC L⁻¹) (Figure 1 A, C).
213 Acetate was completely exhausted in less than 1.5 days and less than 2 days, for *C.*
214 *sorokiniana* and *A. protothecoides*, respectively (Figure 1 B, D). The end of the biomass

215 growth occurred when acetate was completely exhausted. These results showed that
216 acetate did not inhibit microalgae growth even for concentrations as high as 1 gC L⁻¹.
217 This might be due to the composition of the medium where nitrogen and phosphorus
218 were provided in non-limiting concentrations using a very favorable C:N:P molar ratio
219 of 48 : 16 : 1 (Stevenson et al., 1996). Positive controls (cultivation in light with
220 inorganic substrate addition) showed that inoculum was always viable. In negative
221 controls (cultivation in darkness without organic substrate addition but inorganic
222 substrate), a slight growth was observed showing that microalgae carbon reserves were
223 slightly able to support cell growth. It was therefore concluded that the biomass growth
224 was only due to organic carbon assimilation by heterotrophic microalgae. These results
225 are consistent with previous studies reporting that high concentration of acetate can be
226 used to grow successfully *Chlorella sorokiniana* (up to 6 gC L⁻¹ of acetate) and
227 *Auxenochlorella protothecoides* (up to 8.2 gC L⁻¹ of acetate) under heterotrophic
228 conditions (Heredia-Arroyo et al., 2010; Ogbonna et al., 2000). Growth on acetate was
229 modelled with a Monod equation (equation 2). Dynamics of acetate removal S_a and
230 biomass X were accurately predicted by the model for the two species studied (Figure
231 1). A sensitivity analysis of the model parameters was performed to assess the influence
232 of parameter variation on biomass prediction and substrate removals. This analysis
233 showed that the parameters μ_{a_max} and Y_a were highly sensitive and were accurately
234 estimated for the two studied species (Supplementary Material Figure 1). In contrast,
235 K_{S_a} for *Chlorella sorokiniana*, was not a sensitive parameter (Supplementary Material
236 Figure 2). More precisely, the values of K_{S_a} for *Chlorella sorokiniana* ranged between
237 0.025 and 2.10⁻¹⁰ gC L⁻¹. Therefore, these values were too small to be accurately
238 quantified and identified.

239 **[Figure 1]**

240 The maximal growth rates on acetate, μ_{a_max} , were 2.23 d⁻¹ and 2.05 d⁻¹ for
241 *Chlorella sorokiniana* and *Auxenochlorella protothecoides*, respectively (Table 2). The
242 biomass yields, Y_a , on acetate, i.e. the percentage of carbon that was assimilated into the
243 biomass, were similar with 42 % and 38 % for *Chlorella sorokiniana* and
244 *Auxenochlorella protothecoides*, respectively (Table 2). These values are very
245 consistent with those reported previously by Ogbonna et al. (2000) for *Chlorella*
246 *sorokiniana*. Samejima and Myers (1958) reported lower values with a maximal growth
247 rate of 0.5 d⁻¹ and a carbon yield of 26 % for *Chlorella sorokiniana* growing on acetate
248 (0.1 gC L⁻¹) and using nitrate as nitrogen source.

249 **[Table 2]**

250 **3.2 Cell growth inhibition with butyrate**

251 For *Chlorella sorokiniana*, cell growth and complete butyrate exhaustion were
252 observed after 14 days of cultivation only for an initial butyrate concentration of 0.1 gC
253 L⁻¹ (Fig 2 A-B). For butyrate concentrations above 0.1 gC L⁻¹, neither growth of
254 *Chlorella sorokiniana* nor butyrate removal was observed after 23 days of cultivation
255 (Fig 2 A-B). *Auxenochlorella protothecoides* was able to grow on butyrate for
256 concentrations as high as 0.25 gC L⁻¹ over an incubation period of 23 days (Figure 2
257 C,D). At the initial concentration of 0.1 gC L⁻¹ and 0.25 gC L⁻¹, butyrate was exhausted
258 in 7.5 days and 20 days, respectively. For initial butyrate concentration at 0.5 and 1 gC
259 L⁻¹, neither growth nor butyrate removal was observed suggesting a strong inhibition of
260 microalgae growth in the presence of butyrate at concentration higher than 0.1 gC L⁻¹,
261 for *Chlorella sorokiniana* and 0.25 gC L⁻¹ for *Auxenochlorella protothecoides*. Growth

262 on butyrate was modeled with a Haldane equation including an inhibitory constant
263 (Equation 4). Dynamics of butyrate removal S_b and biomass X were accurately predicted
264 by the model for the two species (Figure 2). A sensitivity analysis of the model
265 parameters was performed and showed that the parameters μ_{b_max} , S_{bopt} and Y_b were
266 highly sensitive and were accurately estimated for the two species (Supplementary
267 Material Figure 1- 2). In contrast, α was not a sensitive parameter (Supplementary
268 Material Figure 2). The maximal growth rate on butyrate, μ_{b_max} , was 0.16 d^{-1} and 0.22
269 d^{-1} for *Chlorella sorokiniana* and *Auxenochlorella protothecoides*, respectively (Table
270 2). The biomass yield, Y_b , on butyrate, was 56 % and 48 % for *Chlorella sorokiniana*
271 and *Auxenochlorella protothecoides*, respectively (Table 2). The optimal butyrate
272 concentration, S_{bopt} , was 0.046 and 0.047 gC L^{-1} for *Chlorella sorokiniana* and
273 *Auxenochlorella protothecoides*, respectively (Table 2). Similarly, Samejina and Myers
274 (1958) and Chang *et al* (2012) found that *Chlorella pyrenoidosa* and *Chlorella vulgaris*
275 growth in heterotrophic conditions were inhibited by butyrate concentrations higher
276 than 0.05 gC L^{-1} . In this study, maximal growth rates observed on butyrate were 10
277 times lower than those found on acetate. It was therefore tested whether microalgae
278 could acclimate to the presence of butyrate and improve their growth rates.
279 *Auxenochlorella protothecoides* was successively cultivated on 0.1 gC L^{-1} of butyrate
280 during 4 weeks. The experimental data were compared with a model simulation. The
281 simulations did not fit the experimental data well (data not shown). New estimation of
282 the model parameters was therefore performed and the maximal growth rate was
283 improved from 0.25 , 0.36 to 0.58 d^{-1} after 1, 2 and 3 successive cultures on butyrate,
284 respectively (Table 3). Therefore, the maximum growth rate was three times higher
285 (0.58 d^{-1}) than the value found when the microalgae were not acclimated (0.25 d^{-1}). The

286 biomass yields remained constant around 26%, 31% and 25 % after 1, 2 and 3
287 successive cultures on butyrate, respectively (Table 3). The biomass yields were lower
288 (25 – 30 %) than what was observed when the microalgae were not acclimated (48 %),
289 suggesting a probable exhaustion of some internal carbon reserves through the
290 acclimation process. A sensitivity analysis was carried out and indicated that μ_{b_max} and
291 Y_b were two sensitive parameters but α and S_{bopt} were not. Since S_{bopt} was no more a
292 sensitive parameter, butyrate inhibition might have been partially reduced. The result
293 showed the possibility of enhancing butyrate removal by acclimatizing specifically the
294 microalgae to this inhibitory substrate.

295 **[Figure 2]**

296 **[Table 3]**

297 **3.3 Diauxic growth in the presence of acetate and butyrate**

298 Three different mixtures of acetate and butyrate, ranging from 0.25 to 0.9 gC L⁻¹ and 0.1
299 gC L⁻¹ to 0.25 and butyrate, 0.25, 0.1 and 0.1 gC L⁻¹, respectively, were tested (Table 1).
300 When acetate and butyrate were provided in equal amount, 0.25 gC L⁻¹ each, biomass
301 growth and complete substrates exhaustion were observed for both species (Figure 3 A
302 and D). Acetate was consumed in 1.5 and 1.85 days, by *Chlorella sorokiniana* and
303 *Auxenochlorella protothecoides*, respectively. Butyrate removal started after complete
304 acetate exhaustion for both species. It was completely consumed after 10 days and 7
305 days by *Chlorella sorokiniana* and *Auxenochlorella protothecoides*, respectively. For
306 the two other tested mixtures, the same pattern was observed, i.e. rapid acetate removal
307 followed by slow butyrate removal starting only after complete acetate exhaustion
308 (Supplementary Material Figure 3).

309 Interestingly, a sequential assimilation of acetate and butyrate was observed. Butyrate
310 removal seemed inhibited by the presence of acetate in the medium. This phenomenon
311 is known as diauxie or diauxic effect and has been widely reported in many microbial
312 species, but has never been clearly reported for microalgae grown on VFAs (Kovárová-
313 kovar and Egli, 1998). Narang and Pilyugin (2005) pointed out that when
314 microorganisms are grown with two substitutable substrates, e.g. two carbon sources,
315 they tend to preferentially exhaust the one that sustains a higher growth rate which is
316 consistent with this study where acetate removal is faster and more efficient than
317 butyrate removal.

318 **[Figure 3]**

319 In addition, 0.25 gC L⁻¹ of butyrate was completely exhausted by *Chlorella sorokiniana*
320 in only 10 days when 0.25 gC L⁻¹ of acetate was also added, whereas butyrate, as sole
321 carbon source, was not consumed after 23 days (Figure 2 and 3). Similar results were
322 observed for *Auxenochlorella protothecoides*. Acetate was first taken up by microalgae
323 with a subsequent biomass increase that led to significant butyrate consumption even at
324 inhibitory concentrations when added alone. In a previous study on mixotrophic
325 cultivation of microalgae, Liu *et al* (2012) reported that butyrate removal was enhanced
326 by increasing the initial biomass concentration. Similarly, high acetate:butyrate ratios of
327 8:1 and 3:1 (in g L⁻¹) were used to enhance the biomass production of the oleaginous
328 fungus, *Cryptococcus albidus*, and the oleaginous yeast, *Yarrowia lipolytica*,
329 respectively (Fei et al., 2011; Fontanille et al., 2012).

330 The diauxic behavior was modelled by adding an inhibitory term in the Haldane
331 equation (equation 4). When acetate and butyrate were used in mixtures, the estimated

332 maximal growth rates and biomass yields were similar to the ones estimated when
333 acetate and butyrate were used as single substrates. This result supports that butyrate
334 consumption was favored only by the increase of biomass concentration in a mixture of
335 acetate:butyrate. The low values of K_D , which corresponds to the half inhibitory
336 constant associated with the diauxic growth, $2 \cdot 10^{-10}$ and 0.025 gC L^{-1} , showed that the
337 diauxic effect was very strong for the two species studied. Moreover, a sensitivity
338 analysis was carried out and showed that K_D was not a sensitive parameter
339 (Supplementary Material Figure 2). Thereafter, these kinetic parameters of *Chlorella*
340 *sorokiniana* and *Auxenochlorella protothecoides* growth were compared to literature
341 data by simulating the growth of microalgae on a mixture of different carbon sources. In
342 Liu *et al* (2013), a mixture of acetate (0.12 gC L^{-1}) and butyrate (0.43 gC L^{-1}) was used
343 to sustain the heterotrophic growth of *Chlorella vulgaris*. *Chlorella vulgaris* completely
344 consumed acetate but only 12 % of the initial butyrate concentration in approximately
345 5 days. According to the model predictions made under similar conditions, about 27 %
346 and 35 % of butyrate should be consumed in 5 days for *Chlorella sorokiniana* and
347 *Auxenochlorella protothecoides*, respectively. Therefore, the two species used in the
348 present study seemed more efficient than *Chlorella vulgaris* for heterotrophic
349 cultivation on acetate and butyrate. The results of this study support that the addition of
350 a second carbon source such as acetate cultivation may be a promising strategy to
351 enhance butyrate removal under heterotrophic conditions.

352

353

354 **3.4 No influence of lactate on growth**

355 One concentration of lactate as single substrate, 0.5 gC L⁻¹, was tested for both species.
356 Neither growth nor apparent lactate removal was observed (data not shown). A mixture
357 composed of 0.25 gC L⁻¹ of lactate and 0.25 gC L⁻¹ of acetate was tested. For both
358 species, acetate was completely exhausted and was the sole substrate supporting the
359 biomass growth (Figure 3 B,E). Lactate removal did not occur during the cultivation
360 periods for the two species. A mixture composed of acetate (0.25 gC L⁻¹), butyrate (0.45
361 gC L⁻¹) and lactate (0.16 gC L⁻¹), a ratio similar to effluent composition of real dark
362 fermenter, was tested (Rafrafi et al., 2013). Acetate and butyrate removals occurred
363 during the cultivation periods, while lactate was not degraded (Figure 3 C, F). All
364 results showed that lactate was not used as carbon source to sustain heterotrophic
365 growth for the two species. Nevertheless, the presence of lactate did not influence
366 neither acetate nor butyrate removals. It was therefore concluded that its presence in
367 fermentation effluents would not have any influence on final biomass growth in the
368 range of the tested concentrations. Lactate removal and assimilation by *Chlorella*-like
369 species does not reach a consensus in literature. Lactate removal is likely species-
370 specific and depends highly on culture conditions. As an illustration, it was previously
371 reported that *Chlorella vulgaris* could not use lactate for growth in mixotrophic
372 conditions, and its presence was inhibitory to biomass growth (Liu et al., 2012). In
373 contrast, the use of lactate by the same microalgal species was observed in heterotrophic
374 conditions by Perez-Garcia and al (2011a). Therefore, the influence of lactate on
375 biomass growth and on other substrates removal remains unclear for *Chlorella* sp.

376 **4 Conclusion**

377 Microalgal heterotrophy should provide a mean to successfully produce biofuels if low
378 cost carbon sources are used. The growth of microalgae on synthetic dark fermentation
379 effluent, composed mainly of acetate, butyrate and lactate, was evaluated and modeled.
380 Defining the optimal butyrate concentration and acetate: butyrate ratio should promote
381 microalgae growth. Acclimation of microalgae (during four weeks) to the inhibitory
382 butyrate may substantially improve biomass growth. The use of raw fermentation
383 effluents, with bacteria, for microalgal cultivation will be the next step in assessing the
384 feasibility of coupling dark fermentation with microalgal heterotrophy.

385 **Acknowledgments**

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387 University of Montpellier II, France.

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470

471 **Figure captions**

472 **Figure 1. Growth of *Chlorella sorokiniana* and *Auxenochlorella protothecoides* on**
473 **acetate in heterotrophic conditions.**

474 A – B and C – D represent the growth of, and removal of VFA by, *Chlorella*
475 *sorokiniana* and *Auxenochlorella protothecoides*, respectively. A and C, B and D,
476 represent the biomass (g L^{-1}) growth, the substrate removal (gC L^{-1}), respectively, of
477 microalgae on 1 gC L^{-1} (■), 0.5 gC L^{-1} (◆), 0.25 gC L^{-1} (▲) and 0.1 gC L^{-1} (●) of
478 acetate. The associated model predictions (dashed lines) are also represented. One set of
479 data per triplicate are represented, the model fitted the other two sets of data as well.

480 **Figure 2. Growth of *Chlorella sorokiniana* and *Auxenochlorella protothecoides***
481 **butyrate in heterotrophic conditions.**

482 A – B and C – D represent the growth of, and removal of VFA by, *Chlorella*
483 *sorokiniana* and *Auxenochlorella protothecoides*, respectively. A and C, B and D,
484 represent the biomass (g L^{-1}) growth, the substrate removal (gC L^{-1}), respectively, of
485 microalgae on 1 gC L^{-1} (■), 0.5 gC L^{-1} (◆), 0.25 gC L^{-1} (▲) and 0.1 gC L^{-1} (●) of
486 butyrate. The associated model predictions (dashed lines) are also represented. One set
487 of data per triplicate are represented, the model fitted the other two sets of data as well.

488 **Figure 3. Growth of *Chlorella sorokiniana* and *Auxenochlorella protothecoides* on**
489 **mixtures of acetate, butyrate and lactate in heterotrophic conditions.**

490 A – C and D – F represent the growth of, and removal of organic acids by, *Chlorella*
491 *sorokiniana* and *Auxenochlorella protothecoides*, respectively. Biomass (g L^{-1}) growth (■
492), acetate removal (◆), butyrate removal (▲) and lactate removal (●), as long with the
493 associated model predictions (dashed lines) are represented. A and D represent the

494 experimental and predicted data for microalgae grown on 0.25 gC L⁻¹ of acetate and
495 0.25 gC L⁻¹ of butyrate. B and E represent the experimental and predicted data for
496 microalgae grown on 0.25 gC L⁻¹ of acetate and 0.25 gC L⁻¹ of lactate. C and F
497 represent the experimental and predicted data for microalgae grown on a mix of acetate
498 (0.25 gC L⁻¹), butyrate (0.45 gC L⁻¹) and lactate (0.16 gC L⁻¹) according to published
499 datas found in a fermentative digestate (Rafrafi et al., 2013). One set of data per
500 triplicate are represented, the model fitted the other two sets of data as well.

Highlights

- A diauxic effect of acetate over butyrate was observed.
- A kinetic model for biomass growth, acetate and butyrate removals was built.
- Lactate did not promote or inhibit algal growth.
- Butyrate is key for coupling dark fermentation and microalgal heterotrophy.

Table 1.

List of all the conditions tested for *Chlorella sorokiniana* and *Auxenochlorella protothecoides* to estimate and validate model parameters.

| Tested Experiments | Acetate (gC L ⁻¹) | Butyrate (gC L ⁻¹) | Lactate (gC L ⁻¹) | Estimation (E) or Validation (V)* |
|---|-------------------------------|--------------------------------|-------------------------------|-----------------------------------|
| Growth on acetate | 0.1 | 0 | 0 | E |
| | 0.25 | 0 | 0 | V |
| | 0.5 | 0 | 0 | V |
| | 1 | 0 | 0 | E |
| Growth on butyrate | 0 | 0.1 | 0 | E |
| | 0 | 0.25 | 0 | V |
| | 0 | 0.5 | 0 | V |
| | 0 | 1 | 0 | E |
| Growth on acetate and butyrate mixtures | 0.25 | 0.25 | 0 | E |
| | 0.4 | 0.1 | 0 | V |
| | 0.9 | 0.1 | 0 | E |
| Growth on lactate | 0 | 0 | 0.5 | ** |
| | 0.25 | 0 | 0.25 | V |
| | 0.25 | 0.45 | 0.16 | V |

*: Data used for Estimation (E) or Validation (V) of model parameters

** : the experimental data were not used to build the model due to the absence of lactate removal during the experiments.

Table 1.

Estimated parameters values for the growth of *Chlorella sorokiniana* and *Auxenochlorella protothecoides* on organic acids in heterotrophic culture. Values in italics indicate the range of parameters values for which the sensitivity analysis showed that less than 5% variation of the error between simulation and data occurred.

| Species studied | Growth on acetate | | | Growth on butyrate | | | Diauxie | |
|------------------------|--------------------------------------|-----------------------------------|--------------------------------------|--------------------------------------|-------------------------------------|-------------------------------------|--------------------------------------|--------------------------------|
| | μ_{a_max} (d ⁻¹) | K_{Sa} (gC L ⁻¹) | Y_a (g gC ⁻¹ and %*) | μ_{b_max} (d ⁻¹) | α (L.d gC ⁻¹) | S_{bopt} (gC L ⁻¹) | Y_b (g gC ⁻¹ and %*) | K_D (gC L ⁻¹) |
| <i>Chlorella</i> | 2.23 | 0.002 | 0.84 – 42 % | 0.16 | 15.1 | 0.046 | 1.12 - 56 % | 2.10⁻¹⁰ |
| <i>sorokiniana</i> | 2.18 - 2.27 | 5.10 ⁻⁵ - 0.005 | 0.79 - 0.87 | 0.15 - 0.17 | 9.4 - 19.4 | 0.038 - 0.049 | 1.03 - 1.23 | N/A |
| <i>Auxenochlorella</i> | 2.05 | 0.05 | 0.75 - 38 % | 0.22 | 13.1 | 0.047 | 0.95 - 48 % | 0.025 |
| <i>protothecoides</i> | 2.01 - 2.13 | 0.04 - 0.06 | 0.71 - 0.78 | 0.21 - 0.24 | 12.9 - 13.6 | -0.048 | 0.89 - 1 | 5.10 ⁻⁴ - 0.05 |

*: estimated carbon assimilation for a cell composition of 50 % carbon (Chen and Johns, 1996).

Table 1.

Estimated parameters values for the successive growth of *Auxenochlorella protothecoides* on 0.1 gC L⁻¹ of butyrate. Values in italic indicate the range of parameters values for which the sensitivity analysis indicated that less than 5% variation of the error between simulation and data occurred.

| Kinetic parameters | Culture 0* | Culture 1* | Culture 2* | Culture 3* |
|---|--------------------|--------------------|--------------------|--------------------|
| μ_{b_max} | 0.22 | 0.25 | 0.36 | 0.58 |
| (d ⁻¹) | <i>0.21 – 0.24</i> | - | <i>0.35 – 0.38</i> | <i>0.57 – 0.60</i> |
| Y_b | 0.95 – 48 % | 0.52 – 26 % | 0.61 – 30.5 % | 0.49 – 24.5 % |
| (g gC ⁻¹ and % ^{**}) | <i>0.89 – 1</i> | <i>0.51 – 0.53</i> | <i>0.59 – 0.66</i> | <i>0.47 – 0.50</i> |

*: Cultures 0, 1, 2, 3 refer to acclimation experiments. Culture 0, 1, 2 and 3, was not acclimated, acclimated once (until complete butyrate exhaustion), twice and three times on 0.1 gC L⁻¹ of butyrate, respectively.

** : estimated carbon assimilation for a cell composition of 50 % carbon (Chen and Johns, 1996).

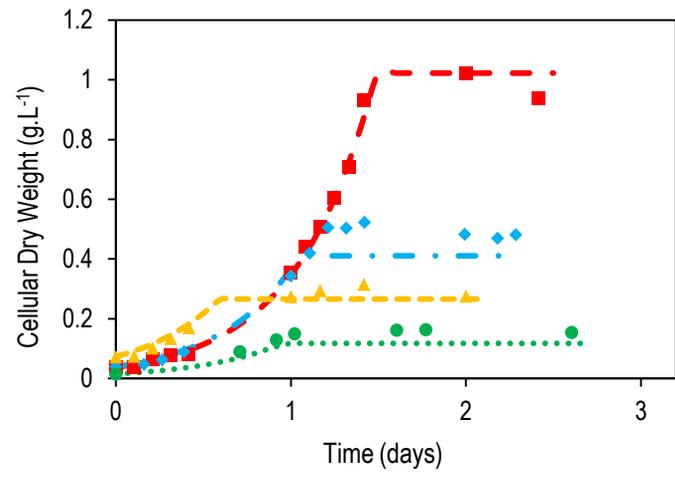
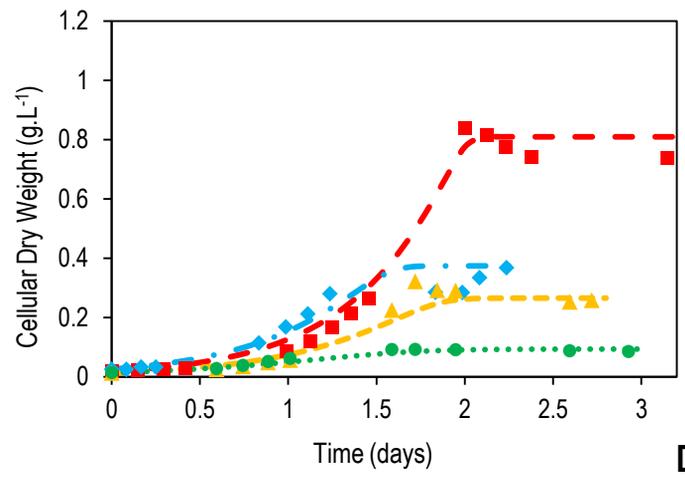
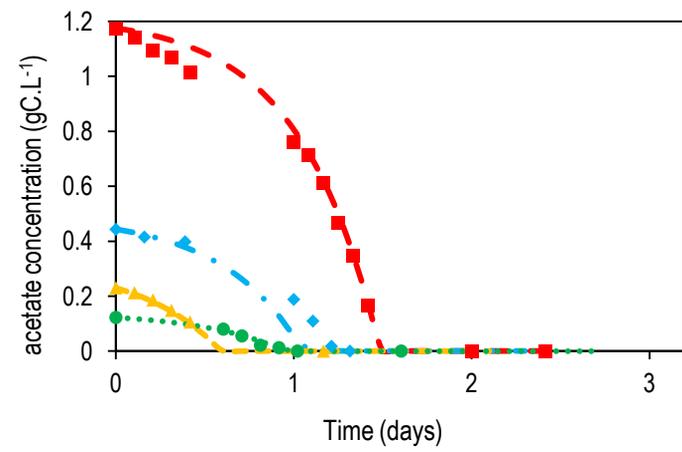
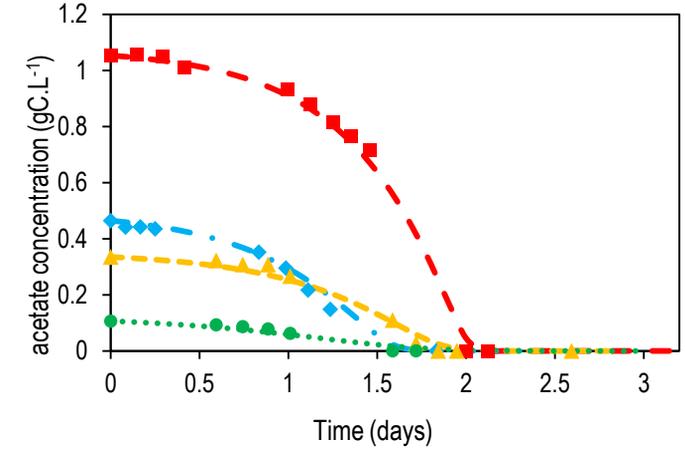
Figure 1*Chlorella sorokiniana***A***Auxenochlorella protothecoides***C****B****D**

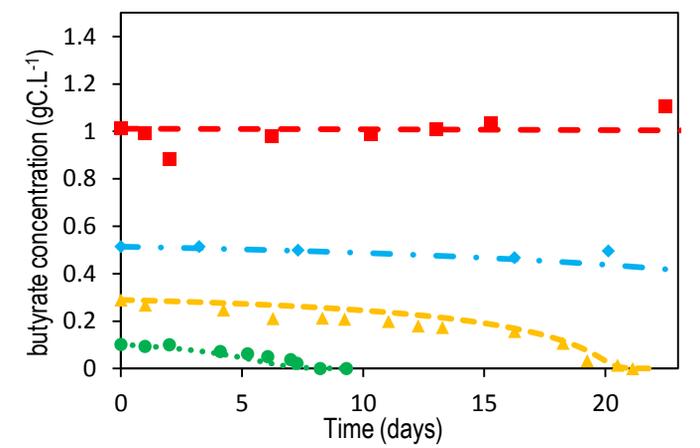
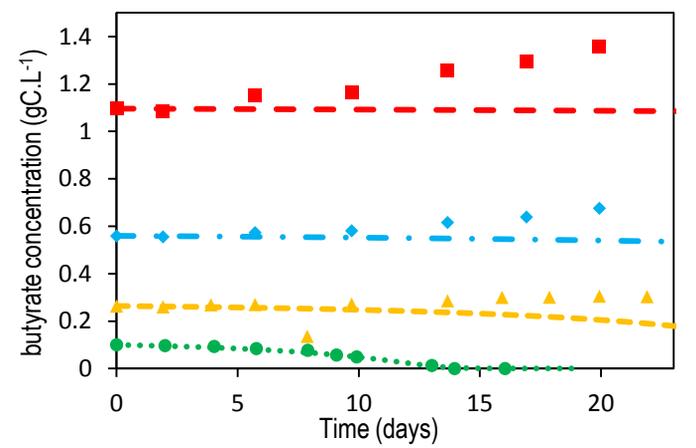
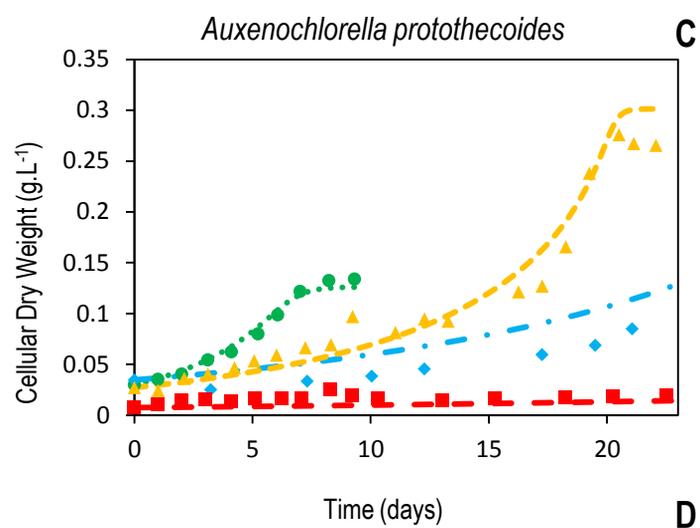
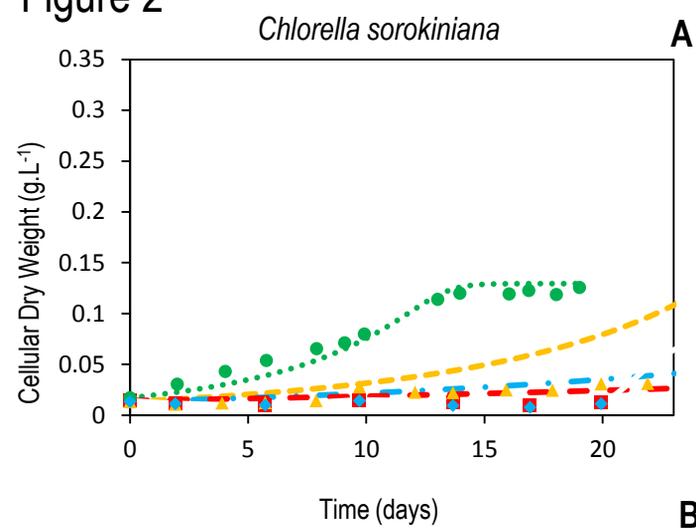
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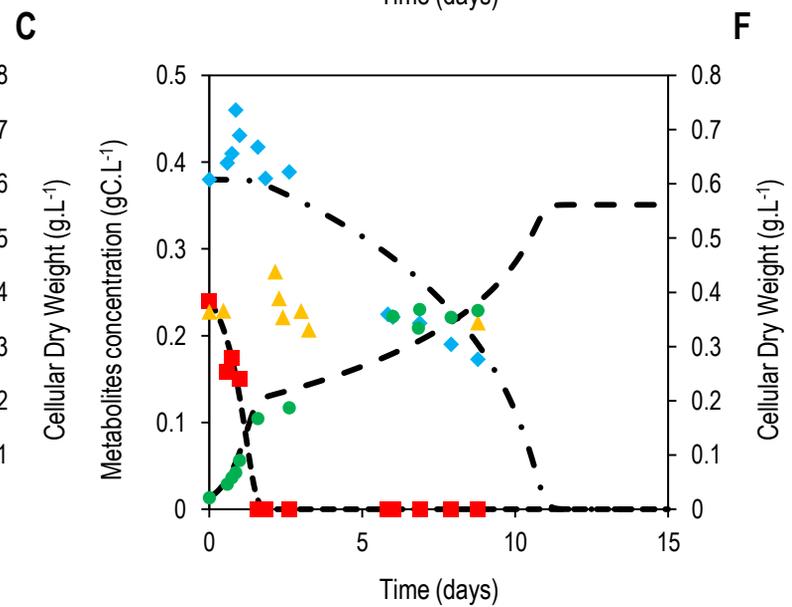
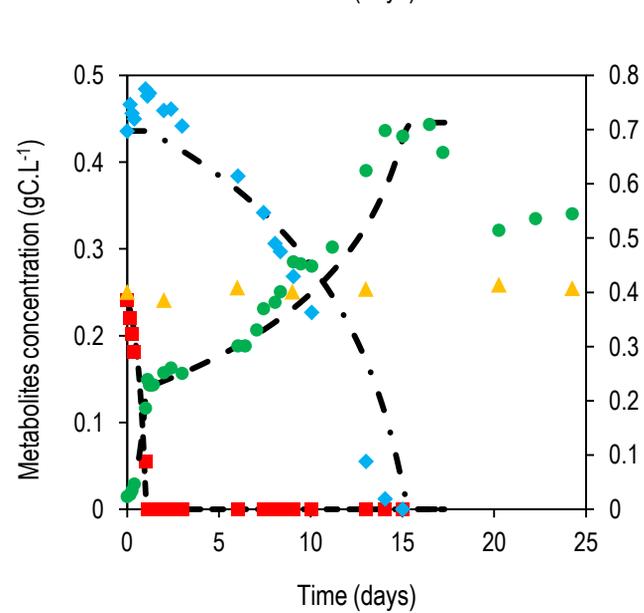
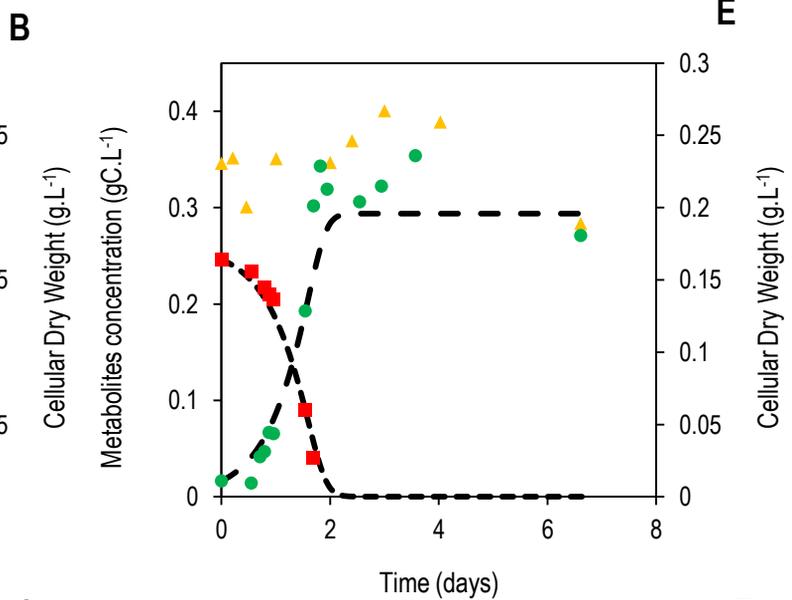
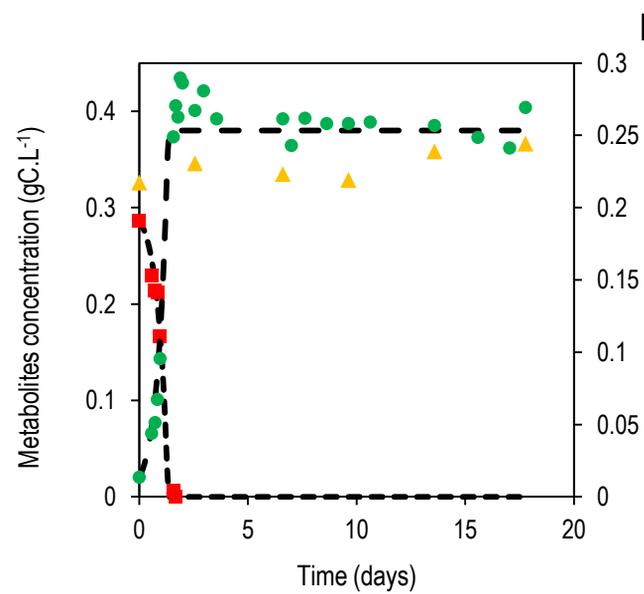
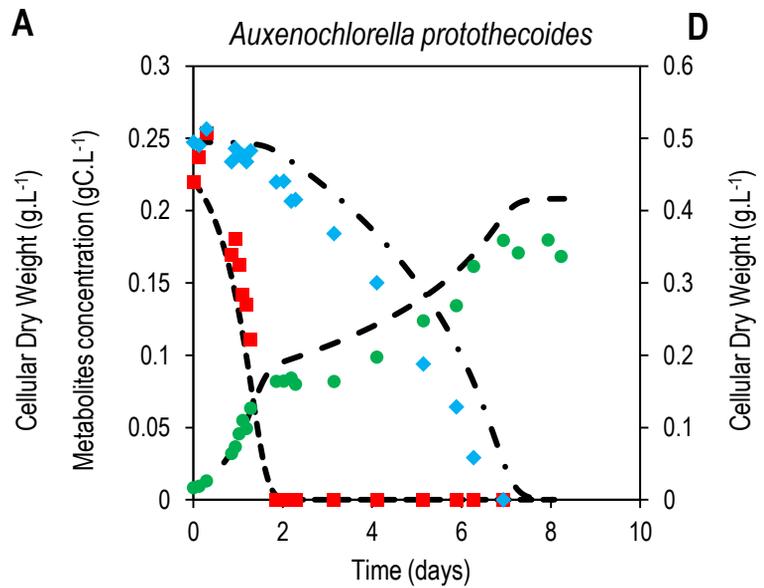
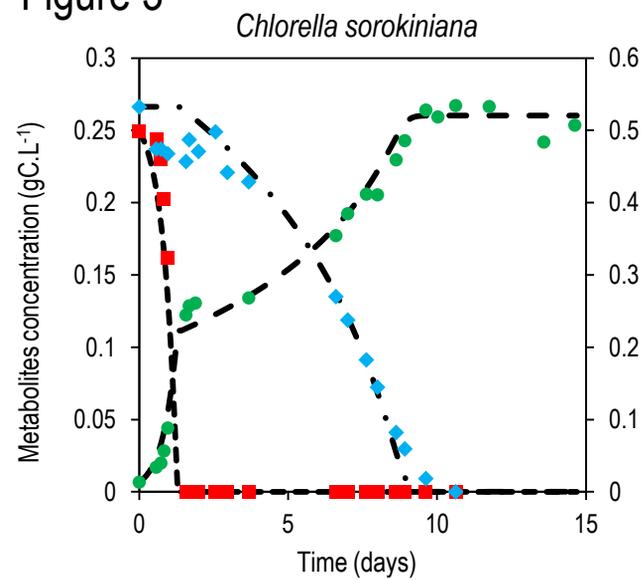
Figure 3

Figure 1, 2, 3 in Black and White

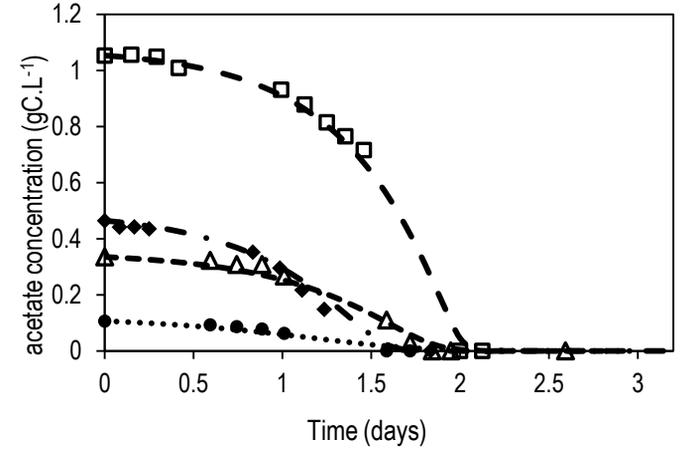
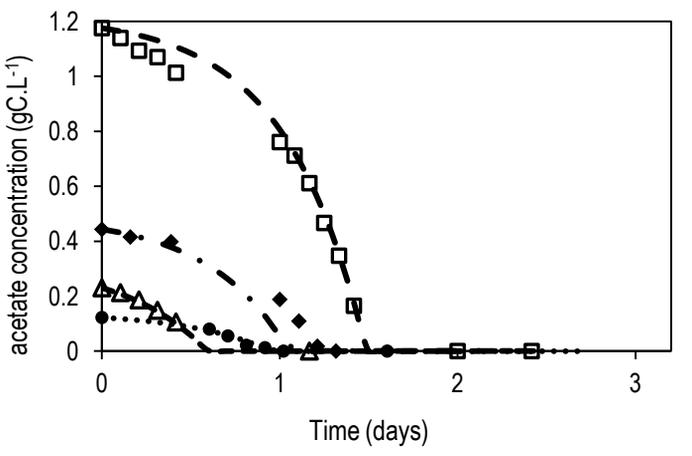
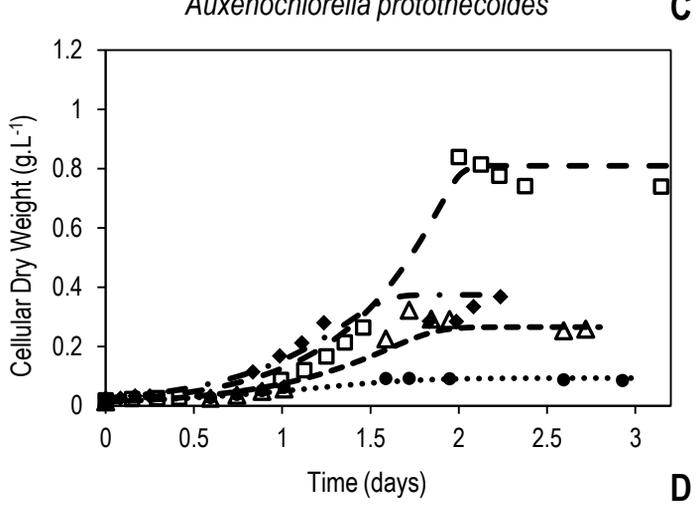
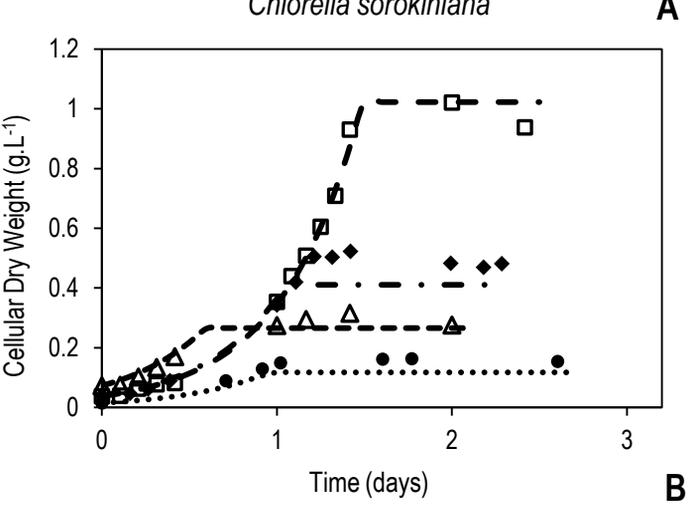
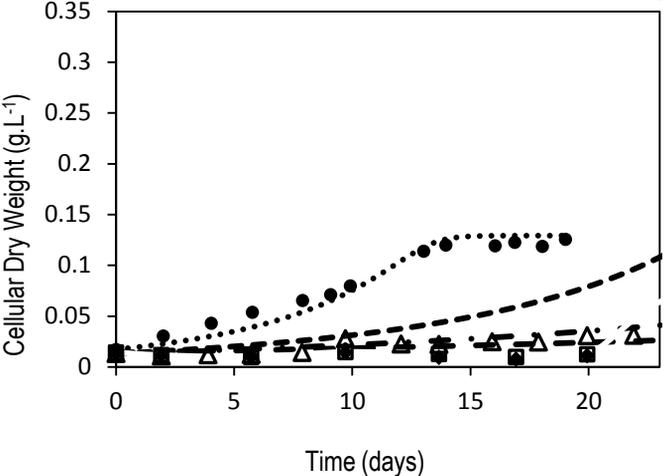


Figure 2

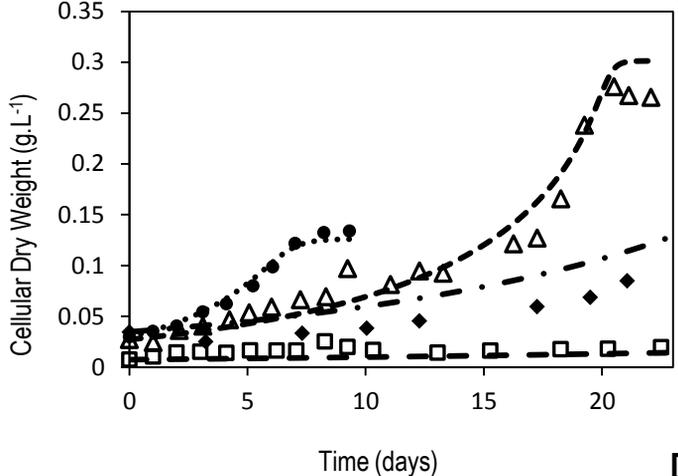
Chlorella sorokiniana

A

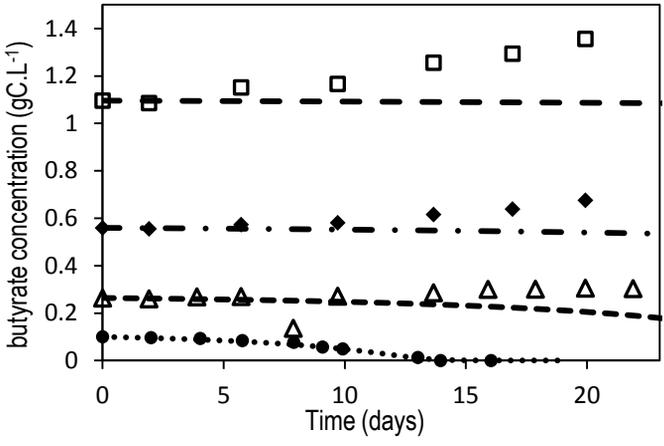


Auxenochlorella protothecoides

C



B



D

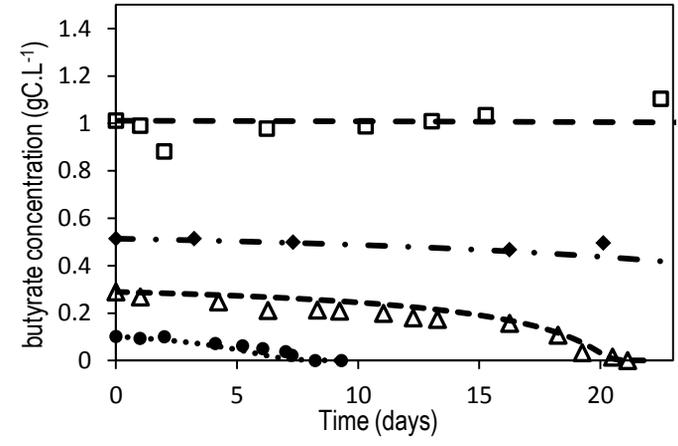
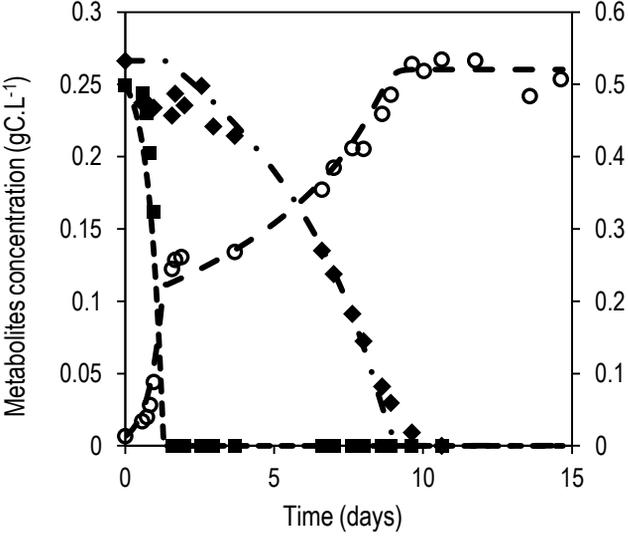


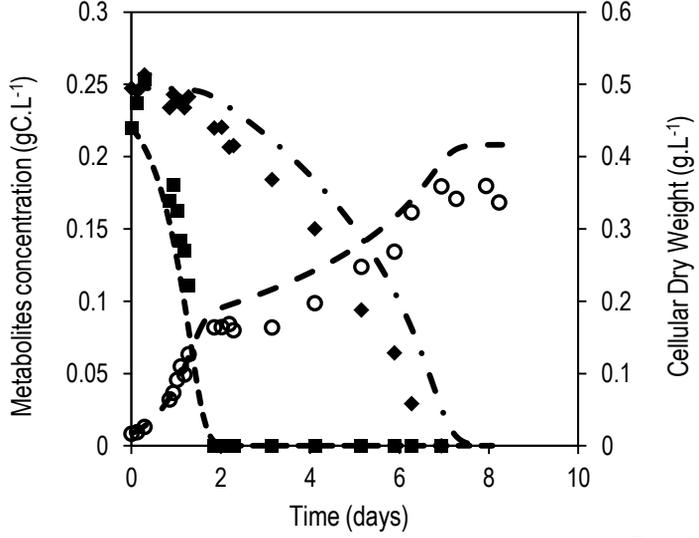
Figure 3

Chlorella sorokiniana

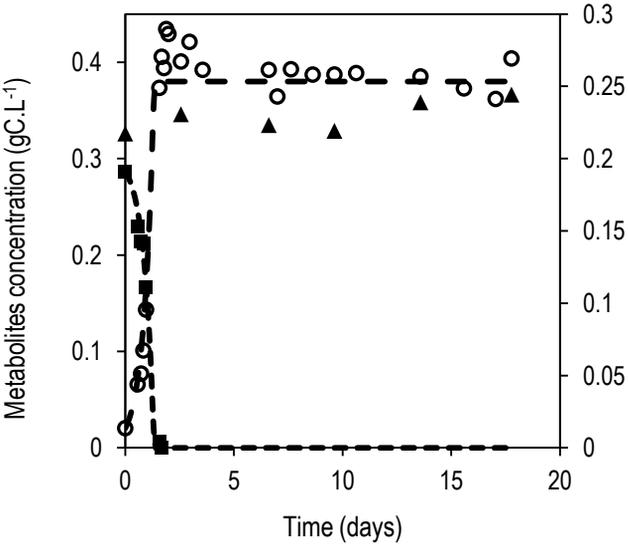


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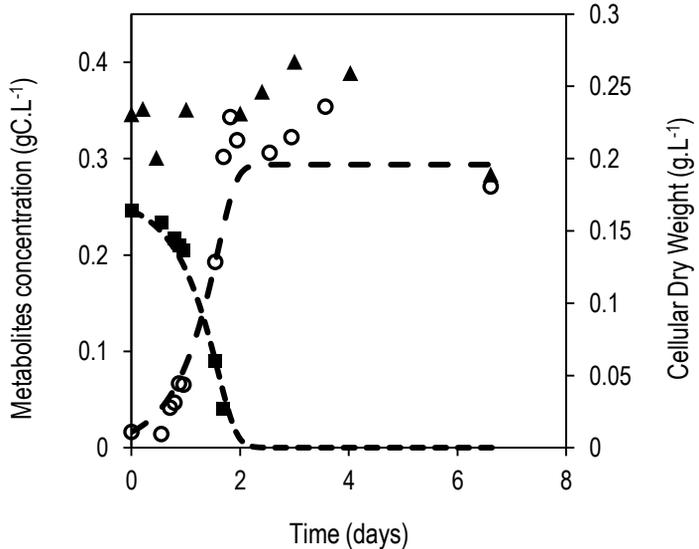
Auxenochlorella protothecoides



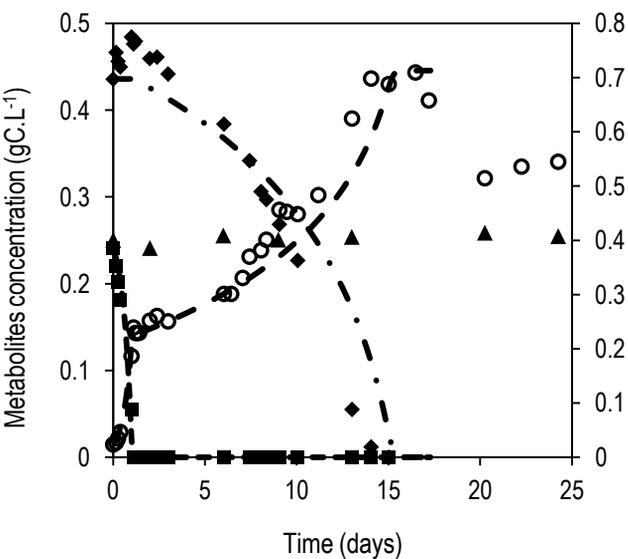
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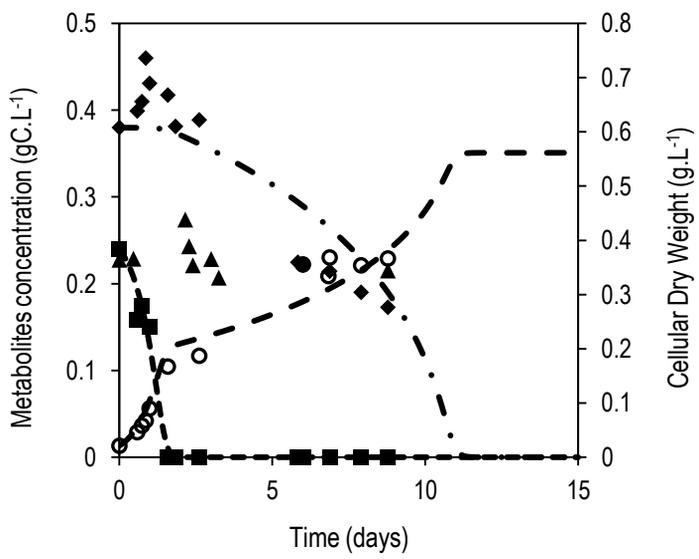
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F