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1           **Growth of *Chlorella sorokiniana* on a mixture of volatile fatty**  
2           **acids: the effects of light and temperature**

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10           **Abstract**

11   The feasibility of coupling of dark fermentation to produce biohydrogen, with  
12   heterotrophic cultivation of microalgae, to produce biolipids from fermentation by-  
13   products, is limited by butyrate which inhibits the growth of the microalgae. This study  
14   investigated the influence of light and temperature on *Chlorella sorokiniana* grown on a  
15   mixture of acetate and butyrate, two of the volatile fatty acids produced by dark  
16   fermentation. Exposure to light caused autotrophic biomass production (56% of the final  
17   biomass) and reduced the time to reach butyrate exhaustion to 7 days at 25 °C from 10  
18   days in the dark. For growth on acetate at the optimum temperature (35 °C), the  
19   presence of butyrate reduced the growth rate (by 46%) and the carbon yield (by 36%).  
20   For successful microalgae growth on dark fermentation effluent, butyrate inhibition may  
21   be reduced by setting the temperature to 30 °C and providing light.

## 22        **Keywords**

23    *Chlorella sorokiniana*; Dark Fermentation; Heterotrophy; Mixotrophy; Butyrate  
24    inhibition

## 25    **1 Introduction**

26    Many studies and industrial projects have shown the value of heterotrophic cultivation  
27    of microalgae for producing high added value compounds, such as docosahexaenoic acid  
28    (DHA), and commodity compounds, such as lipids for biofuels (Lowrey et al., 2015).  
29    When microalgae are grown on organic carbon sources in the dark, they tend to grow  
30    faster with higher biomass and lipid yields than when they are grown using conventional  
31    autotrophic cultivation (Liang, 2013). However, in order to reduce production costs, an  
32    alternative to glucose, the most common substrate, must be found, especially for  
33    producing biofuels (Liang, 2013). Acetate has been suggested as one of the best  
34    alternatives since it can be easily incorporated into lipids or carbohydrates by  
35    microalgae and is widely available as a cheap source of carbon (Lowrey et al., 2015).  
36    Moreover, acetate is one of the main end-products of microbial dark fermentation (DF) of  
37    various types of urban, agricultural and industrial waste (Ghimire et al., 2015).  
38    Recently, several studies have shown the benefits and feasibility of coupling DF, which  
39    produces hydrogen as the main product and volatile fatty acids (VFAs) as secondary  
40    metabolites, with microalgae cultivation, which produces both microalgal biomass and  
41    lipids (Chandra et al., 2015; Liu et al., 2013, 2012; Ren et al., 2014; Turon et al., 2015;  
42    Venkata Mohan and Prathima Devi, 2012). During DF, complex organic compounds  
43    originating from waste are converted by anaerobic bacteria into simple VFAs, mainly  
44    acetate and butyrate, that can be further assimilated by microalgae (Ghimire et al., 2015).

45 The effluent from DF provides a low-cost source of carbon which can successfully  
46 sustain heterotrophic microalgae growth (Liu et al., 2013; Ren et al., 2014). For  
47 example, VFAs were efficiently converted into carbohydrates (51% of dry weight (DW))  
48 by *Chlorella vulgaris* grown heterotrophically on diluted DF effluent (Liu et al., 2013)  
49 and acetate was used to produce lipids, up to 41% of DW, by the heterotroph  
50 *Scenedesmus sp.* grown on fermentation effluent (Ren et al., 2014). These studies  
51 reported that butyrate inhibited microalgae growth, at concentrations as low as 0.1 g.L<sup>-1</sup>,  
52 and this is now considered to be one of the main challenges that must be overcome  
53 when coupling DF and heterotrophic cultivation of microalgae (Fei et al., 2014; Liu et al.,  
54 2012; Turon et al., 2015).

55 Butyrate uptake by microalgae is much slower than acetate uptake and can also reduce  
56 microalgae growth when using a mixture of VFAs as a source of carbon (Fei et al.,  
57 2014). Similar differences between acetate and butyrate uptake rates have also  
58 been reported for oleaginous fungi (Vajpeyi and Chandran, 2015). Liu et al.  
59 (2013) reported that growing *C. vulgaris* mixotrophically, with light and carbon dioxide,  
60 could reduce the inhibitory effect of butyrate. For mixotrophic growth on butyrate alone,  
61 it was suggested that microalgae assimilated CO<sub>2</sub> first, with a subsequent increase in the  
62 total biomass, resulting in faster uptake of butyrate (Liu et al., 2013, 2012). However,  
63 these authors suggested that carbon dioxide was probably preferred to butyrate as a  
64 substrate and that strong competition between CO<sub>2</sub> and butyrate uptake combined  
65 with high CO<sub>2</sub> availability may, therefore, lower the butyrate consumption rate (Liu et al.,  
66 2013, 2012).

67 *Chlorella sorokiniana* is considered to be one of the most promising species for lipid and  
68 biomass production (Griffiths and Harrison, 2009; Lizzul et al., 2014; Zheng et al.,

69 2014). When grown heterotrophically at its optimum growth temperature (37 °C) on  
70 glucose in a two-stage fed-batch culture including a first stage for biomass growth and a  
71 second stage for lipid accumulation through nitrogen depletion, *C. sorokiniana*  
72 produced very high biomass of 103.8 g.L<sup>-1</sup> and lipid concentrations of 40.2 g.L<sup>-1</sup> (Zheng  
73 et al., 2013). Between 35 °C and 37 °C, *C. sorokiniana* achieved high growth rates of  
74 3.4 d<sup>-1</sup> under mixotrophic conditions and 6.5 d<sup>-1</sup> under autotrophic conditions (Janssen et  
75 al., 1999; Li et al., 2014; Van Wagenen et al., 2014b). These results suggest that  
76 temperature and light might be key parameters for increasing *C. sorokiniana* growth on  
77 VFAs.

78 Overall, heterotrophic growth of microalgae on a mixture of VFAs seems strongly  
79 dependent on the acetate:butyrate ratio as high concentrations of butyrate can inhibit  
80 algal growth (Fei et al., 2014; Liu et al., 2012; Turon et al., 2015). However, the  
81 inhibition of *C. sorokiniana* growth at high butyrate concentrations may be mitigated by  
82 light and high temperatures. The interactions between acetate, butyrate and light and  
83 their effects on microalgae growth have not yet been determined. *C. sorokiniana* is  
84 known to be thermotolerant and, therefore, cultivating it on a mixture of VFAs at a high  
85 temperature (35 °C) would provide increased enzymatic activity and reduce  
86 the requirements for cellular temperature control.

87 *C. sorokiniana* has already been cultivated heterotrophically on a mixture of VFAs,  
88 giving a high growth rate on acetate, 2.2 d<sup>-1</sup>, and a low growth rate on butyrate, 0.16 d<sup>-1</sup>,  
89 at 25 °C (Turon et al., 2015). This study set out to determine the interaction between  
90 these two VFAs while growing *C. sorokiniana* in presence of light and at different  
91 temperatures. The effects of (i) light (with light and in the dark) (ii) temperature (25 °C,  
92 30 °C, and 35 °C) and (iii) a combination of light and high temperature (35 °C) were

93 tested on the growth rate and carbon yield of *C. sorokiniana* growing on a mixture of  
94 acetate and butyrate at an inhibiting butyrate concentration (both at  $0.3 \text{ g}_C\cdot\text{L}^{-1}$ ). Control  
95 experiments with either acetate or butyrate as single substrate ( $0.3 \text{ g}_C\cdot\text{L}^{-1}$ ) were also  
96 performed to give a better understanding of the interactions between acetate and  
97 butyrate uptake mechanisms.

## 98 **2 Materials and methods**

### 99 **2.1 Microalgae cultivation conditions**

#### 100 **2.1.1 *Chlorella sorokiniana* stock cultivation conditions**

101 *C. sorokiniana* (CCAP 211/8K) was pre-cultivated axenically in 500 mL Erlenmeyer  
102 flasks with a working volume of 200 mL. A modified BG11 medium was used as  
103 described by Turon *et al* (2015). Sodium bicarbonate (10 mM) was used as an inorganic  
104 carbon (C) source, ammonium chloride (5 mM) as a nitrogen (N) source and  
105 dipotassium phosphate (0.31 mM) as a phosphorus (P) source. The flasks and  
106 components of the medium were sterilized by autoclaving at  $121^\circ\text{C}$  for 20 min before  
107 use. Before starting the experiment, the axenic culture was cultivated under autotrophic  
108 conditions (light intensity of  $100 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) at  $25^\circ\text{C}$  for 7 days.

#### 109 **2.1.2 General cultivation conditions**

110 The carbon concentration of each substrate was mainly set to  $0.3 \text{ g}_C\cdot\text{L}^{-1}$  by adding  
111 sodium bicarbonate, for autotrophic growth conditions, or acetic acid (glacial acetic  
112 acid, 27221-Sigma-Aldrich®) and/or butyric acid (B103500-Sigma-Aldrich®) solutions  
113 at 500 mM, for heterotrophic and mixotrophic growth conditions. For some specific  
114 experiments (Supplementary Information) the carbon concentration was set to  $0.2 \text{ g}_C\cdot\text{L}^{-1}$

115 <sup>1</sup>.As high acetate concentrations have been shown to increase the lag phase of *C.*  
116 *sorokiniana*(Qiao et al., 2012), especially in heterotrophic conditions,relatively low  
117 concentrations of acetate (0.3 g<sub>C</sub>.L<sup>-1</sup> equivalent to 0.75 g.L<sup>-1</sup> and 12.5 mM) and butyrate  
118 (0.3 g<sub>C</sub>.L<sup>-1</sup> equivalent to 0.55 g.L<sup>-1</sup> and 6.25 mM) were used.

119 The C:N:P molar ratiowas set to 48:16:1.Ammonium chloride and dipotassium  
120 phosphate were used as N and P sources, respectively. To encourage heterotrophic  
121 metabolism, sodium bicarbonate was not added to the media for mixotrophicand  
122 heterotrophicgrowth conditions. Only CO<sub>2</sub> from the air dissolved in the media was  
123 available for mixotrophic growth. To maintain the same pH throughout the experiments,  
124 the media were buffered with 100 mM of 2-(*N*-morpholino) ethanesulfonic acid(MES).  
125 The initial pH was set to between 6 and 6.5.Prior to sterilization using a 0.2 μm pore  
126 filter, the working solutions of acetate and butyrate were adjusted to pH 6.5 with NaOH.  
127 The flasks and all components of the medium were sterilized by autoclaving at 121°C  
128 for 20 min before use.The flasks were inoculated with *C.sorokiniana*stock cultures at  
129 10% V/V.

130 *C.sorokiniana*wascultivated in 125 mL black (heterotrophy) or transparent (autotrophy  
131 and mixotrophy) Erlenmeyer flasks containing 40 mL of medium and sealed with cotton  
132 plugs. The flasks were incubatedin the dark (heterotrophy) or under a non-saturating  
133 light intensity of  $123 \pm 10 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (autotrophy and mixotrophy) (Liu et al.,  
134 2012; Van Wagenen et al., 2014a)at different temperatures as described in sections 2.1.4  
135 and 2.1.5. The flasks wereshakenon a rotary shaker (150 rpm) for a maximum of 10  
136 days until the substrate was completely exhausted. All experiments and controls were  
137 performedin triplicate. During the experiment, axeny was checkeddaily by DAPI

138 staining and phase contrast microscopy as well as by spreading the cultures on  
139 ATCC5 solid media (ATCC, <http://www.lgcstandards-atcc.org/>).

### 140 **2.1.3 Cultivation at 25 °C**

#### 141 *2.1.3.1 Using DCMU to inhibit autotrophic growth*

142 A stock solution of 100 mM of 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU),  
143 diluted in ethanol, was used at a final nontoxic concentration of 10 µM for cultivation  
144 under mixotrophic, heterotrophic and autotrophic conditions (Zheng et al., 2014). The  
145 temperature was set to 25°C and light to 123 µmol photons.m<sup>-2</sup>.s<sup>-1</sup> when required. For  
146 the three growth conditions, a control with no DCMU was also carried out in a single  
147 flask.

#### 148 *2.1.3.2 Cultivation on a mixture of VFAs in the presence of light*

149 The mixotrophic growth of *Chlorella sorokiniana* on a mixture of acetate and butyrate at  
150 25 °C was compared to the mixotrophic growth on either acetate or butyrate, as single  
151 substrates (acetate-control and butyrate-control) and to the autotrophic growth  
152 (autotrophic control). The results obtained from a predictive model, as previously  
153 described by Turon *et al* (2015), on VFAs in the dark at 25°C were used to make  
154 assumptions about the heterotrophic growth (Turon et al., 2015). A Monod equation was  
155 used to describe the heterotrophic growth on acetate and a Haldane equation was used  
156 for butyrate. The diauxic growth pattern on acetate and butyrate was also included in the  
157 model. The acetate and butyrate concentrations tested in this study were in the range of  
158 concentrations used to build and validate the model. This model was developed to  
159 predict heterotrophic growth at 25 °C on acetate, butyrate or both acetate and butyrate.  
160 Since the lag phase was not considered when building the model, the microalgae

161 biomass and the acetate and butyrate concentrations, measured at the start of the  
162 microalgal growth curve, were used to initialize the Scilab simulations  
163 (<http://www.scilab.org>).

#### 164 ***2.1.4 Heterotrophic cultivation at 30 °C and 35 °C***

165 The microalgae growth on acetate and butyrate, as single substrates, and on a mixture of  
166 acetate and butyrate in the dark at 30°C and at 35°C was compared to the heterotrophic  
167 growth simulated at 25°C as described in sub-section 2.1.3.2.

#### 168 ***2.1.5 Cultivation at 35 °C under light***

169 The microalgae growth on acetate and butyrate, as single substrates, and on a  
170 combination of acetate and butyrate under light, set to  $123 \pm 10 \mu\text{mol photons.m}^{-2}.\text{s}^{-1}$ , at  
171 35°C was compared to autotrophic (with bicarbonate as the sole carbon source) and  
172 heterotrophic growth at 35°C (sub-section 2.1.4), to mixotrophic growth at 25°C (sub-  
173 section 2.1.3.2) and to predicted heterotrophic growth at 25 °C (sub-section 2.1.3.2).

## 174 **2.2 Analytical methods**

### 175 ***2.2.1 Biomass measurement***

176 The biomass growth was quantified by measuring the optical density at 800 nm ( $\text{OD}_{800}$ )  
177 to minimize pigment interference (Schmidt et al., 2005). Culture samples of 300  $\mu\text{L}$   
178 were dispensed into a 96 well BD Falcon® microplate and analyzed using an  
179 Infinite®M200 NanoQuant spectrophotometer (Tecan). DW was determined after  
180 filtering 15 mL of algal samples onto a pre-weighed GF/F Whatman® filter that was  
181 then dried overnight at 105°C. The calibration curve between DW and  $\text{OD}_{800}$  was  
182 determined using various dilutions of algal biomass for a wide range of dry weight  
183 values ( $0 - 1.4 \text{ g.L}^{-1}$ ). Three calibration curves were determined to allow for the

184 difference in microalgae cell shapes during heterotrophic and mixotrophic/autotrophic  
185 cultivation(Kumar et al., 2014).The equations were:

- 186 •  $DW (g.L^{-1}) = 1.24*OD_{800} (R^2 = 0.95)$  for heterotrophic cultivation,
- 187 •  $DW (g.L^{-1}) = 1.07*OD_{800} (R^2 = 0.94)$  for mixotrophic and autotrophic  
188 cultivation at 25 °C,
- 189 •  $DW (g.L^{-1}) = 1.15*OD_{800} (R^2 = 0.95)$  for mixotrophic and autotrophic  
190 cultivation at 35 °C.

191 The apparent growth rates,  $\mu_{app} (d^{-1})$ , during exponential growth were calculated as  
192 follows (Eq 1):

$$\mu_{app} = \frac{\ln(B_f) - \ln(B_0)}{t_f - t_0} \quad \text{Equation 1}$$

193

194 where  $t_0$  and  $t_f$  are the start and end of the exponential growth phase and  $B_0$  and  $B_f$  are the  
195 DWs ( $g.L^{-1}$ ) at  $t_0$  and  $t_f$ , respectively.

196 The apparent linear production rates of biomass,  $r_{app\_lin} (g.L^{-1}.d^{-1})$ , during linear growth  
197 were calculated as follows (Eq 2):

$$r_{app\_lin} = \frac{B_f - B_0}{t_f - t_0} \quad \text{Equation 2}$$

198 where  $t_0$  and  $t_f$  are the start and end of the exponential growth phase and  $B_0$  and  $B_f$  are the  
199 DWs ( $g.L^{-1}$ ) at  $t_0$  and  $t_f$ , respectively.

200 Under mixotrophic conditions, the mixotrophic carbon yields,  $Y_{Mixo}^{Mixo}$  ( $g_C$  of biomass per  
201  $g_C$  of substrate), on acetate and butyrate separately were calculated as follows (Eq 3):

$$Y_{Mixo}^{Mixo} = \frac{(X_f - X_0) * \alpha}{S_i} \quad \text{Equation 3}$$

202 where  $X_f$  and  $X_0$  are the DWs ( $\text{g.L}^{-1}$ ) at the start and the end of substrate exhaustion,  $\alpha$  is  
 203 the estimated content, 50%, of carbon in microalgae DW (Chen and Johns, 1996),  
 204  $S_i$  ( $\text{g.C.L}^{-1}$ ) is the initial concentration of substrate.

205 Under mixotrophic conditions, the heterotrophic carbon yields,  $Y_{Het}^{Mixo}$  ( $\text{g}_C$  of estimated  
 206 heterotrophic biomass per  $\text{g}_C$  of substrate), on acetate and butyrate separately were  
 207 calculated as follows (Eq 4):

$$Y_{Het}^{Mixo} = \frac{(X_f - X_0 - X_{ctrl\_auto}) * \alpha}{S_i} \quad \text{Equation 4}$$

208 where  $X_f$  and  $X_0$  are the DWs ( $\text{g.L}^{-1}$ ) at the start and the end of substrate exhaustion,  
 209  $X_{ctrl\_auto}$  is the DW in the strict autotrophic control at the same time as substrate  
 210 exhaustion,  $\alpha$  is the estimated content, 50%, of carbon in microalgae DW (Chen and  
 211 Johns, 1996),  $S_i$  ( $\text{g.C.L}^{-1}$ ) is the initial concentration of substrate.

212 Under mixotrophic conditions, the fraction of mixotrophic biomass due to heterotrophic  
 213 growth on acetate and/or butyrate,  $X_{Het}^{Mixo}$  (%), was calculated as follows (Eq 5):

$$X_{Het}^{Mixo} = \frac{Y_{Het}^{Mixo}}{Y_{Mixo}^{Mixo}} * 100 \quad \text{Equation 5}$$

214 Under mixotrophic conditions, the fraction of mixotrophic biomass due to autotrophic  
 215 growth on  $\text{CO}_2$ ,  $X_{Auto}^{Mixo}$  (%), was calculated as follows (Eq 6):

$$X_{Auto}^{Mixo} = 100 - X_{Het}^{Mixo} \quad \text{Equation 6}$$

216

## 217 **2.2.2 Measuring organic acids**

218 Volatile fatty acids (VFAs), e.g. acetate and butyrate, were quantified using a gas  
219 chromatograph (GC 3900 Varian) equipped with a flame ionization detector as  
220 previously described by Rafrafi *et al* ( 2013).

221 The errors associated with OD, DW and organic acid measurements were 2%, 6% and  
222 5%, respectively.

## 223 **2.3 Statistical analysis**

224 Pairwise comparisons of all results were performed by a one-way ANOVA and Tukey's  
225 post-hoc analysis. All statistical analyses were carried out using the Rcmdr package 1.9-  
226 6, R version 2.15.2 (R Development Core Team, 2012).

## 227 **3 Results and discussion**

### 228 **3.1 Effect of light on *C. sorokiniana* growth**

#### 229 **3.1.1 Mixotrophic conditions: a combination of autotrophic and heterotrophic** 230 **conditions**

231 DCMU is a specific inhibitor of electron transport between Photosystem I (PSI) and  
232 Photosystem II (PSII). DCMU was used to estimate the growth due to heterotrophic  
233 metabolism only, by organic carbon fixation from acetate, during mixotrophic growth by  
234 inhibiting autotrophic inorganic carbon fixation (Li et al., 2015). DCMU inhibits the  
235 transport of electrons from PSII to plastoquinone which further blocks the generation of  
236 NADPH and ATP in the chloroplast (Li et al., 2014). CO<sub>2</sub> fixation is subsequently  
237 hampered by the lack of both NADPH and ATP. The production of ATP via the cyclic  
238 electron flow in photosystem I is not affected (Li et al., 2014).

239 As shown in Figure 1-A, almost no growth was observed when microalgae  
240 werecultivated autotrophically in the presence of DCMU,confirming that theautotrophic  
241 metabolismwas inhibited and that no growth on cellular reserves was possible.

242 Heterotrophic growth on acetate only (acetate-control) was not inhibited by DCMU  
243 (Figure 1-A). Inthe presence of DCMU under mixotrophic conditions, ie. acetate and  
244 light, the pattern of microalgae growth was similar to the pattern under heterotrophic  
245 conditions (Figure 1-A). However, at day 1.9 (i.e., when the acetate was exhausted), the  
246 mixotrophic biomass ( $0.68 \text{ g.L}^{-1}$ ) was slightly higher (by 10%) than the sum of the  
247 heterotrophic ( $0.39 \text{ g.L}^{-1}$ ) and autotrophic ( $0.21 \text{ g.L}^{-1}$ ) biomasses. This suggests a  
248 synergistic interaction between the two metabolisms. Positive interactions could  
249 theoretically increase microalgae growth during mixotrophic metabolism: (i) through  
250 cellular energy(ATP), produced by photophosphorylation in the chloroplast that could  
251 be used to boost organic carbon uptake, (ii)by the  $\text{O}_2$ released during photo-oxidation of  
252 water in the chloroplast that could increase the respiration rate in the mitochondria and  
253 (iii) by the  $\text{CO}_2$ released during respiration on organic carbon that could be recycled  
254 through the Calvin cycle and increase the biomass yield (Wan et al., 2011; Yang et al.,  
255 2000). Li *et al* (2014) obtained similar results under mixotrophic conditions with light  
256 intensitiesranging from 100 to 200  $\mu\text{mol photons.m}^{-2}.\text{s}^{-1}$  and glucose as the substrate.In  
257 theirstudy, the*C. sorokiniana* mixotrophic growth rate was 20 to 40% higher than the  
258 sum of the growth rates obtained under heterotrophic and autotrophic conditions.

259 In order to provide further information on the heterotrophic fraction of the mixotrophic  
260 biomass, a strict autotrophic experiment (autotrophic control) was always run in parallel  
261 to the mixotrophic experiments. This control was used to assessthe heterotrophic carbon  
262 yield, $Y_{Het}^{Mixo}$ , associated with butyrate or acetate uptake during mixotrophic growth. The

263 biomass reached under autotrophic conditions can be subtracted from the observed  
264 mixotrophic biomass to assess the fraction of microalgae growth due to organic carbon  
265 assimilation, as described in Van Wageningen et al. (2014a). The excess biomass due to the  
266 positive interaction between the two metabolisms was considered as a boost to the  
267 biomass generated by heterotrophic growth.

### 268 ***3.1.2 Increase in the butyrate uptake rate in the presence of acetate under*** 269 ***mixotrophic conditions***

270 The effect of light on *C. sorokiniana* cultivated on a mixture of acetate and butyrate was  
271 studied. The strict autotrophic control (without organic substrate) was used to give a  
272 better explanation for the mixotrophic growth observed in Figure 1. During the  
273 exponential phase (first two days), the apparent autotrophic growth rate was  $1.04 \pm 0.05$   
274  $\text{d}^{-1}$ . During the linear phase (from day 2 to day 8), the biomass production rate was  $0.11$   
275  $\pm 0.01 \text{ g.L}^{-1}.\text{d}^{-1}$ . With limited light availability (low light intensities and cell self-  
276 shading) or  $\text{CO}_2$  limitation (no air or additional  $\text{CO}_2$ ), the exponential growth phase in  
277 autotrophic batch cultivation will be short and rapidly followed by linear growth  
278 (Ogbonna et al., 1995; Smith et al., 2015). The growth rates during autotrophic growth  
279 were consistent with previously reported results obtained under similar conditions with  
280 *C. sorokiniana* (Kim et al., 2013; Li et al., 2013; Rosenberg et al., 2014).

281 During mixotrophic growth on a mixture of acetate and butyrate (Figure 1-B),  
282 assimilation of acetate and butyrate was diauxic under mixotrophic conditions since  
283 butyrate uptake started only after the acetate had been completely exhausted, as  
284 previously observed in heterotrophic conditions, (Turon et al., 2015). The growth rates  
285 on acetate and butyrate were, therefore, analyzed separately.

286 The growth rate on acetate was slightly higher ( $2.7 \pm 0.1 \text{ d}^{-1}$ ) under mixotrophic  
287 conditions than estimated by modeling under heterotrophic conditions ( $2.21 \text{ d}^{-1}$  - see  
288 Table 1)(Turon et al., 2015). The total biomass accumulated just after acetate  
289 exhaustion in mixotrophic conditions was higher than the biomass predicted by the  
290 model in heterotrophic conditions (Figure 1-B). Furthermore, the mixotrophic carbon  
291 yield on acetate,  $Y_{Mixo}^{Mixo}$ , (Eq 3), was almost twice as high ( $0.79 \pm 0.04 \text{ d}^{-1}$ ) under  
292 mixotrophic conditions than predicted under heterotrophic conditions ( $0.42 \text{ g}_C \cdot \text{g}_C^{-1}$ )  
293 (Table 1). These results confirmed that the presence of light increased both the apparent  
294 growth rate and the mixotrophic carbon yield on acetate compared to those  
295 under heterotrophic conditions at  $25^\circ\text{C}$ . Under mixotrophic conditions, the heterotrophic  
296 carbon yield,  $Y_{Het}^{Mixo}$  - see Eq. 4, was calculated by subtracting the carbon yield  
297 for autotrophic growth (autotrophic control) from the mixotrophic carbon yield ( $Y_{Het}^{Mixo} =$   
298  $0.48 \pm 0.05 \text{ g}_C \text{ biomass per g}_C \text{ acetate}$ , see Table 1). Where there was uptake of both  
299 organic and inorganic carbon, only 39% of the microalgal biomass obtained after  
300 acetate exhaustion was due to  $\text{CO}_2$  assimilation ( $X_{Auto}^{Mixo}$ , see Eq 6 and Table 1). In the  
301 acetate control (with no butyrate), the fraction of biomass due to  $\text{CO}_2$  assimilation  
302 ( $X_{Auto}^{Mixo}$ , 30%) was statistically similar ( $p > 0.05$ ) (see Table 1 and Supplementary  
303 material Figure S1) but the mixotrophic growth rate on acetate reached  $4.1 \pm 0.4 \text{ d}^{-1}$ .  
304 When using mixtures of VFAs, there may be a high ATP demand to deal with the  
305 inhibitory effects of butyrate, such as cytosolic pH acidification, resulting in lower ATP  
306 availability for fast growth on acetate (Tromballa, 1978). In conclusion, the growth rate  
307 and carbon yield on acetate were higher in the presence of light than under heterotrophic  
308 conditions, suggesting that the mixotrophic growth on acetate probably relied on a  
309 synergy between heterotrophic and autotrophic conditions.

310 After a one-day delay after the acetate had been completely exhausted, there was linear  
311 butyrate uptake during the linear growth phase (Figure 1-B). Butyrate exhaustion in  
312 mixotrophic conditions was 3 days shorter than predicted for heterotrophic conditions  
313 (Figure 1-B). Based on the difference between the mixotrophic ( $Y_{Mixo}^{Mixo}$ , Eq 3), and  
314 heterotrophic ( $Y_{Het}^{Mixo}$ , Eq 4) carbon yields on butyrate, 62% of the biomass reached after  
315 butyrate exhaustion was probably due to CO<sub>2</sub> assimilation ( $X_{Auto}^{Mixo}$ , see Eq 6 and Table  
316 1). Similarly, in the butyrate control (without acetate - see Figure 1-C), 74% of the  
317 biomass obtained after butyrate exhaustion was probably due to CO<sub>2</sub> assimilation  
318 ( $X_{Auto}^{Mixo}$  - see Table 1). The model predicted that at 25 °C no heterotrophic growth would  
319 have been observed at such initial butyrate concentration (with no acetate - see Figure 1-  
320 C). Furthermore, the linear butyrate uptake rate measured after acetate exhaustion was  
321 1.5 times higher than measured for the butyrate control. It can, therefore, be concluded  
322 that mixotrophic conditions can substantially accelerate the apparent butyrate uptake  
323 through the production of algal biomass by CO<sub>2</sub> fixation.

## 324 **3.2 Effect of temperature on heterotrophic growth on VFAs**

### 325 **3.2.1 Inhibition by butyrate on heterotrophic growth on acetate at high temperature** 326 **(35°C)**

327 *C. sorokiniana* was grown heterotrophically on acetate as a single substrate (acetate  
328 control), on butyrate as single substrate (butyrate control) and on a mixture of acetate  
329 and butyrate, at 35°C known to be the optimum temperature (Janssen et al., 1999; Li et  
330 al., 2014; Van Wagenen et al., 2014b). On acetate (Supplementary material, Figure S2),  
331 the heterotrophic growth rate reached 5.88 d<sup>-1</sup> which was consistent with previously  
332 reported values at 35-37°C (Van Wagenen et al., 2014b).

333 For heterotrophic growth on a mixture of acetate and butyrate (Figure 2-A), the apparent  
334 growth rate on acetate, at 35°C ( $3.17 \pm 0.45 \text{ d}^{-1}$ ) was higher than at 25°C ( $2.23 \text{ d}^{-1}$  - see  
335 Table 2). However, microalgae biomass concentrations after acetate exhaustion were  
336 similar at 25°C and 35°C (Figure 2). The carbon yields on acetate at 25°C and at 35 °C  
337 were also similar (Table 2). However, the growth rate and carbon yield on acetate in the  
338 acetate control (Supplementary material, Figure S2) were almost 2 and 1.6 times higher  
339 than on the mixture of acetate and butyrate (Table 2). Even though the growth rate on  
340 acetate was highest at 35 °C in the acetate control, the presence of butyrate inhibited the  
341 increase growth rate on acetate at the higher temperature. At 25 °C, the presence of  
342 butyrate did not reduce the growth rate on acetate for butyrate concentrations up to 0.5  
343  $\text{g}_C \cdot \text{L}^{-1}$  (Turón et al., 2015). Ugwu et al (2000) reported that when one abiotic parameter  
344 (irradiance) was set to the optimum, the negative effects of another parameter (such as  
345 high dissolved oxygen concentration or temperature) were aggravated (Ugwu et al.,  
346 2007). Thus, when one growth factor is set at its optimum, the fast metabolism will, in  
347 particular, reduce energy storage and the microalgae might be less able to protect  
348 themselves from any adverse conditions. The negative effect of butyrate on  
349 heterotrophic growth on acetate at 35°C was reduced when the butyrate concentration  
350 was lowered to 0.2  $\text{g}_C \cdot \text{L}^{-1}$  (Supplementary material Figure S4). At this concentration,  
351 the growth rate ( $4.71 \pm 0.24 \text{ d}^{-1}$ ) and carbon yield ( $0.65 \pm 0.02 \text{ g}_C \cdot \text{g}_C^{-1}$ ) on acetate were  
352 higher than with 0.3  $\text{g}_C \cdot \text{L}^{-1}$  of butyrate. As a consequence, these results confirmed that  
353 butyrate inhibition of heterotrophic growth depended on the concentration, as previously  
354 suggested (Liu et al., 2012; Turón et al., 2015).

355 The apparent growth rate on butyrate was lower at 35 °C ( $0.11 \text{ d}^{-1}$ ) than the maximum  
356 growth rate at 25 °C ( $0.16 \text{ d}^{-1}$ ) (Table 2). However, when acetate was completely

357 exhausted, the butyrate was taken up and was exhausted after 9 days at 35 °C whereas  
 358 acetate was not predicted to be completely exhausted after 10 days at 25°C (Figure 2).  
 359 The growth rate associated with butyrate uptake,  $\mu_b(S_b)(d^{-1})$ , at 25°C, was described by  
 360 Turon *et al* (2015) as following a modified Haldane equation (Eq 7).

$$\mu_b(S_b) = \mu_{b\_max} * \frac{K_D}{K_D + S_a} * \frac{S_b}{S_b + \frac{\mu_{b\_max}}{\alpha} * \left(\frac{S_b}{S_{b\_opt}} - 1\right)^2} \quad \text{Equation 7}$$

361

362 where  $S_b$  is the concentration of butyrate ( $g_C.L^{-1}$ ),  $S_{b\_opt}(0.05 g_C.L^{-1})$  is the concentration  
 363 of butyrate when  $\mu_b(S_b)$  is maximum and equivalent to  $\mu_{b\_max}(0.16 d^{-1})$ , the maximum  
 364 growth rate associated with butyrate assimilation,  $\alpha(15.1 L.d.g_C^{-1})$  is the initial slope  
 365 and  $K_D(2.10^{-10} g_C.L^{-1})$  is the half inhibitory constant associated with the diauxic growth.

366 The predicted growth rate on butyrate at 25°C varied with the butyrate concentration  
 367 and reached its maximum,  $\mu_{b\_max}$ , after 9.5 days of cultivation when the butyrate  
 368 concentration reached  $S_{b\_opt}(0.05 g_C.L^{-1})$  (Supplementary Material Figure S3). At 35°C,  
 369 the apparent growth rate was calculated for a butyrate concentration of  $0.23 g_C.L^{-1}$  which  
 370 was reached after 5.7 days of cultivation (Figure 2-B). Consequently, the time to reach  
 371 butyrate exhaustion was shorter at 35 °C than at 25 °C despite a higher  
 372 maximum growth rate at 25 °C than the apparent growth rate at 35 °C (Figure 2). The  
 373 carbon yield on butyrate at 35°C was half that at 25°C. Contrary to the hypothesis  
 374 suggesting that the butyrate inhibition might be reduced at 35°C, butyrate inhibition was  
 375 stronger at 35°C than at 25°C. Furthermore, no microalgae growth was observed at  
 376 either 25 °C or 35 °C in the butyrate control (no acetate). As for growth on acetate in  
 377 mixture, butyrate inhibition at 35 °C depended on the concentration since the butyrate

378 uptake rate was faster at 35 °C than 25 °C when butyrate concentration was reduced to  
379 0.2 g<sub>C</sub>.L<sup>-1</sup> (Supplementary material Figure S4).

### 380 **3.2.2 *Reduced butyrate inhibition at 30 °C***

381 As shown in Figure 2-A and Table 2, the growth rate and carbon yield on acetate in  
382 mixture were both higher at 30 °C than at 25 °C or 35 °C. However, there was no  
383 significant difference (p>0.05) between these growth rates and carbon yields and those in  
384 the acetate control (Table 2, Supplementary material Figure S2). The presence of butyrate  
385 did not appear to inhibit microalgae growth on acetate at 30 °C.

386 Similarly, when butyrate was taken up (in mixture), the apparent growth rate and the  
387 microalgae biomass yield were higher at 30 °C (0.16 d<sup>-1</sup> and 0.56 g<sub>C</sub>.g<sub>C</sub><sup>-1</sup> respectively)  
388 than at 35 °C (0.11 d<sup>-1</sup> and 0.28 g<sub>C</sub>.g<sub>C</sub><sup>-1</sup> respectively) (Table 2). The apparent growth rate  
389 at 30 °C was calculated for a butyrate concentration of 0.29 g<sub>C</sub>.L<sup>-1</sup> which was reached  
390 after 2 days of cultivation (Figure 2 and Table 2). As explained in the previous  
391 paragraph (3.2.1), the maximum growth rate at 25 °C (0.16 d<sup>-1</sup>) could only be reached at  
392 a low butyrate concentration (0.05 g<sub>C</sub>.L<sup>-1</sup>). These results suggest that there was less  
393 butyrate inhibition at 30 °C than at 25 °C. Furthermore, microalgae growth was observed  
394 in the butyrate control whereas no growth was observed at 25 °C or 35 °C. A cultivation  
395 temperature of 30 °C thus successfully reduced butyrate inhibition and consequently  
396 butyrate exhaustion occurred more than 3 days earlier than at 25 °C (Figure 2-A). At  
397 30 °C, enzymatic reactions countering butyrate inhibition may have been encouraged.  
398 Temperatures higher than 25 °C increased heterotrophic growth on both acetate and  
399 butyrate. However, the near-optimum temperature for acetate was 35 °C while for  
400 butyrate it was 30 °C. Cultivation on a mixture of acetate and butyrate at a

401 suboptimum temperature for growth on acetate alone may have reduced butyrate  
402 inhibition.

### 403 **3.3 Combined effects of temperature and light on growth of *C. sorokiniana*** 404 **on a mixture of acetate and butyrate**

#### 405 **3.3.1 At 35 °C in the presence of light, microalgae growth on acetate or on butyrate** 406 **relied more on heterotrophic growth than at 25 °C**

407 A strict autotrophic control (bicarbonate as the sole carbon source) was carried out at  
408 35 °C to assess the effect of temperature in autotrophic conditions. In the autotrophic  
409 control, the autotrophic production rate of biomass ( $0.09 \text{ g.L}^{-1}.\text{d}^{-1}$ ) at 35 °C (Figure 3-A)  
410 was similar to that observed at 25 °C ( $0.11 \text{ g.L}^{-1}.\text{d}^{-1}$  - see Figure 1-B). Temperature  
411 appeared to have no significant effect on autotrophic growth.

412 Under mixotrophic conditions for the acetate control (no butyrate), the growth rate was  
413 significantly higher ( $p < 0.05$ ) at 35 °C ( $5.65 \text{ d}^{-1}$ ) than at 25 °C ( $4.14 \text{ d}^{-1}$ ) in the presence  
414 of light but was not significantly different from the growth rate observed at 35 °C with  
415 no light ( $5.88 \text{ d}^{-1}$ ) ( $p > 0.05$  - Tables 1 and 3). About 85% of the biomass content ( $X_{Het}^{Mixo}$ ,  
416 Eq 5) at the time of acetate exhaustion was due to acetate uptake (Table 3). These results  
417 suggest that *C. sorokiniana* followed a heterotrophic type of metabolism at 35 °C  
418 despite the presence of light.

419 The combined effects of temperature and light on microalgae growth for the  
420 butyrate control (no acetate) was also studied (Figure 3-A). During the first six days, the  
421 biomass in the butyrate control was lower than the biomass in the autotrophic control.  
422 The presence of butyrate seemed to inhibit autotrophic growth under mixotrophic  
423 conditions at 35 °C. This inhibition depended on the concentrations since autotrophic

424 growth was inhibited only during the first three days when the initial butyrate  
425 concentration was 0.2 g<sub>C</sub>.L<sup>-1</sup>(Supplementary material Figure S5-B).However, the  
426 butyrate uptake rate was significantly higher (p < 0.05) at 35 °C (88 mg<sub>C</sub>.L<sup>-1</sup>.d<sup>-1</sup>) than at  
427 25 °C (47.5 mg<sub>C</sub>.L<sup>-1</sup>.d<sup>-1</sup>) in the presence of light (Tables 1 and 3). Moreover, the fraction  
428 of biomass production due to autotrophic growth ( $X_{Auto}^{Mixo}$ , Eq 6) was lower (55%) at  
429 35°C than at 25°C (74%). As for growth on acetate, it was concluded that growth on  
430 butyrate at 35 °C with light relied more on heterotrophic growth than at 25 °C.

### 431 **3.3.2 At 35 °C, light reduced butyrate inhibition of growth on butyrate but not on** 432 **acetate**

433 The combined effect of temperature and light on *C. sorokiniana* growth on a mixture of  
434 acetate and butyrate, was studied to assess the interactions between acetate and butyrate  
435 (Figure 3-B). In the presence of butyrate, both the growth rate and the heterotrophic  
436 carbon yield on acetate (2.53 d<sup>-1</sup> and 0.36 g<sub>C</sub>.g<sub>C</sub><sup>-1</sup>, respectively) were half those  
437 measured in the acetate control (5.65 d<sup>-1</sup> and 0.60 g<sub>C</sub>.g<sub>C</sub><sup>-1</sup>, respectively – see Table 3).  
438 The growth rate on acetate was not statistically different (p > 0.05) from that measured  
439 with no light at 35 °C (3.17 d<sup>-1</sup>) (Tables 2 and 3). Consequently, butyrate inhibition of  
440 acetate uptake was not reduced by the presence of light at 35 °C. The fraction of  
441 biomass due to acetate uptake ( $X_{Het}^{Mixo}$ , Eq 5) was estimated at 60% (Table 3). This  
442 suggests that *C. sorokiniana* growth on acetate in a mixture of acetate and butyrate relied  
443 mostly on heterotrophic growth as was also observed for the acetate control.

444 Inhibition of autotrophic growth on butyrate which was observed in the  
445 butyrate control (paragraph 3.3.1) did not appear after acetate exhaustion (Figure 3-B). The  
446 fraction of biomass due to autotrophic growth ( $X_{Auto}^{Mixo}$ , Eq 6) at 35 °C was estimated at

447 62% (Table 3). The time taken to exhaust butyrate completely was 3 days less than under  
448 heterotrophic conditions at 25 °C and 35°C, probably because of the high biomass  
449 reached after acetate exhaustion and because of the autotrophic biomass growth at 35°C.  
450 Light increased butyrate uptake at 35°C for cultivation on a mixture of acetate and  
451 butyrate. At 35 °C, the presence of butyrate reduced the apparent growth rate on acetate  
452 under both heterotrophic and mixotrophic conditions and also inhibited autotrophic  
453 growth in the butyrate control under mixotrophic conditions. Further investigation on the  
454 effect of butyrate on the respiration rate and/or photosynthetic activity may provide  
455 further information on the negative effect of butyrate on mixotrophic and heterotrophic  
456 growth observed in this study at high temperature.

#### 457 **4 Conclusions**

458 The previously accepted optimum cultivation temperature (35°C) did not provide the  
459 best conditions for heterotrophic or mixotrophic growth of *C. sorokiniana* on a mixture  
460 of acetate and butyrate. The apparent heterotrophic growth rate on acetate was highest at  
461 30 °C ( $4.1 \text{ d}^{-1}$ ). At 25 °C light improved the apparent butyrate uptake ( $71 \text{ mg C} \cdot \text{L}^{-1} \cdot \text{d}^{-1}$ )  
462 because simultaneous heterotrophic and autotrophic growth increased the biomass  
463 (reaching  $1.14 \text{ g} \cdot \text{L}^{-1}$ ). In conclusion, *C. sorokiniana* may be cultivated successfully on  
464 DF effluents, at a temperature lower than that previously considered to be optimum  
465 (30°C) and with exposure to light.

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469 **6 References**

470 1 Chandra, R., Arora, S., Rohit, M.V., Venkata Mohan, S., 2015. Lipid metabolism in response to  
471 individual short chain fatty acids during mixotrophic mode of microalgal cultivation: Influence  
472 on biodiesel saturation and protein profile. *Bioresour. Technol.* 188, 169–176.

473 2 Chen, F., Johns, M.R., 1996. Relationship between substrate inhibition and maintenance energy  
474 of *Chlamydomonas reinhardtii* in heterotrophic culture. *J. Appl. Phycol.* 8, 15–19.

475 3 Fei, Q., Fu, R., Shang, L., Brigham, C.J., Chang, H.N., 2014. Lipid production by microalgae  
476 *Chlorella protothecoides* with volatile fatty acids (VFAs) as carbon sources in heterotrophic  
477 cultivation and its economic assessment. *Bioprocess Biosyst. Eng.* 691–700.

478 4 Ghimire, A., Frunzo, L., Pirozzi, F., Trably, E., Escudie, R., Lens, P.N.L., Esposito, G., 2015. A  
479 review on dark fermentative biohydrogen production from organic biomass: Process parameters  
480 and use of by-products. *Appl. Energy* 144, 73–95.

481 5 Griffiths, M.J., Harrison, S.T.L., 2009. Lipid productivity as a key characteristic for choosing  
482 algal species for biodiesel production. *J. Appl. Phycol.* 21, 493–507.

483 6 Janssen, M., Kuijpers, T.C., Veldhoen, B., Ternbach, M.B., Tramper, J., Mur, L.R., Wijffels,  
484 R.H., 1999. Specific growth rate of *Chlamydomonas reinhardtii* and *Chlorella sorokiniana* under  
485 medium duration light/dark cycles: 13-87 s. *Prog. Ind. Microbiol.* 35, 323–333.

486 7 Kim, S., Lee, Y., Hwang, S.-J., 2013. Removal of nitrogen and phosphorus by *Chlorella*  
487 *sorokiniana* cultured heterotrophically in ammonia and nitrate. *Int. Biodeterior. Biodegradation*  
488 85, 511–516.

489 8 Kumar, V., Muthuraj, M., Palabhanvi, B., Ghoshal, A.K., Das, D., 2014. High cell density lipid  
490 rich cultivation of a novel microalgal isolate *Chlorella sorokiniana* FC6 IITG in a single-stage  
491 fed-batch mode under mixotrophic condition. *Bioresour. Technol.* 170, 115–24.

492 9 Li, T., Gargouri, M., Feng, J., Park, J.-J., Gao, D., Miao, C., Dong, T., Gang, D.R., Chen, S.,  
493 2015. Regulation of Starch and Lipid Accumulation in a Microalga *Chlorella sorokiniana*.  
494 *Bioresour. Technol.* 180, 250–257.

495 10 Li, T., Zheng, Y., Yu, L., Chen, S., 2013. High productivity cultivation of a heat-resistant  
496 microalga *Chlorella sorokiniana* for biofuel production. *Bioresour. Technol.* 131, 60–7.

497 11 Li, T., Zheng, Y., Yu, L., Chen, S., 2014. Mixotrophic cultivation of a *Chlorella sorokiniana*  
498 strain for enhanced biomass and lipid production. *Biomass and Bioenergy* 66, 204–213.

499 12 Liang, Y., 2013. Producing liquid transportation fuels from heterotrophic microalgae. *Appl.*  
500 *Energy* 104, 860–868.

501 13 Liu, C.-H., Chang, C.-Y., Liao, Q., Zhu, X., Chang, J.-S., 2012. Photoheterotrophic growth of  
502 *Chlorella vulgaris* ESP6 on organic acids from dark hydrogen fermentation effluents. *Bioresour.*  
503 *Technol.* 145, 331–336.

504 14 Liu, C.-H., Chang, C.-Y., Liao, Q., Zhu, X., Liao, C.-F., Chang, J.-S., 2013. Biohydrogen  
505 production by a novel integration of dark fermentation and mixotrophic microalgae cultivation.  
506 *Int. J. Hydrogen Energy* 38, 15807–15814.

507 15 Lizzul, a M., Hellier, P., Purton, S., Baganz, F., Ladommatos, N., Campos, L., 2014. Combined  
508 remediation and lipid production using *Chlorella sorokiniana* grown on wastewater and exhaust  
509 gases. *Bioresour. Technol.* 151, 12–8.

510 16 Lowrey, J., Brooks, M.S., McGinn, P.J., 2015. Heterotrophic and mixotrophic cultivation of  
511 microalgae for biodiesel production in agricultural wastewaters and associated challenges—a  
512 critical review. *J. Appl. Phycol.* 27, 1485–1498.

513 17 Ogbonna, J.C., Yada, H., Tanaka, H., 1995. Kinetic study on light-limited batch cultivation of  
514 photosynthetic cells. *J. Ferment. Bioeng.* 80, 259–264.

515 18 Qiao, H., Wang, G., Liu, K., Gu, W., 2012. Short-Term Effects of Acetate and Microaerobic  
516 Conditions on Photosynthesis and Respiration in *Chlorella Sorokiniana* Gxnn 01 (Chlorophyta)1.  
517 *J. Phycol.* 48, 992–1001.

518 19 R Development Core Team, 2012. R: A Language and Environment for Statistical Computing (R  
519 Foundation for Statistical Computing, Vienna, Austria).

520 20 Rafrafi, Y., Trably, E., Hamelin, J., Latriille, E., Meynial-Salles, I., Benomar, S., Giudici-  
521 Ortoni, M.-T., Steyer, J.-P., 2013. Sub-dominant bacteria as keystone species in microbial  
522 communities producing bio-hydrogen. *Int. J. Hydrogen Energy* 38, 4975–4985.

- 523 21 Ren, H.-Y., Liu, B.-F., Kong, F., Zhao, L., Xing, D., Ren, N.-Q., 2014. Enhanced energy  
524 conversion efficiency from high strength synthetic organic wastewater by sequential dark  
525 fermentative hydrogen production and algal lipid accumulation. *Bioresour. Technol.* 157, 355–  
526 359.
- 527 22 Rosenberg, J.N., Kobayashi, N., Barnes, A., Noel, E.A., Betenbaugh, M.J., Oyler, G.A., 2014.  
528 Comparative analyses of three *Chlorella* species in response to light and sugar reveal distinctive  
529 lipid accumulation patterns in the microalga *C. sorokiniana*. *PLoS One* 9, e92460.
- 530 23 Schmidt, R.A., Wiebe, M.G., Eriksen, N.T., 2005. Heterotrophic high cell-density fed-batch  
531 cultures of the phycocyanin-producing red alga *Galdieria sulphuraria*. *Biotechnol. Bioeng.* 90,  
532 77–84.
- 533 24 Smith, R.T., Bangert, K., Wilkinson, S.J., Gilmour, D.J., 2015. Synergistic carbon metabolism in  
534 a fast growing mixotrophic freshwater microalgal species *Micractinium inermum*. *Biomass and*  
535 *Bioenergy* In press.
- 536 25 Trombala, H.W., 1978. Influence of permeant acids and bases on net potassium uptake by  
537 *Chlorella*. *Planta* 138, 243–8.
- 538 26 Turon, V., Baroukh, C., Trably, E., Latrille, E., Fouilland, E., Steyer, J.-P., 2015. Use of  
539 fermentative metabolites for heterotrophic microalgae growth: Yields and kinetics. *Bioresour.*  
540 *Technol.* 175, 342–349.
- 541 27 Ugwu, C.U., Aoyagi, H., Uchiyawa, H., 2007. Influence of irradiance, dissolved oxygen  
542 concentration, and temperature on the growth of *Chlorella sorokiniana*. *Photosynthetica* 45, 309–  
543 311.
- 544 28 Vajpeyi, S., Chandran, K., 2015. Microbial conversion of synthetic and food waste-derived  
545 volatile fatty acids to lipids. *Bioresour. Technol.* 188, 49–55.
- 546 29 Van Wagenen, J., De Francisci, D., Angelidaki, I., 2014a. Comparison of mixotrophic to cyclic  
547 autotrophic/heterotrophic growth strategies to optimize productivity of *Chlorella sorokiniana*. *J.*  
548 *Appl. Phycol.*
- 549 30 Van Wagenen, J., Holdt, S.L., De Francisci, D., Valverde-Pérez, B., Plósz, B.G., Angelidaki, I.,  
550 2014b. Microplate-based method for high-throughput screening of microalgae growth potential.  
551 *Bioresour. Technol.* 169, 566–572.
- 552 31 Venkata Mohan, S., Prathima Devi, M., 2012. Fatty acid rich effluent from acidogenic  
553 biohydrogen reactor as substrate for lipid accumulation in heterotrophic microalgae with  
554 simultaneous treatment. *Bioresour. Technol.* 123, 627–35.
- 555 32 Wan, M., Liu, P., Xia, J., Rosenberg, J.N., Oyler, G. a, Betenbaugh, M.J., Nie, Z., Qiu, G., 2011.  
556 The effect of mixotrophy on microalgal growth, lipid content, and expression levels of three  
557 pathway genes in *Chlorella sorokiniana*. *Appl. Microbiol. Biotechnol.* 91, 835–44.
- 558 33 Yang, C., Hua, Q., Shimizu, K., 2000. Energetics and carbon metabolism during growth of  
559 microalgal cells under photoautotrophic, mixotrophic and cyclic light-autotrophic/dark-  
560 heterotrophic conditions. *Biochem. Eng. J.* 6, 87–102.
- 561 34 Zheng, Y., Li, T., Yu, X., Bates, P.D., Dong, T., Chen, S., 2013. High-density fed-batch culture  
562 of a thermotolerant microalga *Chlorella sorokiniana* for biofuel production. *Appl. Energy* 108,  
563 281–287.
- 564 35 Zheng, Y., Yu, X., Li, T., Xiong, X., Chen, S., 2014. Induction of D-xylose uptake and  
565 expression of NAD(P)H-linked xylose reductase and NADP<sup>+</sup>-linked xylitol dehydrogenase in  
566 the oleaginous microalga *Chlorella sorokiniana*. *Biotechnol. Biofuels* 7, 125.

567

## 568 7 Figure captions

569 **Figure 1. Effect of DCMU and light on growth of *C. sorokiniana* cultivated on**

570 **butyrate and acetate at 25 °C.**

571 (A) Dry weight of *C. sorokiniana* cultivated without DCMU under autotrophic  
572 conditions (with 0.3 g<sub>C</sub>.L<sup>-1</sup> of NaHCO<sub>3</sub> and under 123 ± 10 μmol photons.m<sup>-2</sup>.s<sup>-1</sup>)(●),  
573 mixotrophic conditions (with 0.3 g<sub>C</sub>.L<sup>-1</sup> of acetate and under 123 ± 10 μmol photons.m<sup>-2</sup>.s<sup>-1</sup>) (◆)  
574 and heterotrophic conditions (with 0.3 g<sub>C</sub>.L<sup>-1</sup> of acetate in darkness) (■). Dry  
575 weight of *C. sorokiniana* cultivated with 10 μM DCMU under autotrophic conditions  
576 (with 0.3 g<sub>C</sub>.L<sup>-1</sup> of NaHCO<sub>3</sub> and under 123 ± 10 μmol photons.m<sup>-2</sup>.s<sup>-1</sup>) (●),  
577 mixotrophic conditions (with 0.3 g<sub>C</sub>.L<sup>-1</sup> of acetate and under 123 ± 10 μmol photons.m<sup>-2</sup>.s<sup>-1</sup>) (◆)  
578 and heterotrophic conditions (with 0.3 g<sub>C</sub>.L<sup>-1</sup> of acetate in darkness) (■). (B)  
579 and (C) *C. sorokiniana* cultivated under mixotrophic conditions at 25 °C. Dry weight (●  
580 ), butyrate concentration (◆) and acetate concentration (■) during cultivation (B) on a  
581 mixture of butyrate and acetate, 0.3 g<sub>C</sub>.L<sup>-1</sup> of each and (C) on 0.3 g<sub>C</sub>.L<sup>-1</sup> of butyrate as  
582 single substrate (butyrate control). The dry weight for autotrophic cultivation (●) and the  
583 predicted values for heterotrophic cultivation at 25 °C - dry weight (green dashed lines),  
584 acetate concentration (red dashed lines) and butyrate concentration (blue dashed lines)-  
585 are shown for comparison.

586 **Figure 2. Effect of increasing temperature, from 25 °C to 35 °C, on heterotrophic**  
587 **growth of *Chlorella sorokiniana* cultivated on a mixture of acetate and butyrate.**

588 (A) Dry weight of *C. sorokiniana* cultivated under heterotrophic conditions on a mixture  
589 of acetate and butyrate at 30 °C (●) and 35 °C (●). (B) Acetate and butyrate  
590 concentrations for cultivation at 30 °C (■ and ◆) and 35 °C (■ and ◆). The  
591 predicted values for heterotrophic cultivation at 25 °C - dry weight (green dashed lines),  
592 acetate concentration (red dashed lines) and butyrate concentration (blue dashed lines) at  
593 25 °C are shown for comparison.

594 **Figure 3. Effect of butyrate on growth of *C. sorokiniana* cultivated on acetate and**  
595 **butyrate at 35 °C under mixotrophic conditions.**

596 Dry weight of *C. sorokiniana* (●), butyrate concentration (◆) and acetate concentration  
597 (■) during cultivation on (A) 0.3 g<sub>C</sub>.L<sup>-1</sup> of butyrate (butyrate control) and (B) on a  
598 mixture of 0.3 g<sub>C</sub>.L<sup>-1</sup> butyrate and 0.3 g<sub>C</sub>.L<sup>-1</sup> acetate. The dry weight for autotrophic  
599 cultivation (●) and the predicted values for heterotrophic cultivation at 25 °C - dry  
600 weight (green dashed lines), acetate concentration (red dashed lines) and butyrate  
601 concentration (blue dashed lines) - are shown for comparison.

Figure 1

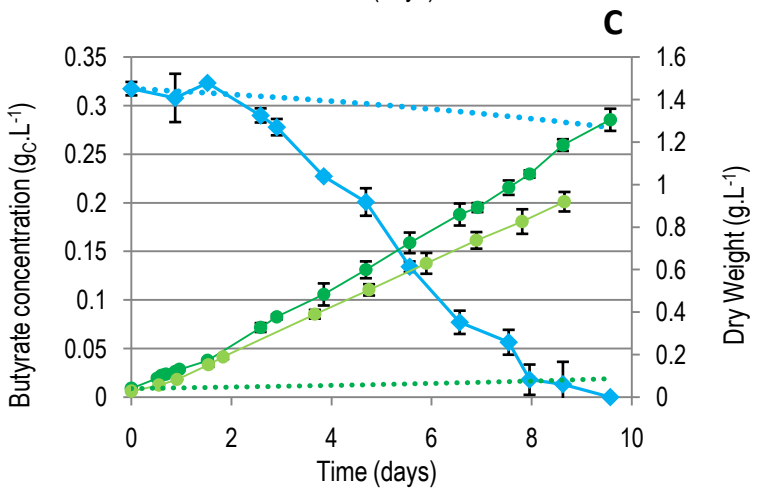
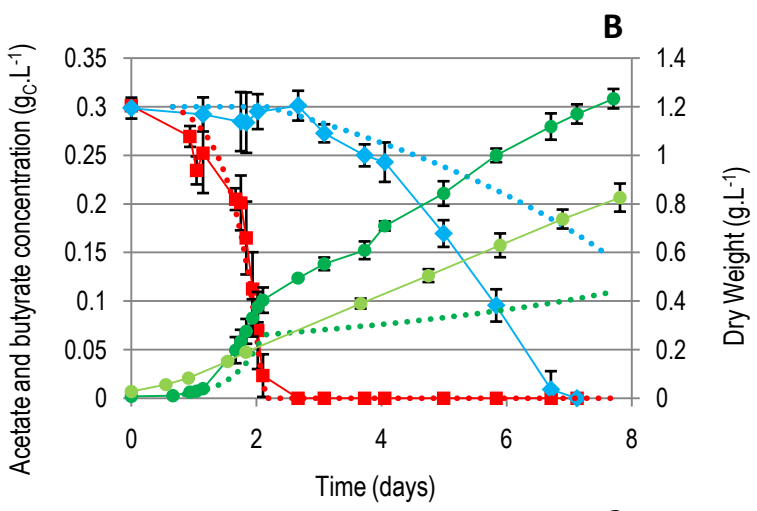
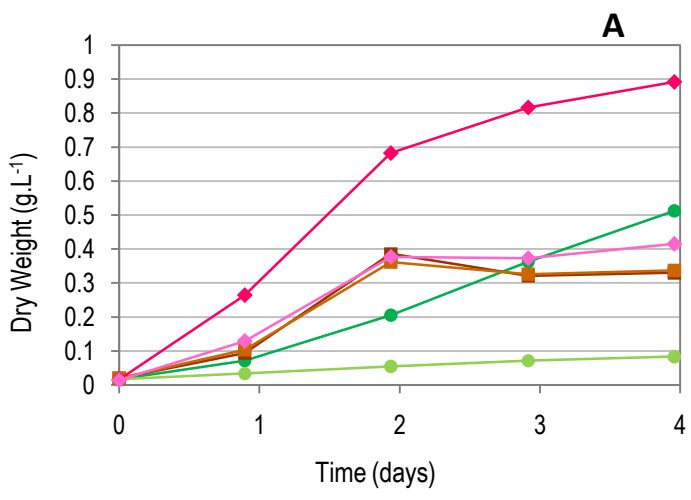


Figure 2

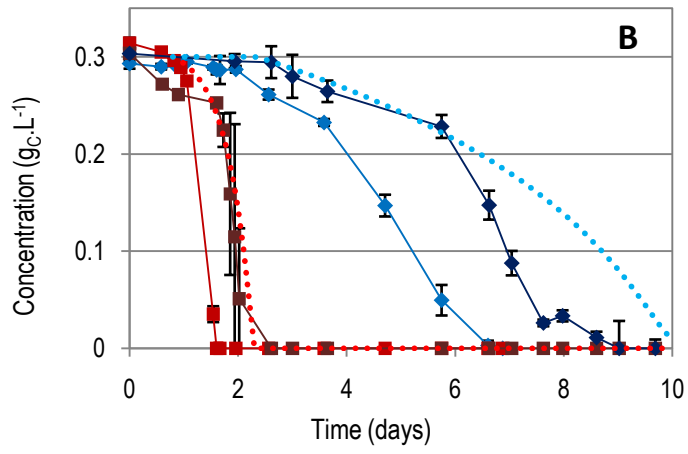
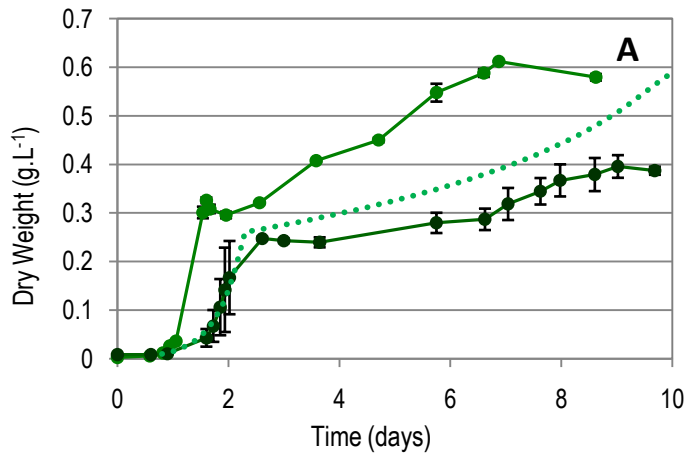
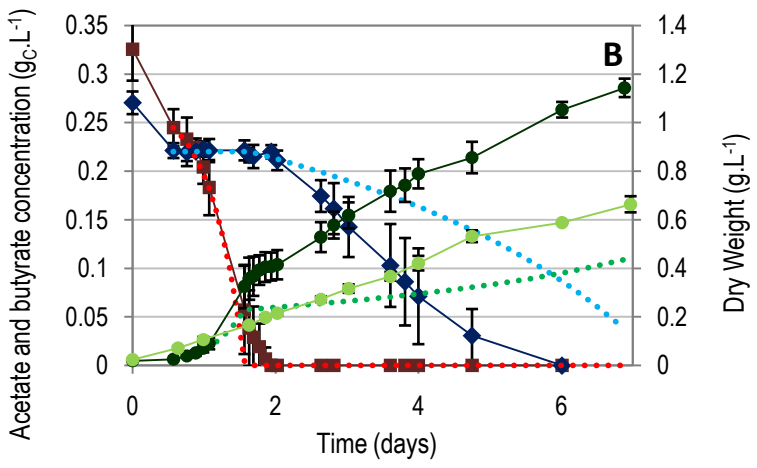
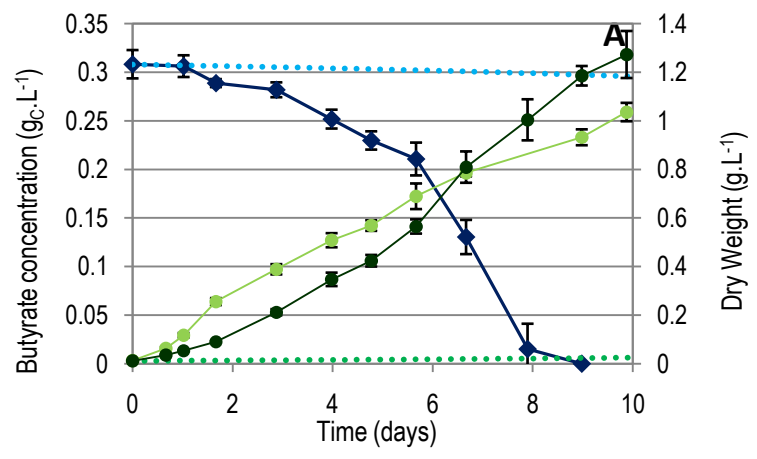


Figure 3



## Supplementary Material

### Figure S1. Effect of light on *C. sorokiniana*'s growth on acetate ( $0.3 \text{ g}_C\cdot\text{L}^{-1}$ ) at 25

°C. Microalgae concentration ( $\text{g}_C\cdot\text{L}^{-1}$ ) (●) and acetate concentration (■) are presented.

Microalgae concentration ( $\text{g}_C\cdot\text{L}^{-1}$ ) (●) during autotrophic growth is presented. The simulated heterotrophic microalgae concentration (green dashed lines) and acetate concentration (red dashed lines) (blue dashed lines) at 25 °C are represented.

### Figure S2. Effect of temperature on microalgae heterotrophic growth on acetate

( $0.3 \text{ g}_C\cdot\text{L}^{-1}$ ). Microalgae concentration, in  $\text{g}_C\cdot\text{L}^{-1}$ , during heterotrophic growth on acetate

at 30 °C (●) and 35 °C (●) are represented in subfigure A. Acetate concentrations, in

$\text{g}_C\cdot\text{L}^{-1}$ , during growth at 30 °C (■) and 35 °C (■) are represented in subfigure B. The

simulated heterotrophic microalgae concentration (green dashed lines) and acetate concentration (red dashed lines) (blue dashed lines) at 25 °C are represented.

### Figure S3. Variation of the growth rate on butyrate ( $\mu_b(Sb)$ ) according to the simulations of the model representing heterotrophic growth at 25°C.

### Figure S4. Heterotrophic growth of *Chlorella sorokiniana* on mixtures of acetate

and butyrate ( $0.2 \text{ g}_C\cdot\text{L}^{-1}$  each) at 25 °C, 30 °C and 35 °C. Microalgae concentration,

in  $\text{g}_C\cdot\text{L}^{-1}$ , during heterotrophic growth on mixtures of acetate and butyrate at 30 °C (●)

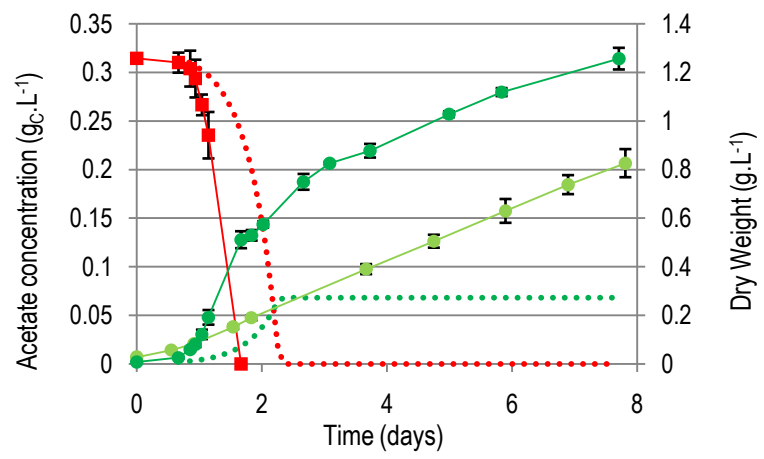
and 35 °C (●) are represented in subfigure A. Acetate and butyrate removals, in  $\text{g}_C\cdot\text{L}^{-1}$ ,

during growth at 30 °C (■ and ◆) and 35 °C (■ and ◆) are represented in subfigure B.

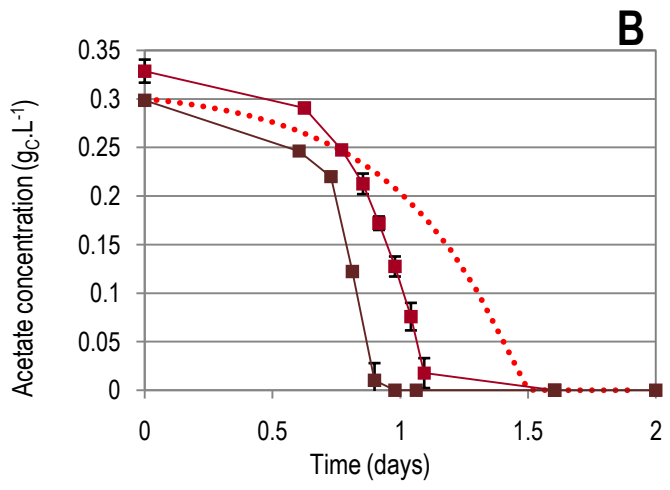
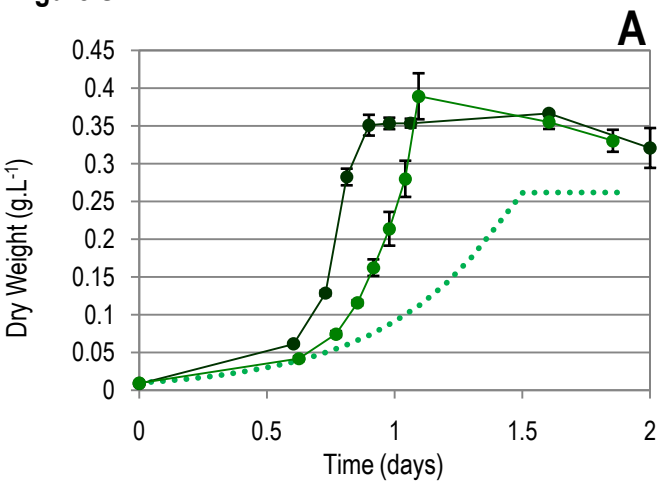
The simulated heterotrophic microalgae concentration (green dashed lines), acetate concentration (red dashed lines) and butyrate concentration (blue dashed lines) at 25 °C are represented.

**Figure S5. Comparison of autotrophic and mixotrophic growth of *C. sorokiniana* on 0.3 g<sub>C</sub>.L<sup>-1</sup> of acetate (A) and 0.2 g<sub>C</sub>.L<sup>-1</sup> of butyrate (B) at 35 °C.** Microalgae concentration (g.L<sup>-1</sup>) (●), butyrate uptake (■) and acetate uptake (◆) are presented. Microalgae concentration (g.L<sup>-1</sup>) (●) during autotrophic growth is presented. The simulated heterotrophic microalgae concentration (green dashed lines), acetate concentration (red dashed lines) and butyrate concentration (blue dashed lines) at 25 °C are represented.

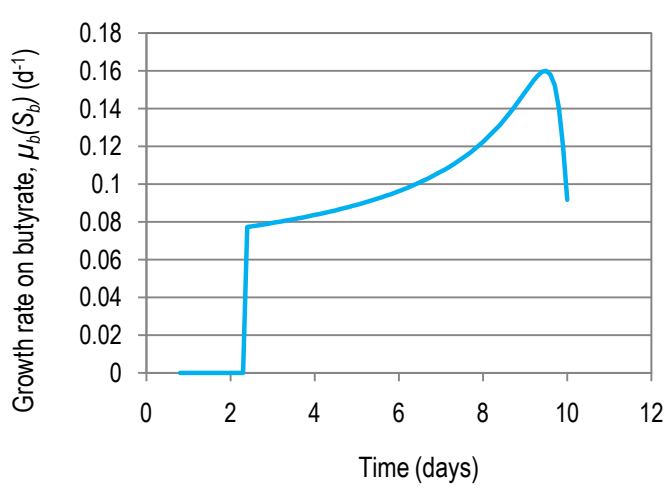
**Figure S1**



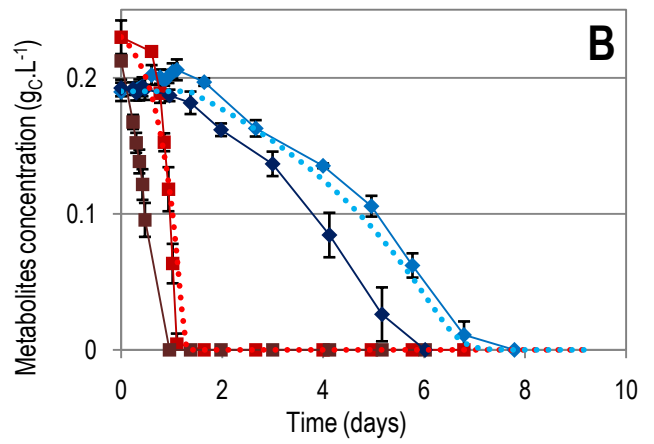
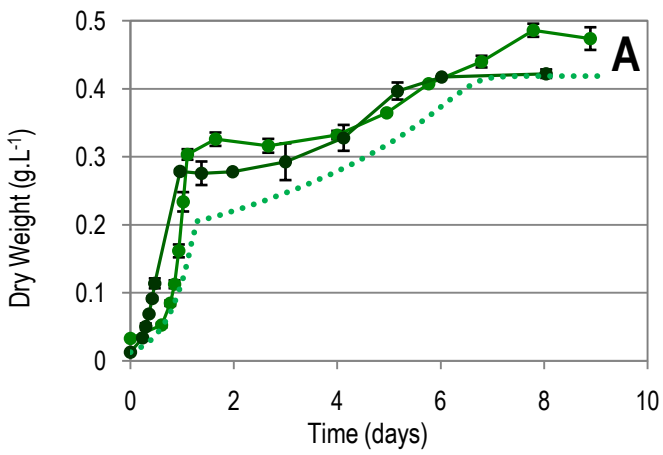
**Figure S2**



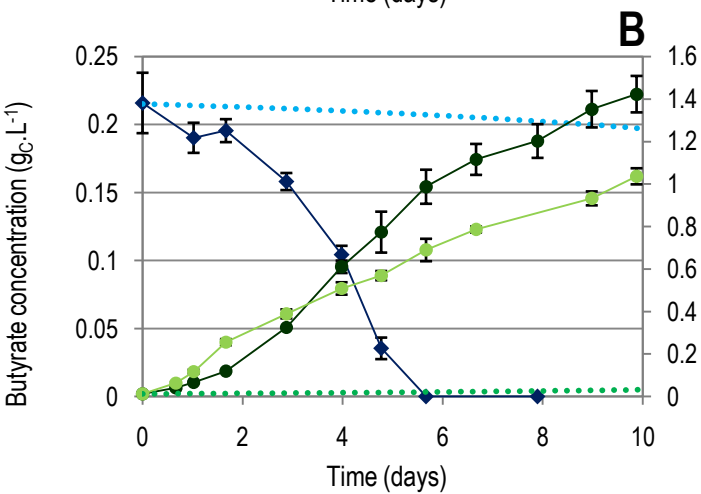
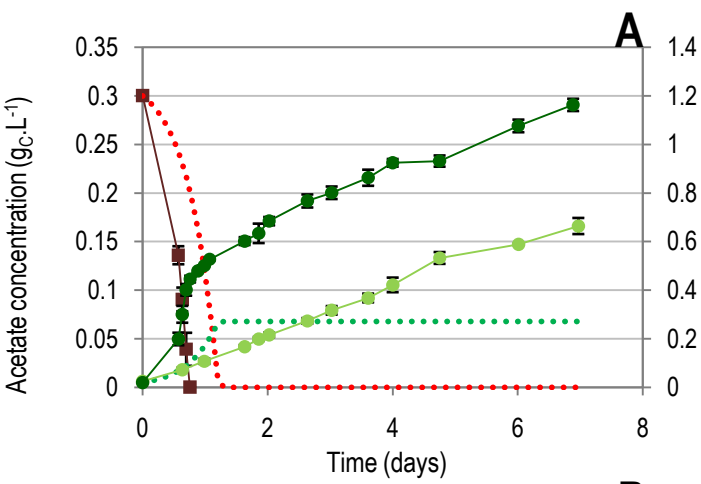
**Figure S3**



**Figure S4**



**Figure S5.**



**Table 1**

**Effect of light on growth and production rates ( $\mu_{app}$  and  $r_{lin}$ ) and yields of *C. sorokiniana* for cultivation at 25 °C on acetate (A), butyrate (B) and a mixture of butyrate and acetate (A + B). Mean values and standard deviations calculated from triplicates are given.**

	Growth on acetate				Growth on butyrate				
	$\mu_{app}$ (d <sup>-1</sup> )	$Y_{Mixo}^{Mixo}$ (g <sub>c</sub> .g <sub>c</sub> <sup>-1</sup> )	$Y_{Het}^{Mixo}$ (g <sub>c</sub> .g <sub>c</sub> <sup>-1</sup> ) <sup>a</sup>	$X_{Auto}^{Mixo}$ (%) <sup>b</sup>	$r_{app\_lin}$ (g.L <sup>-1</sup> .d <sup>-1</sup> )	Uptake rate (mg <sub>c</sub> .L <sup>-1</sup> .d <sup>-1</sup> )	$Y_{Mixo}^{Mixo}$ (g <sub>c</sub> .g <sub>c</sub> <sup>-1</sup> )	$Y_{Het}^{Mixo}$ (g <sub>c</sub> .g <sub>c</sub> <sup>-1</sup> ) <sup>a</sup>	$X_{Auto}^{Mixo}$ (%) <sup>b</sup>
A	4.14 ± 0.35	0.8 ± 0.05	0.56 ± 0.06	30					
B					0.14 ± 0.00	47.5 ± 0.5	1.69 ± 0.02	0.44 ± 0.03	74
A + B	2.68 ± 0.12	0.79 ± 0.04	0.48 ± 0.05	39	0.16 ± 0.01	71 ± 2.7	1.19 ± 0.11	0.45 ± 0.05	62

<sup>a</sup>: The heterotrophic carbon yield ( $Y_{Het}^{Mixo}$ ) was calculated by subtracting the carbon yield associated with autotrophic growth from the mixotrophic carbon yield ( $Y_{Mixo}^{Mixo}$ ).

<sup>b</sup>: The fraction of mixotrophic biomass due to autotrophic growth on CO<sub>2</sub> ( $X_{Auto}^{Mixo}$ ) was calculated as follows:

$$X_{Auto}^{Mixo} = \frac{Y_{Mixo}^{Mixo} - Y_{Het}^{Mixo}}{Y_{Mixo}^{Mixo}} * 100$$

**Table 2**

**Effect of temperature on apparent growth rate ( $\mu_{app}$ ) and heterotrophic carbon yield of *Chlorella sorokiniana* under heterotrophic conditions on acetate (A), butyrate (B) and a mixture of butyrate and acetate (B + A). The figures at 25 °C are taken from a previous study for heterotrophic growth of *C. sorokiniana*. For 30 °C and 35 °C, the mean values and standard deviations calculated from triplicates are given. Values with different letters are statistically different ( $p \leq 0.05$ , one-way ANOVA and Tukey's post-hoc analysis). The carbon yield was estimated for a microalgae cell composition of 50% of carbon [12].**

Temperature	Conditions tested	Growth on acetate		Growth on butyrate	
		$\mu_{app}$ (d <sup>-1</sup> )	$Y_{Het}^{Het}$ (gc.gc <sup>-1</sup> )	$\mu_{app}$ (d <sup>-1</sup> )	$Y_{Het}^{Het}$ (gc.gc <sup>-1</sup> )
25 °C	A; B and A + B	2.23	0.42	0.16*	0.56
30 °C	A	4.65 ± 0.16 <sup>a</sup>	0.58 ± 0.04 <sup>a, b</sup>		
	B			0.13 ± 0.01 <sup>a, b</sup>	0.42 ± 0.03 <sup>a</sup>
	A + B	4.12 ± 0.19 <sup>a</sup>	0.51 ± 0.01 <sup>a</sup>	0.16 ± 0.01 <sup>b</sup>	0.56 ± 0.01 <sup>b</sup>
35 °C	A	5.88 ± 0.39 <sup>b</sup>	0.64 ± 0.06 <sup>b</sup>		
	B			No growth	
	A + B	3.17 ± 0.45 <sup>c</sup>	0.41 ± 0.02 <sup>c</sup>	0.11 ± 0.02 <sup>a</sup>	0.28 ± 0.03 <sup>c</sup>