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

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Abstract

The feasibility of coupling of dark fermentation to produce biohydrogen, with heterotrophic cultivation of microalgae, to produce biolipids from fermentation by-products, is limited bybutyrate which inhibits the growth of themicroalgae. This study investigated the influence of light and temperature on*Chlorella sorokiniana*grown on a mixture of acetateand butyrate, two of the volatile fatty acids produced by dark fermentation.Exposure to light caused autotrophicbiomass production (56% of the final biomass)and reduced thetime to reach butyrate exhaustionto 7 days at 25 °C from 10 days in the dark. For growth on acetate at the optimum temperature (35 °C), the presence of butyratereduced the growth rate (by 46%) and the carbon yield (by 36%). For successful microalgae growth on dark fermentation effluent, butyrate inhibition may be reduced by setting the temperature to 30 °Cand providing light.

Keywords

Chlorella sorokiniana;Dark Fermentation; Heterotrophy; Mixotrophy; Butyrate inhibition

1 Introduction

Many studies and industrial projects have shown the value of heterotrophic cultivation of microalgae forproducing high added value compounds, such as docosahexaenoic acid (DHA), andcommodity compounds, such as lipids for biofuels (Lowrey et al., 2015). When microalgae are grown on organic carbon sources in the dark, they tend to grow faster with higher biomass and lipid yields than when they are grown usingconventional autotrophic cultivation (Liang, 2013). However, in order to reduce production costs, an alternative to glucose, the most common substrate, must be found, especially for producing biofuels (Liang, 2013). Acetate has been suggested as one of the best alternatives since it can be easily incorporated into lipids or carbohydratesby microalgaeandis widely available as a cheap source of carbon(Lowrey et al., 2015). Moreover, acetate is one of the main end-productsof microbial dark fermentation (DF)of various types of urban, agricultural and industrial waste (Ghimire et al., 2015).

Recently, several studies have shown the benefits and feasibility of coupling DF, which produces hydrogen as the main product and volatile fatty acids (VFAs) as secondary metabolites, with microalgae cultivation, which produces bothmicroalgal biomass and lipids(Chandra et al., 2015; Liu et al., 2013, 2012; Ren et al., 2014; Turon et al., 2015; Venkata Mohan and Prathima Devi, 2012).During DF, complex organic compounds originating from waste are converted by anaerobic bacteria into simple VFAs, mainly acetate and butyrate, that can be further assimilated by microalgae(Ghimire et al., 2015).

The effluent from DF providesa low-cost source of carbon which can successfully sustain heterotrophic microalgae growth(Liu et al., 2013; Ren et al., 2014).For example,VFAs were efficiently converted into carbohydrates (51% of dry weight (DW)) by *Chlorella vulgaris* grown heterotrophically on diluted DF effluent (Liu et al., 2013) andacetate was used to produce lipids, up to 41% of DW, by the heterotroph *Scenedesmus sp.* grown on fermentation effluent (Ren et al., 2014). These studies reported thatbutyrate inhibitedmicroalgae growth, at concentrations as low as 0.1 $g.L^{-1}$, and thisis now considered to beone of the main challengesthat mustbe overcome whencouplingDF and heterotrophic cultivation of microalgae(Fei et al., 2014; Liu et al., 2012; Turon et al., 2015).

Butyrate uptake by microalgae is much slower than acetate uptake and can also reduce microalgae growth when using amixture of VFAs as a source of carbon (Fei et al., 2014). Similar differences between acetate and butyrate uptake rates havealso beenreported for oleaginous fungi (Vajpeyi and Chandran, 2015). Liu et al. (2013)reported that growing *C. vulgaris* mixotrophically, with light and carbon dioxide, could reducethe inhibitory effect of butyrate. For mixotrophic growth on butyrate alone, it was suggested that microalgae assimilated CO_2first, with a subsequent increase in the total biomass,resulting infaster uptake of butyrate(Liu et al., 2013, 2012). However, these authors suggested that carbon dioxide was probably preferred to butyrate as a substrate and that strong competition between CO_2 and butyrate uptakecombined withhigh CO_2availabilitymay, therefore,lower the butyrate consumption rate(Liu et al., 2013, 2012).

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

2014). When grown heterotrophically at its optimum growth temperature (37 °C) on glucose in a two-stage fed-batch culture including a first stage for biomass growth and a second stage for lipid accumulation through nitrogen depletion, *C. sorokiniana* produced very high biomass of 103.8 $g.L^{-1}$and lipid concentrationsof 40.2 $g.L^{-1}$(Zheng et al., 2013).Between35 °C and 37 °C,*C. sorokiniana* achieved high growth rates of 3.4 d^{-1}under mixotrophic conditions and 6.5 d^{-1}under autotrophic conditions(Janssen et al., 1999; Li et al., 2014; Van Wagenen et al., 2014b). These results suggest that temperature and light might be key parameters for increasing*C. sorokiniana* growth on VFAs.

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

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

testedon the growth rate and carbon yield of *C. sorokiniana* growing on a mixture of acetate and butyrate at an inhibiting butyrate concentration (both at 0.3 $g_C.L^{-1}$).Control experiments with either acetate or butyrate as single substrate (0.3 $g_C.L^{-1}$) were also performed to give a better understanding of the interactions between acetate and butyrate uptake mechanisms.

2 Materials and methods

2.1 Microalgae cultivation conditions

2.1.1 Chlorella sorokiniana stock cultivation conditions

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

2.1.2 General cultivation conditions

The carbon concentration of each substrate was mainly set to 0.3 $g_C.L^{-1}$ by adding sodium bicarbonate, for autotrophic growth conditions, or acetic acid (glacial acetic acid, 27221-Sigma-Aldrich®) and/or butyric acid (B103500-Sigma-Aldrich®) solutions at 500 mM, for heterotrophic and mixotrophic growth conditions. For somespecific experiments (Supplementary Information) the carbon concentration was set to 0.2 $g_C.L^{-}$

[1].As high acetate concentrations have been shown to increase the lag phase of *C. sorokiniana*(Qiao et al., 2012), especially in heterotrophic conditions,relatively low concentrations of acetate (0.3 $g_C.L^{-1}$ equivalent to 0.75 $g.L^{-1}$ and 12.5 mM) and butyrate (0.3 $g_C.L^{-1}$ equivalent to 0.55 $g.L^{-1}$ and 6.25 mM) were used.

The C:N:P molar ratiowas set to 48:16:1.Ammonium chloride and dipotassium phosphate were used as N and P sources, respectively. To encourage heterotrophic metabolism, sodium bicarbonate was not added to the media for mixotrophicand heterotrophicgrowth conditions. Only CO_2 from the air dissolved in the media was available for mixotrophic growth. To maintain the same pH throughout the experiments, the media were buffered with 100 mM of 2-(*N*-morpholino) ethanesulfonic acid(MES). The initial pH was set to between 6 and 6.5.Prior to sterilization using a 0.2 µm pore filter, the working solutions of acetate and butyrate were adjusted to pH 6.5 with NaOH. The flasks and all components of the medium were sterilized by autoclaving at 121°C for 20 min before use.The flasks were inoculated with *C.sorokiniana*stock cultures at 10% V/V.

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

staining and phase contrast microscopy as well as byspreading the cultures on ATCC5solid media (ATCC, http://www.lgcstandards-atcc.org/).

2.1.3 Cultivation at 25 °C

2.1.3.1 Using DCMU to inhibit autotrophic growth

A stock solution of 100 mMof 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU), diluted in ethanol, was used at a final nontoxic concentration of 10 µMfor cultivation under mixotrophic, heterotrophic and autotrophic conditions (Zheng et al., 2014).The temperature was set to 25°C and light to123 µmol photons.m^{-2}.s^{-1} when required. For the three growth conditions, a control with no DCMU was also carried out ina single flask.

2.1.3.2 Cultivation on a mixture of VFAs in the presence of light

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

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

2.1.4 Heterotrophic cultivation at 30 °C and 35 °C

The microalgae growth on acetate and butyrate, as single substrates, and on a mixture of acetate and butyrate in the dark at 30°C and at 35°C wascompared to the heterotrophic growth simulatedat 25°C as described in sub-section 2.1.3.2.

2.1.5 Cultivation at 35 °C under light

The microalgae growth on acetate and butyrate, as single substrates, and on a combination of acetate and butyrate under light, set to 123 ± 10 µmol photons.m^{-2}.s^{-1},at 35°C was compared to autotrophic (with bicarbonate as the sole carbon source) and heterotrophic growth at 35°C (sub-section 2.1.4), to mixotrophicgrowth at 25°C (sub-section 2.1.3.2)and to predicted heterotrophic growth at 25 °C (sub-section 2.1.3.2).

2.2 Analytical methods

2.2.1 Biomass measurement

The biomass growth was quantified by measuring the optical densityat 800 nm (OD_{800}) to minimize pigment interference (Schmidt et al., 2005). Culture samples of 300µL were dispensed into a 96 well BD Falcon® microplate and analyzed using an Infinite®M200 NanoQuant spectrophotometer (Tecan). DW was determined after filtering 15 mL of algal samples onto a pre-weighed GF/F Whatman® filter that was then dried overnight at 105°C. The calibration curve between DW and $OD_{800}$was determined usingvarious dilutions of algal biomass for a wide range of dry weight values (0 – 1.4 $g.L^{-1}$).Three calibration curves weredetermined 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, μ_{app} (d^{-1}), during exponential growth were calculated as
192 follows (Eq 1):

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

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):

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

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):

$$Y_{Mixo}^{Mixo} = \frac{(X_f - \mathrm{X}_0) * \alpha}{S_i} \quad \text{Equation 3}$$

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

Under mixotrophic conditions, the heterotrophic carbon yields, Y_{Het}^{Mixo} (g_C of estimated heterotrophic biomass per g_C of substrate), on acetate and butyrate separately were calculated as follows (Eq 4):

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

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

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

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

Under mixotrophic conditions, the fraction of mixotrophic biomass due to autotrophic growth on CO_2, X_{Auto}^{Mixo} (%), was calculated as follows (Eq 6):

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

2.2.2 Measuring organic acids

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

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

2.3 Statistical analysis

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

3 Results and discussion

3.1 Effect of light on*C. sorokiniana* growth

3.1.1 Mixotrophic conditions: a combination of autotrophic and heterotrophic conditions

DCMU is a specific inhibitor of electron transport between Photosystem I (PSI) and Photosystem II (PSII). DCMU was used to estimate the growth due to heterotrophic metabolism only,by organic carbon fixationfrom acetate,during mixotrophic growth by inhibiting autotrophic inorganic carbon fixation (Li et al., 2015). DCMU inhibits the transport of electrons from PSII to plastoquinone which further blocks the generation of NADPH and ATP in the chloroplast (Li et al., 2014). CO_2 fixation is subsequently hampered by the lack of both NADPH and ATP. The production of ATP via the cyclic electron flow in photosystem I is not affected(Li et al., 2014).

As shown in Figure 1-A, almost no growth was observed when microalgae werecultivated autotrophically in the presence of DCMU,confirming that theautotrophic metabolismwas inhibited and that no growth on cellular reserves was possible. Heterotrophic growth on acetate only (acetate-control) was not inhibited by DCMU (Figure 1-A). Inthe presence of DCMU under mixotrophic conditions, ie. acetate and light, the pattern of microalgae growth was similar to the pattern under heterotrophic conditions (Figure 1-A). However, at day 1.9 (i.e., when the acetate was exhausted), the mixotrophic biomass (0.68 $g.L^{-1}$) was slightly higher (by 10%) than the sum of the heterotrophic (0.39 $g.L^{-1}$) and autotrophic (0.21 $g.L^{-1}$) biomasses. This suggests a synergistic interaction between the two metabolisms. Positive interactions could theoretically increase microalgae growth during mixotrophic metabolism: (i) through cellular energy(ATP), produced by photophosphorylation in the chloroplast that could be used to boost organic carbon uptake, (ii)by the O_2released during photo-oxidation of water in the chloroplast that could increase the respiration rate in the mitochondria and (iii) by the CO_2released during respiration on organic carbon that could be recycled through the Calvin cycle and increase the biomass yield (Wan et al., 2011; Yang et al., 2000). Li *et al* (2014) obtained similar results under mixotrophic conditions with light intensitiesranging from 100 to 200 µmol photons.$m^{-2}.s^{-1}$and glucose as the substrate.In theirstudy, the*C. sorokiniana* mixotrophic growth rate was 20 to 40% higher than the sum of the growth rates obtained under heterotrophic and autotrophic conditions.

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

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

3.1.2 Increase in the butyrate uptake rate in the presence ofacetateunder mixotrophic conditions

The effect of light on *C. sorokiniana*cultivatedon a mixture of acetate and butyratewas studied. The strict autotrophic control (without organic substrate) was used to give a better explanation forthe mixotrophic growth observed in Figure 1. During the exponential phase (first two days), the apparent autotrophic growth rate was 1.04 ± 0.05 d^{-1}. During the linear phase (from day 2 to day 8), the biomass production rate was 0.11 ± 0.01 $g.L^{-1}.d^{-1}$. With limited light availability (low light intensities and cell self-shading) or CO_2 limitation (no air or additional CO_2), the exponential growth phase in autotrophic batch cultivation will be short and rapidly followed by linear growth (Ogbonna et al., 1995; Smith et al., 2015). The growth rates during autotrophic growth were consistent with previously reported results obtained under similar conditions with *C. sorokiniana*(Kim et al., 2013; Li et al., 2013; Rosenberg et al., 2014).

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

The growth rate on acetate was slightly higher (2.7 ± 0.1 d^{-1}) under mixotrophic conditions than estimated by modeling under heterotrophic conditions (2.21 d^{-1} - see Table 1)(Turon et al., 2015). The total biomass accumulated just after acetate exhaustion in mixotrophic conditionswas higher than the biomass predicted by the model in heterotrophic conditions (Figure 1-B). Furthermore, the mixotrophic carbon yieldon acetate, Y_{Mixo}^{Mixo} , (Eq 3), was almost twice as high (0.79 ± 0.04 d^{-1}) under mixotrophic conditions thanpredicted under heterotrophic conditions (0.42 $g_C.g_C^{-1}$) (Table 1). These results confirmed that the presence of light increased both the apparent growth rate and the mixotrophic carbon yield on acetate compared to those underheterotrophic conditions at 25°C. Under mixotrophic conditions,the heterotrophic carbon yield,Y_{Het}^{Mixo} - see Eq. 4, was calculated by subtracting the carbon yield forautotrophic growth (autotrophic control) from the mixotrophic carbon yield (Y_{Het}^{Mixo} = 0.48 ± 0.05 g_C biomass per g_C acetate, see Table 1). Wherethere was uptake of both organic and inorganic carbon, only 39% of the microalgal biomass obtained after acetate exhaustion was due to CO_2 assimilation (X_{Auto}^{Mixo} , see Eq 6 and Table 1).In the acetatecontrol (with no butyrate), the fraction of biomass due to CO_2 assimilation (X_{Auto}^{Mixo} , 30%) was statistically similar (p>0.05) (see Table 1 and Supplementary material Figure S1) but the mixotrophic growth rate on acetate reached 4.1 ± 0.4 d^{-1}. When using mixtures of VFAs, there may be a high ATP demand to deal with the inhibitory effects of butyrate, such as cytosolic pH acidification, resulting in lower ATP availability for fast growth on acetate (Tromballa, 1978). In conclusion, the growth rate and carbon yield on acetate were higherin the presence of light than under heterotrophic conditions, suggesting that the mixotrophic growth on acetate probably relied on a synergy between heterotrophicand autotrophic conditions.

After a one-day delay after the acetate had been completely exhausted, there was linear butyrate uptake during the linear growth phase (Figure 1-B).Butyrate exhaustion in mixotrophic conditions was 3 days shorter than predicted for heterotrophic conditions (Figure 1-B). Based on the difference between the mixotrophic (Y_{Mixo}^{Mixo} , Eq 3), and heterotrophic (Y_{Het}^{Mixo} , Eq 4) carbon yields on butyrate, 62% of the biomass reached after butyrate exhaustion was probably due to CO_2 assimilation (X_{Auto}^{Mixo} , see Eq 6 and Table 1). Similarly, in the butyratecontrol (without acetate - see Figure 1-C), 74% of the biomass obtained after butyrate exhaustion was probably due to CO_2 assimilation (X_{Auto}^{Mixo} - seeTable 1). The model predicted thatat 25 °C no heterotrophic growth would have been observed at such initial butyrate concentration (with no acetate - see Figure 1-C). Furthermore,the linear butyrate uptake rate measuredafter acetate exhaustion was 1.5 times higher than measured for the butyratecontrol. It can, therefore, be concluded that mixotrophic conditionscan substantially accelerate the apparent butyrate uptake through the production of algal biomass byCO_2 fixation.

3.2 Effect of temperature on heterotrophic growth on VFAs

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

C.sorokiniana was grown heterotrophically on acetate as a single substrate (acetate control), on butyrate as single substrate (butyrate control) and on a mixture of acetate and butyrate, at 35°Cknown to be the optimum temperature (Janssen et al., 1999; Li et al., 2014; Van Wagenen et al., 2014b). On acetate(Supplementary material, Figure S2), the heterotrophic growth rate reached 5.88 d^{-1}which was consistent with previously reported values at 35-37°C (Van Wagenen et al., 2014b).

Forheterotrophic growth on a mixture of acetate and butyrate (Figure 2-A), the apparent growth rate on acetate, at 35°C (3.17 ± 0.45 d^{-1}) was higher than at 25°C (2.23 d^{-1}- see Table 2). However, microalgae biomass concentrationsafter acetate exhaustionwere similar at 25°C and 35°C (Figure 2). The carbon yields on acetate at 25°C and at 35 °C were also similar (Table 2).However, the growth rate and carbon yield on acetate in the acetatecontrol (Supplementary material, Figure S2) were almost 2 and 1.6 times higher than on the mixture of acetate and butyrate(Table 2). Even though the growth rate on acetate was highest at 35 °C in the acetatecontrol, the presence of butyrate inhibited the increase growth rate on acetate atthe higher temperature. At 25 °C, the presence of butyrate did not reduce the growth rate on acetate for butyrate concentrationsup to 0.5 $g_C.L^{-1}$(Turon et al., 2015).Ugwu et al (2000) reported that when one abiotic parameter (irradiance) was set to the optimum, the negative effects of another parameter (such as high dissolved oxygen concentration or temperature) wereaggravated(Ugwu et al., 2007). Thus, when one growth factor is set at its optimum, the fast metabolism will, in particular, reduce energy storage and the microalgae might be less able to protect themselves from any adverse conditions. The negative effect of butyrate on heterotrophic growth on acetate at 35°C was reduced when the butyrate concentration was lowered to 0.2 $g_C.L^{-1}$ (Supplementary material Figure S4). At this concentration, the growth rate (4.71 ± 0.24 d^{-1}) and carbon yield (0.65 ± 0.02 $g_C.g_C^{-1}$) on acetate were higher than with 0.3 $g_C.L^{-1}$ of butyrate. As a consequence, these results confirmed that butyrate inhibition ofheterotrophic growth depended on theconcentration,as previously suggested (Liu et al., 2012; Turon et al., 2015).

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

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

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

where$S_b$is the concentration of butyrate ($g_C.L^{-1}$), S_{b_opt}(0.05 $g_C.L^{-1}$) is the concentration of butyrate when μ_b (S_b) is maximum and equivalent to μ_{b_max} (0.16 d^{-1}),the maximum growth rate associated with butyrate assimilation, α (15.1 $L.d.g_C^{-1}$) is the initial slope andK_D ($2.10^{-10}g_C.L^{-1}$)is the half inhibitory constant associated with the diauxic growth.

The predicted growth rate on butyrate at 25°C varied with the butyrate concentration and reached its maximum, μ_{b_max}, after 9.5 days of cultivation when the butyrate concentration reached S_{b_opt}(0.05 $g_C.L^{-1}$)(Supplementary Material Figure S3). At 35°C, the apparent growth rate was calculated for a butyrate concentration of 0.23 $g_C.L^{-1}$which was reached after 5.7 days of cultivation (Figure 2-B). Consequently, the time to reach butyrate exhaustion was shorter at 35 °C than at 25 °C despite a higher maximumgrowth rate at 25 °C than the apparent growth rate at 35 °C (Figure 2). The carbon yield on butyrate at 35°C was half that at 25°C. Contrary to the hypothesis suggesting that the butyrate inhibitionmight be reduced at 35°C, butyrate inhibition was stronger at 35°C than at 25°C. Furthermore, no microalgae growth was observed at either25 °C or35 °Cin the butyratecontrol (no acetate). As for growth on acetate in mixture, butyrate inhibition at 35 °C depended on the concentration since the butyrate

uptake rate was faster at 35 °C than 25 °C when butyrate concentration was reduced to 0.2 $g_C.L^{-1}$ (Supplementary material Figure S4).

3.2.2 Reduced butyrate inhibition at 30 °C

As shown in Figure 2-A and Table 2, the growth rate and carbon yield on acetatein mixturewere both higher at 30 °C than at 25 °C or35°C. However, there was no significant difference ($p>0.05$)between these growth rates and carbon yields and thosein the acetatecontrol(Table 2, Supplementary material Figure S2). The presence of butyrate did not appear to inhibit microalgae growth on acetate at 30°C.

Similarly, when butyrate was taken up (in mixture), the apparent growth rate and the microalgae biomass yield were higher at 30 °C (0.16 d^{-1} and 0.56 $g_C.g_C^{-1}$ respectively) than at 35 °C (0.11 d^{-1} and 0.28 $g_C.g_C^{-1}$ respectively) (Table 2).The apparent growth rate at 30°C was calculated for a butyrate concentration of 0.29 $g_C.L^{-1}$ which was reached after 2 days of cultivation (Figure 2 and Table 2).As explained in the previous paragraph (3.2.1), the maximum growth rate at 25°C (0.16 d^{-1}) could only be reached at a low butyrate concentration (0.05 $g_C.L^{-1}$). These results suggest that there was less butyrate inhibition at 30 °C thanat 25 °C. Furthermore, microalgae growth was observed in the butyratecontrol whereas no growth was observed at 25 °C or 35 °C. A cultivation temperature of 30 °C thus successfully reduced butyrate inhibition and consequently butyrate exhaustion occurredmore than 3 days earlier than at 25 °C (Figure 2-A). At 30°C,enzymatic reactions countering butyrate inhibition mayhave beenencouraged.

Temperatures higher than 25 °C increased heterotrophic growth on both acetate and butyrate. However, thenear-optimum temperature for acetate was 35 °C while for butyrate it was 30°C. Cultivation on a mixture of acetate and butyrate ata

suboptimumtemperature for growth on acetate alonemay have reduced butyrate inhibition.

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

3.3.1 At 35 °C in the presence of light, microalgae growth on acetate or on butyrate reliedmoreon heterotrophic growth than at 25°C

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

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

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

growth was inhibited only during the first three days when the initial butyrate concentration was 0.2 $g_C.L^{-1}$(Supplementary material Figure S5-B).However, the butyrate uptake rate was significantly higher ($p < 0.05$) at 35 °C (88 $mg_C.L^{-1}.d^{-1}$) than at 25 °C (47.5 $mg_C.L^{-1}.d^{-1}$) in the presence of light (Tables1 and 3). Moreover, the fraction of biomass production due to autotrophic growth (X_{Auto}^{Mixo} , Eq 6) was lower (55%) at 35°C than at 25°C (74%). As for growth on acetate, it was concluded that growth on butyrate at 35 °C with light relied more on heterotrophic growth than at 25 °C.

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

The combined effect of temperature and light on *C. sorokiniana* growth on a mixture of acetate and butyrate, was studied to assess the interactionsbetween acetate and butyrate (Figure 3-B). In the presence of butyrate, both the growth rate and the heterotrophic carbon yield on acetate (2.53 d^{-1} and 0.36 $g_C.g_C^{-1}$, respectively) were halfthose measuredin the acetatecontrol (5.65 d^{-1} and 0.60 $g_C.g_C^{-1}$, respectively – see Table 3). The growth rate on acetate was not statistically different ($p > 0.05$) from that measured with no light at 35 °C(3.17 d^{-1}) (Tables 2 and 3). Consequently, butyrate inhibition of acetate uptake was not reduced by the presence of light at 35 °C. The fraction of biomass due to acetate uptake (X_{Het}^{Mixo} , Eq 5) was estimated at 60% (Table 3). This suggests that *C. sorokiniana*growth on acetate in a mixture of acetate and butyrate relied mostly on heterotrophic growthas wasalso observedforthe acetatecontrol.

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

62% (Table 3). The time taken to exhaustbutyrate completely was 3 daysless than under heterotrophic conditions at 25 °C and 35°C, probably because ofthehigh biomass reached after acetate exhaustion and because of the autotrophic biomass growth at 35°C. Light increased butyrate uptake at 35°C for cultivation on a mixture of acetate and butyrate. At 35 °C, the presence of butyrate reduced the apparent growth rate on acetate under both heterotrophic and mixotrophic conditions and also inhibited autotrophic growth in the butyratecontrol under mixotrophic conditions. Further investigation on the effect of butyrate on the respiration rate and/or photosynthetic activity mayprovide further information on the negative effect of butyrate on mixotrophic and heterotrophic growth observed in this study at high temperature.

4 Conclusions

The previously accepted optimum cultivation temperature (35°C) did not provide the best conditions forheterotrophic or mixotrophic growth of *C. sorokiniana*on a mixture of acetate and butyrate. The apparent heterotrophic growthrate on acetate was highest at 30 °C(4.1d^{-1}). At 25 °C light improvedthe apparent butyrate uptake(71 $mg_C.L^{-1}.d^{-1}$)because simultaneous heterotrophic and autotrophic growth increased thebiomass (reaching 1.14 $g.L^{-1}$). In conclusion, *C. sorokiniana* may be cultivated successfullyon DF effluents, at a temperature lower than that previously considered to be optimum (30°C) and withexposure to light.

5 Acknowledgements

This study was funded by the National Institute of Agronomic Research (INRA) and the University of Montpellier II, France.

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7 Figure captions

Figure 1. Effect of DCMU and light on growth of *C. sorokiniana* cultivated on butyrate and acetate at 25 °C.

(A) Dry weight of *C. sorokiniana* cultivatedwithout DCMU under autotrophic conditions (with 0.3 $g_C.L^{-1}$ of $NaHCO_3$ and under 123 ± 10 µmol photons.$m^{-2}.s^{-1}$)(●), mixotrophicconditions (with 0.3 $g_C.L^{-1}$ of acetate and under 123 ± 10 µmol photons.$m^{-2}.s^{-1}$) (◆) and heterotrophicconditions (with 0.3 $g_C.L^{-1}$ of acetate in darkness) (■). Dry weight of *C. sorokiniana* cultivatedwith 10µM DCMU under autotrophicconditions (with 0.3 $g_C.L^{-1}$ of $NaHCO_3$ and under 123 ± 10 µmol photons.$m^{-2}.s^{-1}$) (●), mixotrophicconditions (with 0.3 $g_C.L^{-1}$ of acetate and under 123 ± 10 µmol photons.$m^{-2}.s^{-1}$) (◆) and heterotrophicconditions (with 0.3 $g_C.L^{-1}$ of acetate in darkness) (■). **(B) and (C)***C. sorokiniana* cultivated under mixotrophic conditions at 25 °C. Dry weight (●), butyrate concentration (◆) and acetate concentration (■) during cultivation**(B)**on a mixture of butyrate and acetate, 0.3 $g_C.L^{-1}$ of each and **(C)** on 0. 3 $g_C.L^{-1}$ of butyrate as single substrate (butyratecontrol). The dry weight for autotrophic cultivation (●) andthe predicted values for heterotrophic cultivation at 25 °C - dry weight (green dashed lines), acetate concentration(red dashed lines) and butyrate concentration(blue dashed lines)- are shown for comparison.

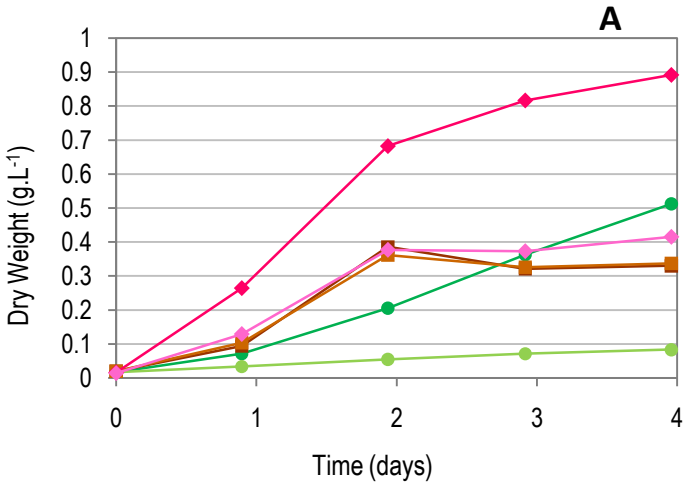
Figure 2. Effect of increasing temperature, from 25 °C to 35 °C, on heterotrophic growth of *Chlorella sorokiniana* cultivated on a mixture of acetate and butyrate.

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

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

Dry weight of *C. sorokiniana* (●), butyrate concentration (◆) and acetate concentration (■) during cultivation on **(A)**0.3 $g_C.L^{-1}$ of butyrate (butyratecontrol) and **(B)**on a mixture of 0.3 $g_C.L^{-1}$butyrate and 0.3 $g_C.L^{-1}$acetate.The dry weight for autotrophic cultivation (●) and the predicted values for heterotrophic cultivation at 25 °C - dry weight (green dashed lines), acetate concentration (red dashed lines) and butyrate concentration (blue dashed lines) - are shown for comparison.

Figure 1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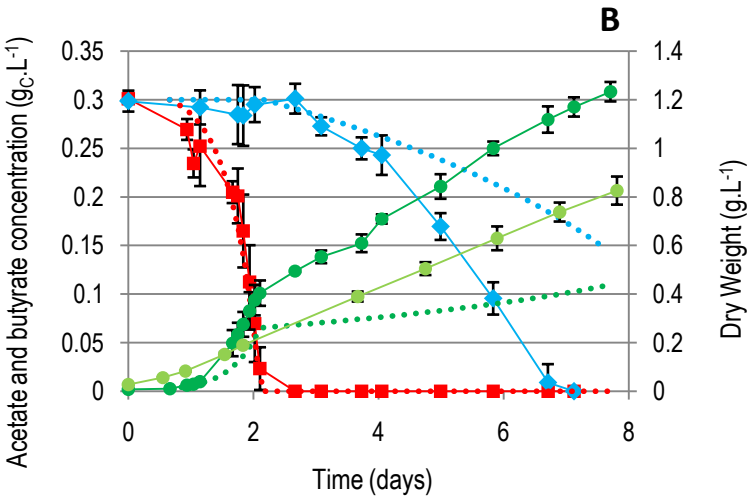
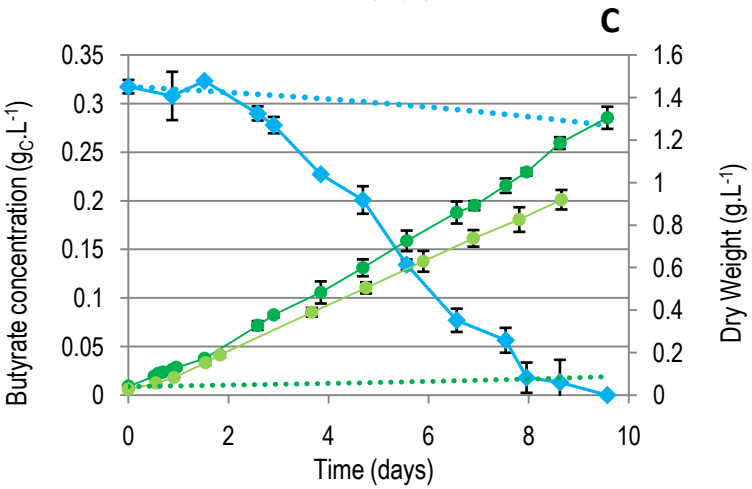
Dry Weight (g.L-1)
Time (days)


B
Acetate and butyrate concentration (gC.L-1)
Dry Weight (g.L-1)
Time (days)


C
Butyrate concentration (gC.L-1)
Dry Weight (g.L-1)
Time (days)

Figure

Figure 2

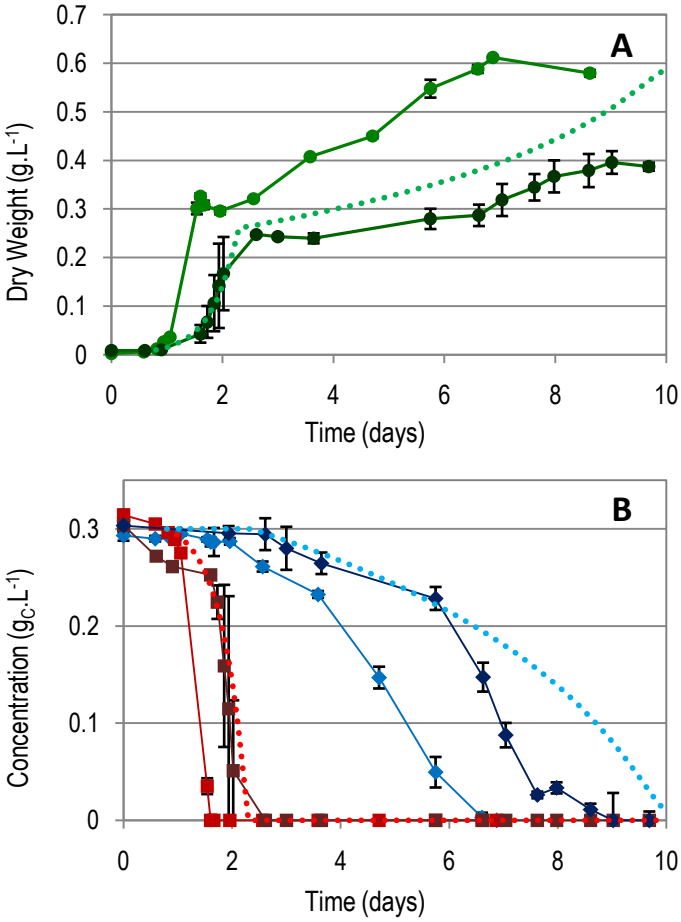


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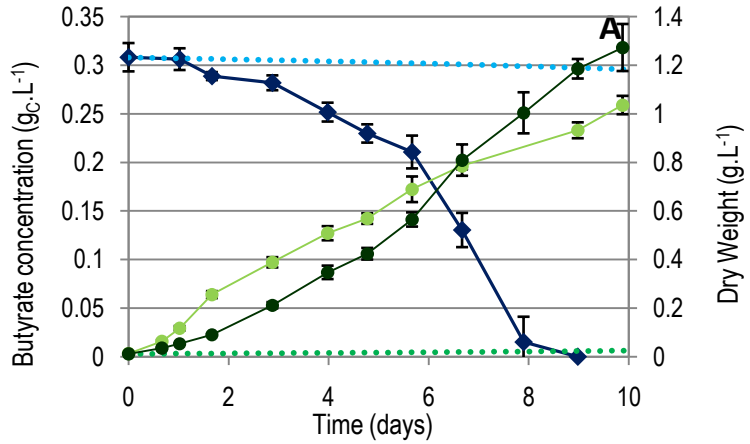
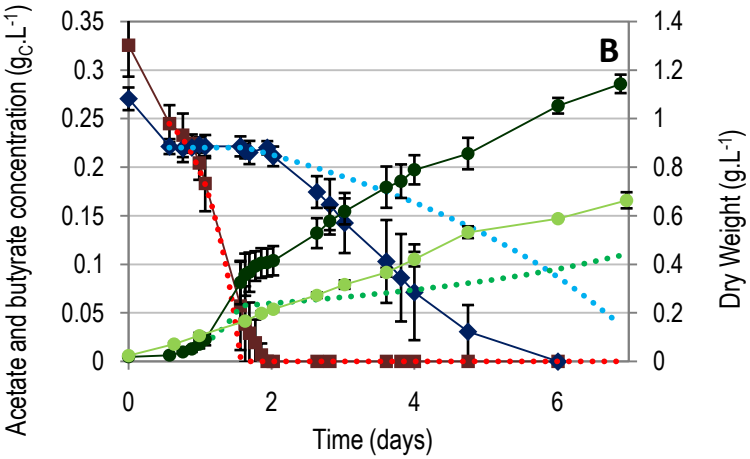
Figure 3





Supplementary Material

Figure S1. Effect of light on *C. sorokiniana*'s growth on acetate (0.3 $g_C.L^{-1}$) at 25 °C. Microalgae concentration ($g.L^{-1}$) (●) and acetate concentration (■) are presented. Microalgae concentration ($g.L^{-1}$) (●) 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 $g_C.L^{-1}$). Microalgae concentration, in $g.L^{-1}$, during heterotrophic growth on acetate at 30 °C (●) and 35 °C (●) are represented in subfigure A. Acetate concentrations, in $g_C.L^{-1}$, 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 (*μb(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 gC.L-1 each) at 25 °C, 30 °C and 35 °C. Microalgae concentration, in $g.L^{-1}$, 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 $g_C.L^{-1}$, 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 $g_C.L^{-1}$ of acetate (A) and 0.2 $g_C.L^{-1}$ of butyrate (B) at 35 °C. Microalgae concentration ($g.L^{-1}$) (●), butyrate uptake (■) and acetate uptake (◆) are presented. Microalgae concentration ($g.L^{-1}$) (●) 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

Figure S1

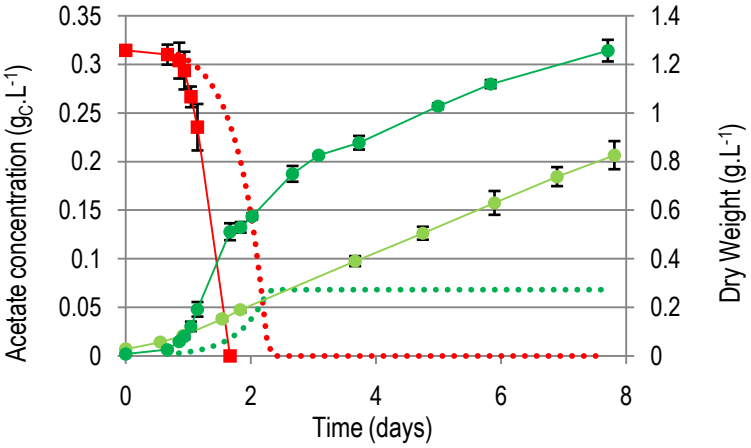
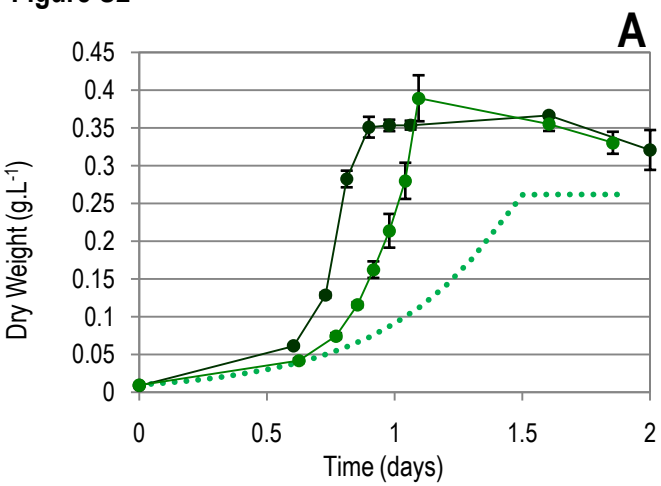


Figure S2



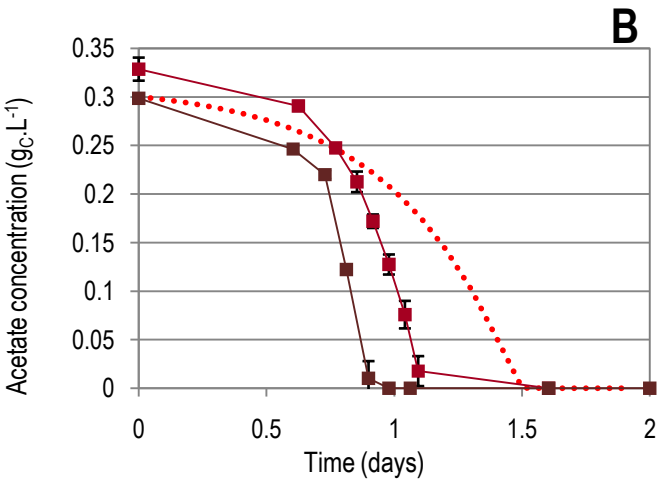


Figure S3

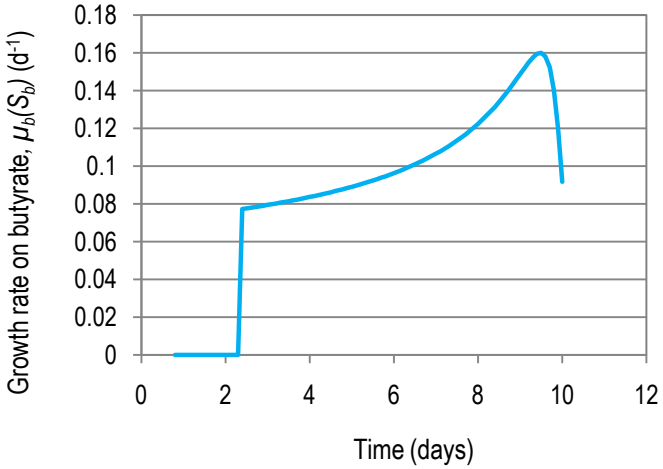


Figure S4

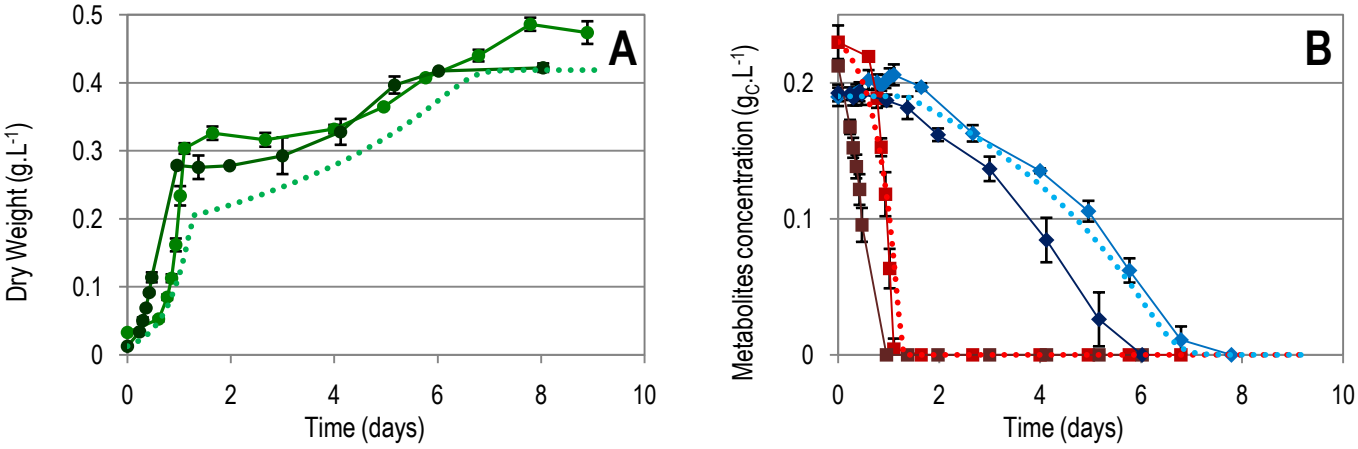


Figure S5.

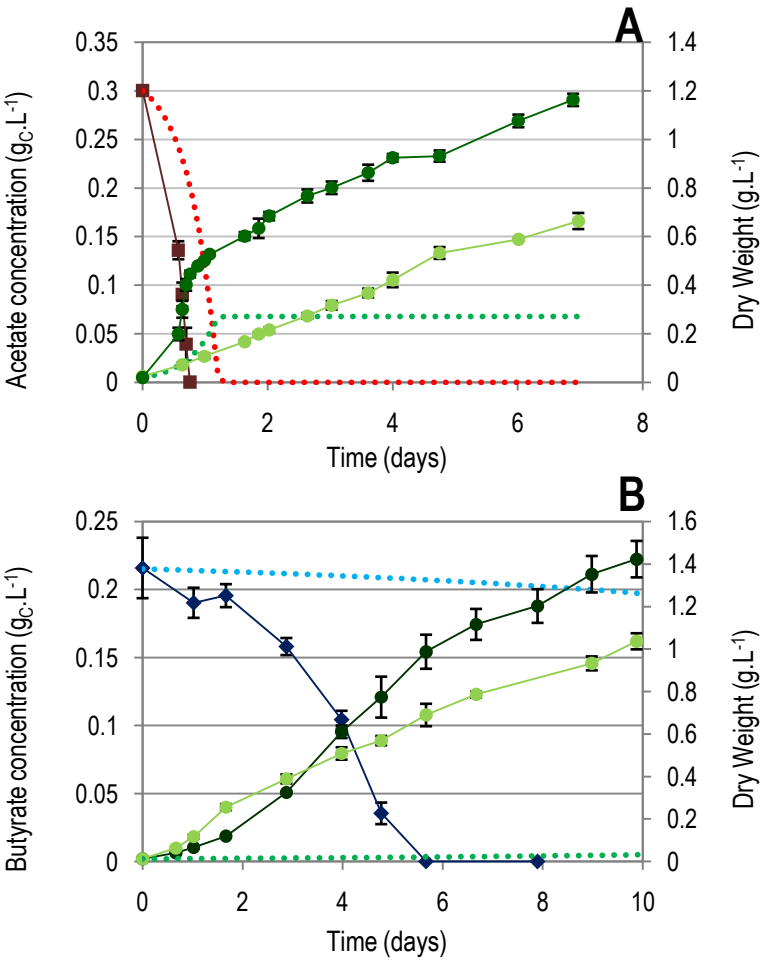


Table 1

Effect of light on growth and productionrates (μ_{app}andr$_{lin}$)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				
	μ_{app} (d^{-1})	Y_{Mixo}^{Mixo} ($g_C.g_C^{-1}$)	Y_{Het}^{Mixo} ($g_C.g_C^{-1}$)[a]	X_{Auto}^{Mixo} (%)[b]	r_{app_lin} ($g.L^{-1}.d^{-1}$)	Uptake rate ($mg_C.L^{-1}.d^{-1}$)	Y_{Mixo}^{Mixo} ($g_C.g_C^{-1}$)	Y_{Het}^{Mixo} ($g_C.g_C^{-1}$)[a]	X_{Auto}^{Mixo} (%)[b]
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

[a]: 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}).

[b]: The fraction of mixotrophic biomass due to autotrophic growth on CO_2 (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 (μ_{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. Valueswith 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	
		μ_{app} (d^{-1})	Y_{Het}^{Het} ($g_C.g_C^{-1}$)	μ_{app} (d^{-1})	Y_{Het}^{Het} ($g_C.g_C^{-1}$)
25 °C	A; B and A + B	2.23	0.42	0.16*	0.56
30 °C	A	4.65 ± 0.16 [a]	0.58 ± 0.04 [a, b]		
	B			0.13 ± 0.01 [a,b]	0.42 ± 0.03 [a]
	A + B	4.12 ± 0.19 [a]	0.51 ± 0.01 [a]	0.16 ± 0.01 [b]	0.56 ± 0.01 [b]
35 °C	A	5.88 ± 0.39 [b]	0.64 ± 0.06 [b]		
	B			No growth	
	A + B	3.17 ± 0.45 [c]	0.41 ± 0.02 [c]	0.11 ± 0.02 [a]	0.28 ± 0.03[c]