Mixotrophic growth of microalgae on volatile fatty acids is determined by their undissociated form

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Abstract

The influence of pH and substrate concentration on the mixotrophic growth of five microalgae species in presence of acetate or butyrate was evaluated. *Acutodesmus obliquus, Auxenochlorella protothecoïdes,* two strains of *Chlamydomonas reinhardtii*, and *Chlorella sorokiniana* were cultivated at pH from 5 to 10. Acetate was efficiently assimilated by all strains while butyrate uptake was greatly strain dependent. Growth rates at pH values above 8 were reduced while values below 5 or 6 inhibited growth on acetate and butyrate respectively. The influence of acetic and butyric acid concentration was tested. It was demonstrated that the main factor affecting microalgae growth is the concentration of undissociated acid, which can be controlled by pH adjustment. The strains exhibited inhibition threshold concentrations of 71 – 207 $mg.L^{-1}$ for acetic acid and of 13 – 25 $mg.L^{-1}$ for butyric acid. These findings are crucial for proper control of processes coupling bacterial fermentation and microalgae cultures.

Keywords: Dark fermentation; microalgae; acetic acid; butyric acid; pH

1. Introduction

Fossil fuel depletion and climate change concerns have raised the interest in microalgae based biotechnologies, as microalgae can accumulate 20-50% of their dry weight as lipids convertible in biofuel [1] as well as other high added value molecules such as carotenoids [2]. Conventional cultivation methods involve growing microalgae under photo-autotrophy, only using CO_2 as carbon source and light as energy in the process of photosynthesis. However, these light dependent systems are limited in productivity, as the increase in biomass density prevents light from penetrating in the bulk of the reactor [3]. Heterotrophic growth has been suggested as a way to get around this constraint by feeding microalgae under dark conditions with organic compounds such as glucose or acetate, used as both carbon and energy sources [4]. When microalgae are cultivated under mixotrophy, organic carbon and CO_2 are both consumed under illumination [5], usually leading to higher growth rates and biomass yields than those achieved by either auto- or heterotrophy [6].

The cost of organic compounds (especially glucose) required for hetero- and mixotrophic cultivation mode may however limit the economics of the process [7]. Therefore, glucose must be substituted by low cost carbon sources, preferentially originating from various waste streams so as to further improve environmental benefits of micro-algae cultivation [8]. In recent years, coupling dark fermentation (DF) with microalgal culture has been suggested as an effective way to treat DF effluents and provide cheap substrates for heterotrophic or mixotrophic micro-algae production [9],[10]. During DF, the organic matter from waste is converted into hydrogen and other metabolites by fermentative bacteria. Most of the initial organic material (expressed as COD) is however retrieved in the effluent in the form of volatile fatty acids (VFA), mainly acetate and butyrate [11]. Consequently, DF must be associated with another

bioprocess, typically anaerobic digestion or photo-fermentation, in order to reduce the effluent COD and to maximize waste valorisation [12]. Although acetate is a suitable substrate for most strains, butyrate is not as easily degraded. As an illustration, *Auxenochlorella protothecoïdes* and *Chlorella sorokiniana* were able to quickly assimilate acetate up to 2.1 $g.L^{-1}$ in heterotrophy, leading to biomass yield of 0.75 $g.g_C.L^{-1}$ and 0.84 $g.g_C.L^{-1}$ respectively [13]. Meanwhile, butyrate was found to be inhibitory at concentrations as low as 0.16 $g.L^{-1}$. Paradoxically, Liu et al (2013) successfully cultivated *C. vulgaris* ESP-6 on a sterilized and diluted DF effluent containing 0.3 $g.L^{-1}$ acetate and 0.8 $g.L^{-1}$ butyrate [14]. Fei et al [15] showed that cultivation of *C. protothecoïdes* was possible on a mixture of VFA with ratio of acetic:propionic:butyric acid of 6:1:3 up to a total concentration of 4 $g.L^{-1}$. The lag phase increased considerably with increasing concentrations of VFA and growth was totally inhibited at 8 $g.L^{-1}$ VFAs.

Because of the variability of strains as well as the growth conditions, it remains unclear whether the microalgal growth is hampered by high concentrations of VFA, pH or is only strain dependant. Acetic and butyric acid are well known for their detrimental effects on bacteria [16] or eukaryotic organisms by interacting with the cell membrane and eventually disrupting essential metabolic activities such as the electron transport chain and oxidative phosphorylation [17]. Zuo et al showed that 1 mM acetic acid at pH 5 induced programmed cell death was inhibitory for *Chlamydomonas reinhardtii* by inducing oxidative stress [18]. The authors suggested this inhibition was dependant on the ROOH concentration more than on the total (un-dissociated and dissociated form) of the acid. Therefore, it was supposed in this work that growth suppression at acidic pH or at high VFA concentration was likely neither due to pH value nor the total organic acid

concentration but rather resulted from the presence of the undissociated acid form (ROOH).

Understanding the causes of inhibition of microalgal growth is crucial to allow an efficient coupling of DF processes with microalgal growth. The performances of five microalgal strains grown on a synthetic medium mimicking a DF effluent were thus evaluated. The strains *Acutodescmus obliquus*, *Auxenochlorella protothecoïdes* and *Chlorella sorokiniana* were chosen for their ability to perform mixotrophic growth on acetate and butyrate and their high growth rates. The model strain *Chlamydomonas reinhardtii* CC-124 was also selected as the most studied model species. The cell wall-less mutant *C. reinhardtii* CC-400 was also tested since its lack of cell wall is of potential biotechnological interest. Using a microplate set-up, pH values of the medium ranging from 5 to 10 were screened. In addition, concentrations of acetic and butyric acid used as single substrates ranging from 0.16 to 8.6 $g.L^{-1}$ of total acetate and from 0.08 to 3.8 $g.L^{-1}$ of total butyrate were tested in order to determine the optimal growth conditions as well as a minimum inhibitory concentration caused by the undissociated form.

2. Material and methods

2.1 General growth medium

A modified TAP medium (named hereinafter HAP medium) was prepared, by replacing the TRIS buffer by a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer. The medium was composed as follows: sodium acetate (1.1 $g.L^{-1}$), potassium phosphate (K_2HPO_4) (1 mM), HEPES (20 mM), 25 $mL.L^{-1}$ Beijerincks (40X) solution, leading to an ammonium (NH_4^+) concentration of 7.5 mM, 1$mL.L^{-1}$ Hutner's trace element solution and 100 $\mu L.L^{-1}$ vitamins solution (vitamin B1 50 mM; Biotin 1

mM; Cyanocobalamin 1 mM). The pH of the medium was adjusted to 7.0 by addition of NaOH 5M prior to sterilization by autoclave at 121°C for 20 min. Vitamins were sterilized by filtration over a 0.2 µm filter and added after autoclaving to avoid their degradation. To obtain a solidified medium, 1.5 % m/v agar were added before sterilization. An HBP medium was also prepared by replacing sodium acetate by sodium butyrate (0.8 $g.L^{-1}$). All chemicals were purchased from Sigma Aldrich, USA.

2.2 Microalgae strains and cultivation conditions

Five strains were used in this study. *Acutodescmus obliquus, Auxenochlorella protothecoïdes* and *Chlorella sorokiniana* were obtained from the SAG culture collection (Goettingen, Germany). The wild-type *Chlamydomonas reinhardtii* CC-124 and the wall-less mutant *Chlamydomonas reinhardtii* CC-400 were obtained from the *Chlamydomonas* Resource Center (University of Minnesota, St Paul, MN, USA). The strains were maintained on agar HAP medium under constant illumination at 25°C. Before performing experiments, few colonies were picked and re-suspended in 40 mL of HAP medium in pre-sterilized 125 mL flasks. Flasks were then incubated at 25°C, under constant light of 100 µE and constant agitation of 130 rpm. After a few days, cultures were resuspended in 40 mL fresh HAP medium and incubated for 1-2 days at 25°C under constant light (100 µE) and constant agitation (130 rpm).

Cells in exponential growth phase were collected, centrifuged at 2500 rpm for 10 min and supernatant was discarded. The pellet was resuspended in PBS so as to reach a final optical density at 750 nm (OD_{750}) around 5. This concentrated culture was used as inoculum for the experiments. Before incubation and during cultivation, cultures were periodically checked for contaminants by microscopy.

2.3 Variable pH or concentration media

Media were prepared as described in section 2.1 except for pH, buffer and substrate composition. The pH was buffered at values between 5 and 10 using 100 mM of an appropriate buffer, ie MES at pH 5 and 6, HEPES at pH 7 and 8, CHES at pH 9 and 10. Substrate was either sodium acetate (1.1 $g.L^{-1}$) or sodium butyrate (0.8 $g.L^{-1}$) with a constant organic carbon concentration of 0.5 $g_C.L^{-1}$. An autotrophic control was also performed for each strain and under each pH condition by omitting organic acids in the medium under atmospheric CO_2 conditions and constant illumination of 100 µE.

Culture media with variable concentrations of the undissociated acid form were prepared as follows. In all experiments, MES was used as buffer at a concentration of 100 mM. The pH was set to 5.5, 6.0 or 6.5. Increasing concentrations of sodium acetate and sodium butyrate were added to the medium to get a range of undissociated acid concentrations from 23.6 to 295 $mg.L^{-1}$ of acetic acid (AcOOH) and 12.5 to 74.7 $mg.L^{-1}$ of butyric acid (BuOOH). The undissociated acid form concentration ([ROOH], $mg.L^{-1}$) was calculated based on the modified Henderson – Hasselbach equation:

$$[ROOH] = \frac{C_t}{1+ 10^{pH - pKa}}$$

where C_t is the total concentration of organic acid (in $mg.L^{-1}$).

The nutrients concentration was adjusted based on the Redfield C/N/P ratio of 106/16/1 by diluting the proper amount of 1 M NH_4Cl and 1 M K_2HPO_4 stock solutions. Beijerincks solution (40X), Hutner's trace solution and vitamins were added as stated in section 2.1. Media were sterilized by autoclaving (121°, 20 min).

Sterile 24-well microplates were used to screen each condition. Up to six conditions in triplicates were tested in one plate, the six remaining wells being blank condition.

Wells were filled with 1 mL of medium and inoculated with 10 µL of culture prepared as stated in section 2.2 (initial DO_{750} = 0.05). Micro-algae were incubated in an AlgaeTron AG 230 shaker incubator (PSI, Drásov, Czech Republic). Agitation was set to 100 rpm to prevent spilling from the wells, at 25°C and under constant illumination of 100 µE. Final pH was measured at the end of cultivation.

2.4 Biomass quantification

Biomass of the microplates cultures was quantified by direct measurement of the optical density at 750 nm (OD_{750}) in an Infinite Nanoquant M200 spectrophotometer (Tecan®, Switzerland). OD_{750} was chosen to avoid interferences with pigments absorbance [19]. Before analysis, microplates lids were aseptically wiped to remove condensation. The measurements were done 1 to 3 times a day depending on the growth phase. To correlate OD_{750} to biomass dry weight (DW), calibration curves were plotted for each strain and each substrate. Briefly, 200 mL of HAP medium or HBP medium in 500 mL flasks were inoculated with cells in the exponential phase. Flasks were incubated as stated in section 2.2. Samples were taken 1 to 3 times a day. A known volume of sample was filtered on pre-weighed GF/C filters (Whatman®), dried overnight at 105°C and weighed again to obtain the DW. The OD_{750} was determined by filling a well of a 24-well microplate with 1 mL of sample, using water as blank. The equation correlating DW to OD_{750} is as follows:

$$DW = C_f * OD_{750}$$

where DW is g dry weight.L^{-1}, C_f is a conversion factor ($g_{DW}.L^{-1}$) and OD_{750} the optical density measured at 750 nm. Values of C_f for growth on acetate were of 2.025 for *A. obliquus* (R^2 = 0.965), 2.622 for *A. protothecoïdes* (R^2 = 0.970), 2.687 for *C. reinhardtii* CC-124 (R^2 = 0.937), 1.101 for *C. reinhardtii* CC-400 (R^2 = 0.922), 2.0745 for *C.*

sorokiniana (R^2 = 0.999). Values of C_f for growth on butyrate were of 2.562 for *A. obliquus* (R^2 = 0.991), 1.864 for *A. protothecoïdes* (R^2 = 0.978), 3.1504 for *C. reinhardtii* CC-124 (R^2 = 0.965), 1.074 for *C. reinhardtii* CC-400 (R^2 = 0.9623) and 1.728 for *C. sorokiniana* (R^2 = 0.985).

2.5 Volatile Fatty Acid (VFA) measurement

The VFA concentrations were quantified at the end of the culture by gas chromatography. Samples were collected, centrifuged at 15 000 rpm and stored at -25°C. Before analysis, 500 µL of supernatant were mixed with 500 µL of internal standard solution (ethyl-2-butyric acid, 1 $g.L^{-1}$). The apparatus was a Perkin Clarus 580 with capillary column Elite-FFAP crossbond®carbowax® (15 m) maintained at 200°C and with N_2 as the gas vector (flow rate of 6 mL.min-1) equipped with a flame ionization detector (FID) maintained at 280°C (PerkinElmer, USA).

2.6 Calculation

The apparent production rate of biomass for r_{app} ($g_{DW}.L^{-1}.d^{-1}$) was calculated according to equation (1):

$$r_{app} = \frac{X_f - X_0}{t_f - t_0} \qquad (1)$$

With X_0 and X_f the biomass concentrations ($g_{DW}.L^{-1}$) measured at the beginning (t_0, d^{-1}) and the end (t_f, d^{-1}) of the exponential phase.

The mixotrophic yield Y_M ($g_{DW}.g_{substrate}^{-1}$) was calculated according to equation (2):

$$Y_M = \frac{X_f - X_0}{S_c} \qquad (2)$$

Where X_0 and X_f are the biomass concentrations ($g_{DW}.L^{-1}$) at the beginning and at the end of the experiment and S_C ($g.L^{-1}$) the substrate consumed.

The estimated heterotrophic yield Y_H ($g_{DW}.g_{substrate}^{-1}$) was calculated according to equation (3):

$$Y_H = \frac{X_f - X_0 - X_{Auto}}{S_c} \quad (3)$$

Where X_0 and X_f are the biomass concentrations ($g_{DW}.L^{-1}$) at the beginning and at the end of the experiment, S_C ($g.L^{-1}$) the substrate consumed and X_{auto} the estimated autotrophic biomass concentration ($g_{DW}.L^{-1}$) calculated as follows (Equation 4):

$$X_{Auto} = t_f * r_{app,\ Auto} \quad (4)$$

Where t_f is the duration of the mixotrophic experiment (d^{-1}) and $r_{app,\ auto}$ ($g_{DW}.L^{-1}.d^{-1}$) the apparent production rate of biomass obtained in the autotrophic control. X_{Auto} and $r_{app,\ Auto}$ were calculated for each pH condition.

3. Results and discussion

3.1 Effect of initial pH on acetate growth

The effect of initial pH on microalgae growth was investigated either in autotrophic conditions (Fig. S1) or in presence of acetate (Fig. S2). As the aim of the study was to compare the ability of the microalgal strain to assimilate organic substrate, no extra CO_2 was added into the medium by bicarbonate addition or bubbling. The pH of the medium affected microalgal growth differently in both conditions with each strain exhibiting a specific behaviour. Final pH was unchanged for all conditions except at pH 10 where it decreased between 0.2 and 0.5 pH unit (data not shown).

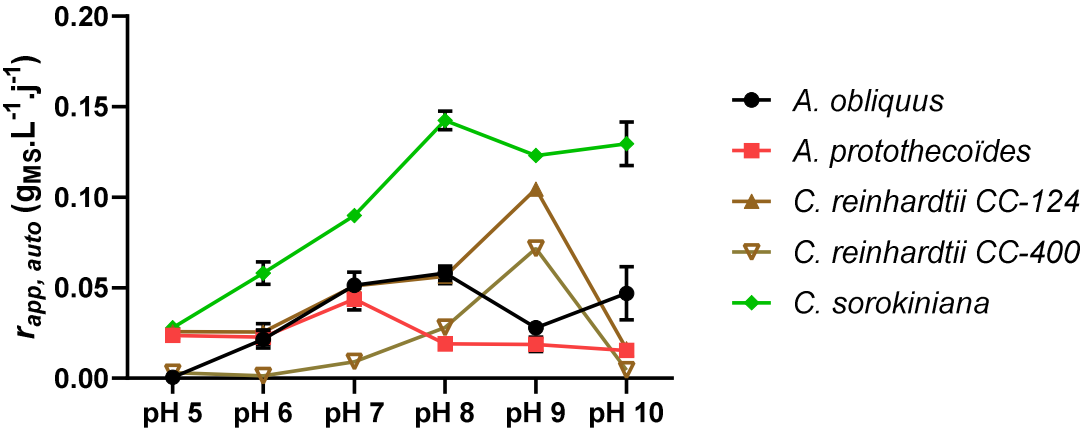

Fig. 1: Apparent autotrophic productivity $r_{app, Auto}$ ($g.L^{-1}.d^{-1}$) of *A. obliquus*, *A. protothecoïdes*, *C. reinhardtii* CC-124, *C. reinhardtii* CC-400, and *C. sorokiniana* at various initial pH and atmospheric CO_2 conditions. Standard deviations are given for 3 biological replicates.

Autotrophic cultivation of all the strains is shown in Fig. 1. For all strains, growth was linear (Fig. SI). The autotrophic biomass productivity $r_{app, Auto}$ of *A. protothecoïdes* remained constant at all tested pH values. For the other strains, $r_{app, Auto}$ increased linearly with initial pH from 5 to 8. For *A. obliquus* and *C. sorokiniana*, $r_{app, Auto}$ was constant at pH 8-10, but was maximal at pH 9 for both *C. reinhardtii* strains. Autotrophic growth of *A. obliquus* and *C. reinhardtii* CC-400 was inhibited at pH 5.

Figure 2 shows that acetate was a suitable substrate for mixotrophic growth of all microalgae. The exponential phase lasted 1 to 3 days at most initial pH values (Fig. S2). The highest apparent production rate on acetate ($r_{app, Ac}$) was found for most strains at initial pH 7.0, except for *C. reinhardtii* CC-400 where $r_{app, Ac}$ is optimal at pH 6. *C. sorokiniana* exhibited the highest apparent growth rate (1.17 ± 0.03 $g.L^{-1}.d^{-1}$) followed by *C. reinhardtii* CC-124 (0.57 ± 0.02 $g.L^{-1}.d^{-1}$) (Fig. 2-A). The $r_{app, Ac}$ of all strains

declined with initial pH increasing from 7 to 10. Growth of all strains was inhibited at pH 5 in presence of acetate, with no apparent biomass increase and acetate consumption. Whenever growth occurred, acetate was almost always totally consumed by all strains except at pH 9 and 10 (Fig. 2-B). *C. sorokiniana* assimilated only 34.4 ± 5.9% of the initial acetate at pH 9. Acetate uptake was completely inhibited at pH 10 for all strains except for *A. protothecoïdes* which consumed 27.1 ± 3.4% of the acetate.

Mixotrophic biomass yield (Y_M in $g_{DW}.g_{substrate}^{-1}$) increased or remained constant with increasing initial pH except for *A. protothecoïdes* (Table 1). *C. sorokiniana* and *C. reinhardtii* CC124 exhibited the highest Y_M at pH 9.0 (3.88 ± 0.30 $g.g^{-1}$ and 1.49 ± 0.10 $g.g^{-1}$ respectively).Y_H remained relatively constant for all strains for each pH tested, except at pH 10.0. *C. sorokiniana* exhibited a higher Y_H at pH 6.0 and 7.0 (1.02 ± 0.04 and 1.19 ± 0.10 $g_{DW}.g_{substrate}^{-1}$) than the other pH.

The linear autotrophic biomass growth indicates that cells were limited. As addition of organic carbon resulted in exponential phase, we can assume that carbon was the limiting nutrient. As cells were grown without bicarbonate nor bubbling air, CO_2 was only coming from diffusion from atmosphere, which probably was the limiting factor.

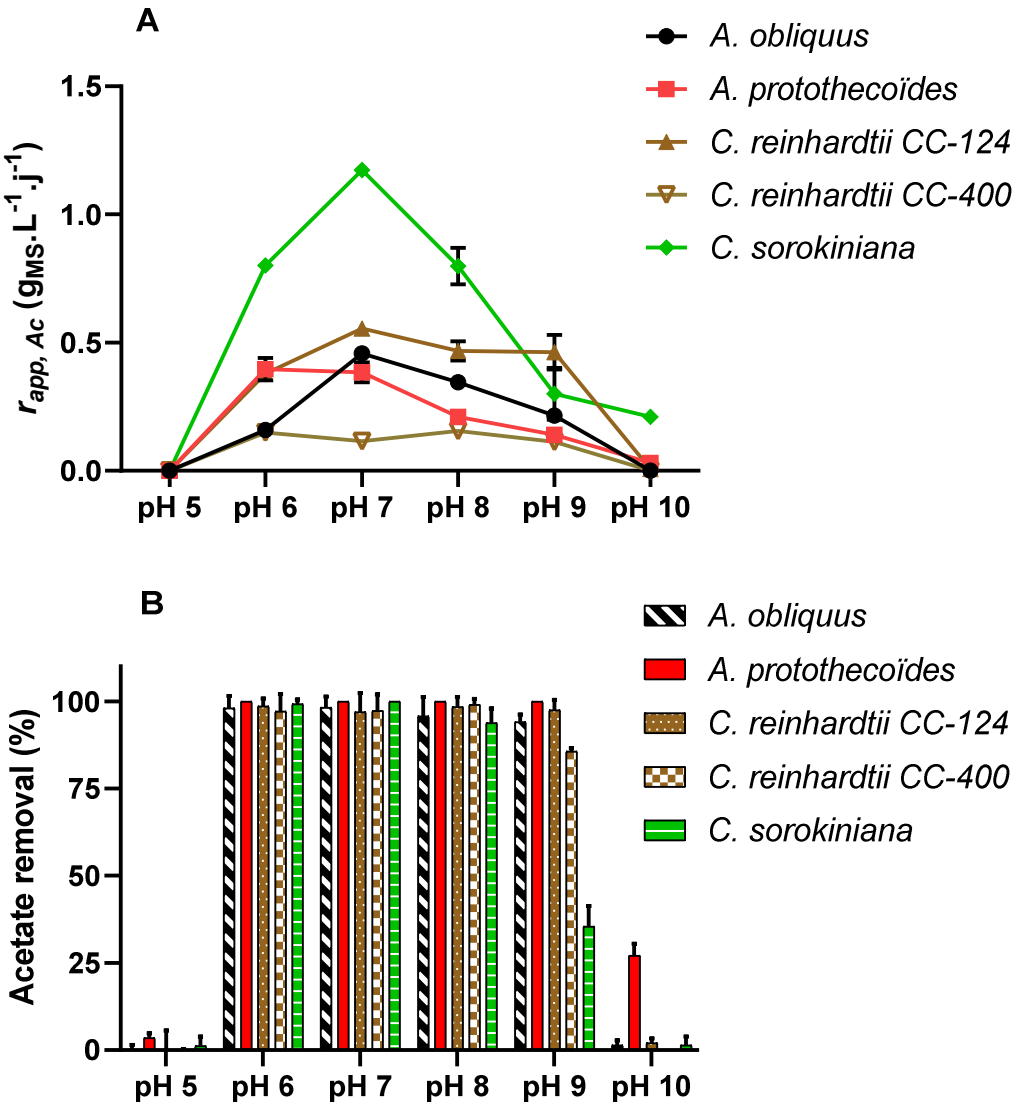

Fig. 2: Mixotrophic cultivation on 1.1 $g.L^{-1}$ acetate and atmospheric CO_2 of *A. obliquus*, *A. protothecoïdes*, *C. reinhardtii* CC-124, *C. reinhardtii* CC-400, and *C. sorokiniana* on 1.1 $g.L^{-1}$ at various initial pH values. (A) Apparent productivity $r_{app, Ac}$ ($g.L^{-1}.d^{-1}$) (B) Acetate removal (% of initial substrate) at the end of the experiment. Standard deviations are given for 3 biological replicates.

Strain	Initial pH	Y_M ($g.g^{-1}$)	Y_H ($g.g^{-1}$)
A. obliquus	5	NA	NA
	6	0.65 ± 0.05	0.54 ± 0.08
	7	1.06 ± 0.03	0.74 ± 0.02
	8	1.02 ± 0.06	0.58 ± 0.03
	9	1.17 ± 0.03	0.72 ± 0.03
	10	NA	NA
A. protothecoïdes	5	NA	NA
	6	1.34 ± 0.06	1.13 ± 0.06
	7	1.46 ± 0.08	0.99 ± 0.08
	8	1.23 ± 0.05	1.06 ± 0.05
	9	1.07 ± 0.03	0.90 ± 0.03
	10	0.81 ± 0.26	0.31 ± 0.23
C. reinhardtii 124	5	NA	NA
	6	1.06 ± 0.04	0.85 ± 0.05
	7	1.16 ± 0.01	0.85 ± 0.03
	8	1.07 ± 0.03	0.73 ± 0.04
	9	1.49 ± 0.10	0.85 ± 0.08
	10	NA	NA
C. reinhardtii 400	5	NA	NA
	6	0.37 ± 0.04	0.37 ± 0.01
	7	0.42 ± 0.04	0.37 ± 0.01
	8	0.49 ± 0.04	0.35 ± 0.01
	9	0.63 ± 0.08	0.22 ± 0.01
	10	NA	NA
C. sorokiniana	5	NA	NA
	6	1.55 ± 0.01	1.02 ± 0.04
	7	2.01 ± 0.05	1.19 ± 0.10
	8	2.17 ± 0.12	0.59 ± 0.05
	9	3.88 ± 0.3	0.79 ± 0.02
	10	NA	NA

Table 1: Biomass yields on 0.5 $g_C.L^{-1}$ acetate. Y_M ($g.g^{-1}$) stands for global mixotrophic yield. Y_H ($g.g^{-1}$) is the estimated yield only due to heterotrophy. Standard deviations are given for 3 biological replicates. When no values are given, there was either no growth observed or no substrate consumed.

This would also explain why autotrophic biomass production increased with rising pH and was maximum between pH 8-10 for most strains. Indeed, between pH 6 and 10, bicarbonate (HCO_3^-), which is more soluble than CO_2, becomes the predominant form of the inorganic carbon species. The increase of initial pH may have resulted in an

increase of dissolved inorganic carbon in the medium, leading to more carbon available for microalgae growth.

For most strains, Y_M was superior to 1. As carbon content of biomass can be estimated to 50% and that algae were fed with 0.5 $g_C.L^{-1}$, this implies that extra carbon (ie. inorganic) was absorbed. This can be inferred from the growth curves (Fig. S2), where a linear growth phase is visible after the exponential phase. As carbon is the limiting nutrient in the used media, this linear growth was most probably due to autotrophic growth on atmospheric CO_2. Many authors reported that mixotrophic biomass production rate is at least the sum of the autotrophic and heterotrophic rates [20]. Thus, heterotrophic yield Y_H (in $g_{DW}.g_{substrate}^{-1}$) at each pH was estimated by subtracting the potential autotrophic biomass obtained during the duration of the mixotrophic cultivation. The autotrophic contributions were estimated using results from figure 1 as suggested by Turon et al [21]. Some biases are induced with this method as autotrophic growth (meaning growth using atmospheric CO_2) was assumed to be constant, independently of the amount of organic substrate present in the medium. This might be a strong assumption taking into account that acetate was shown to reduce CO_2 assimilation in either *C. reinhardtii* [22] or *C. sorokiniana* [23]. Despite these limitations, some conclusions can be drawn from these values.

Firstly, the constant heterotrophic yield from pH 6 to 9 for most strains shows that acetate was efficiently converted into biomass at these pH values. This implies that the constant or decreasing biomass production rates observed at pH 8-10 in the mixotrophic conditions are due to the decrease of the heterotrophic biomass production rates. A decrease in mixotrophic productivity was thus probably caused by a limitation in acetate consumption, potentially at the level of its transport into the cells. The uptake of VFAs involves two mechanisms: (i) passive diffusion of the liposoluble undissociated form of

the acids across the membrane, and (ii) active transport of the anionic forms mediated by membrane carboxylate/proton transporters [24]. Near neutral external pH, the concentration of the undissociated form is less than 1% of the dissociated form ($pK_{a,acetate}$ = 4.75 and $pK_{a,butyrate}$ = 4.8) and the transport of VFA relies mostly on an active uptake at a rate that is $[H^+]$-dependent [25]. At high pH where the anionic acid form is predominant, proton concentration might be limiting, leading to a reduced uptake rate of acetate and thus a reduced $r_{app, Ac}$, up to a complete inhibition of acetate uptake, eg. at pH 10. The results indicate that this limitation of substrate transport, depending on the pH, is also strain dependant, *C. sorokiniana* being limited in acetate uptake before the other strains. This is consistent with previous results for other strains. Hwang et al (2014) showed that increasing the initial pH from 4.9 to 8 increased acetate removal by 90.5% by the strain *Micractinium reisseri* YSW0 growing under photo-heterotrophic hydrogen producing conditions [26]. Increasing the pH further resulted however in only 45% of initial acetate uptake. Cho et al (2015) also showed that VFA removal was enhanced for *C. vulgaris* when pH was controlled below pH 9 in presence of high VFA concentration [27].

Secondly, growth inhibition of all strains at pH 5 in mixotrophy compared to the autotrophic control suggests that organic substrates, especially acetate, is responsible for absence of growth at this pH and not the acidic pH value in itself. Results in the literature tend to confirm this hypothesis: *C. sorokiniana* is able to grow autotrophically from pH 4 to 12 [28] but growth was inhibited at pH 5.5 in presence of acetate [29]. On the other hand *Scenedesmus sp.* R-16 could grow heterotrophically in presence of 10 $g.L^{-1}$ glucose at pH 4 [30]. Buffer toxicity was not evaluated in this study, and some authors reported that organic buffer such as those used in the present paper (MES, HEPES and CHES) could be inhibitory [31]. However, pH 5 and 6 conditions were both

buffered with 100 mM MES. Normal growth was observed at pH 6 and as such, although buffers may have some impact on the obtained growth rates, their presence is unlikely to explain the complete inhibition observed at pH 5.

3.2 Effect of initial pH on butyrate growth

Effect of the initial pH on microalgal growth in presence of butyrate was also investigated (Fig. S3). Compared to acetate, butyrate is a much less appropriate substrate for microalgae and the strains exhibited stronger discrepancies in their behaviour compared to growth on acetate. Interestingly, exponential growth was observed for *A. protothecoïdes* and *C. sorokiniana* while growth was linear for *A. obliquus* and the two *C. reinhardtii* strains. Stationary phase was reached after 6-12 days depending on strains and initial pH. Figure 3 shows that growth on butyrate and its assimilation was strain dependent and more affected by pH values than acetate. When butyrate was used as carbon source, no growth could be observed below pH 7 for most strains. *C. sorokiniana* exhibited the highest productivity on butyrate (0.23 ± 0.007 $g.L^{-1}.d^{-1}$at initial pH 8) followed by *C. reinhardtii* CC-124 (0.20 ± 0.004 $g.L^{-1}.d^{-1}$at pH 9). For *C. sorokiniana* and *A. protothecoïdes*, biomass productivity was increased two-fold at pH 7 and pH 8 respectively compared to the autotrophic control, showing that the strains were able to assimilate and convert butyrate into biomass. On the other hand, biomass production rates of *A. obliquus*, *C. reinhardtii* CC-124 and CC-400 were close to the autotrophic ones and butyrate addition resulted in a small increase in the biomass productivity, indicating that butyrate was poorly converted to biomass.

Biomass production rates of *A. protothecoïdes and C. sorokiniana* decreased above pH 7 and pH 8 respectively, while $r_{app, Bu}$ of both *C. reinhardtii* strains and *A.*

obliquus increased with the pH rising from 7 to 9. For these three strains, growth was totally inhibited at pH 10.

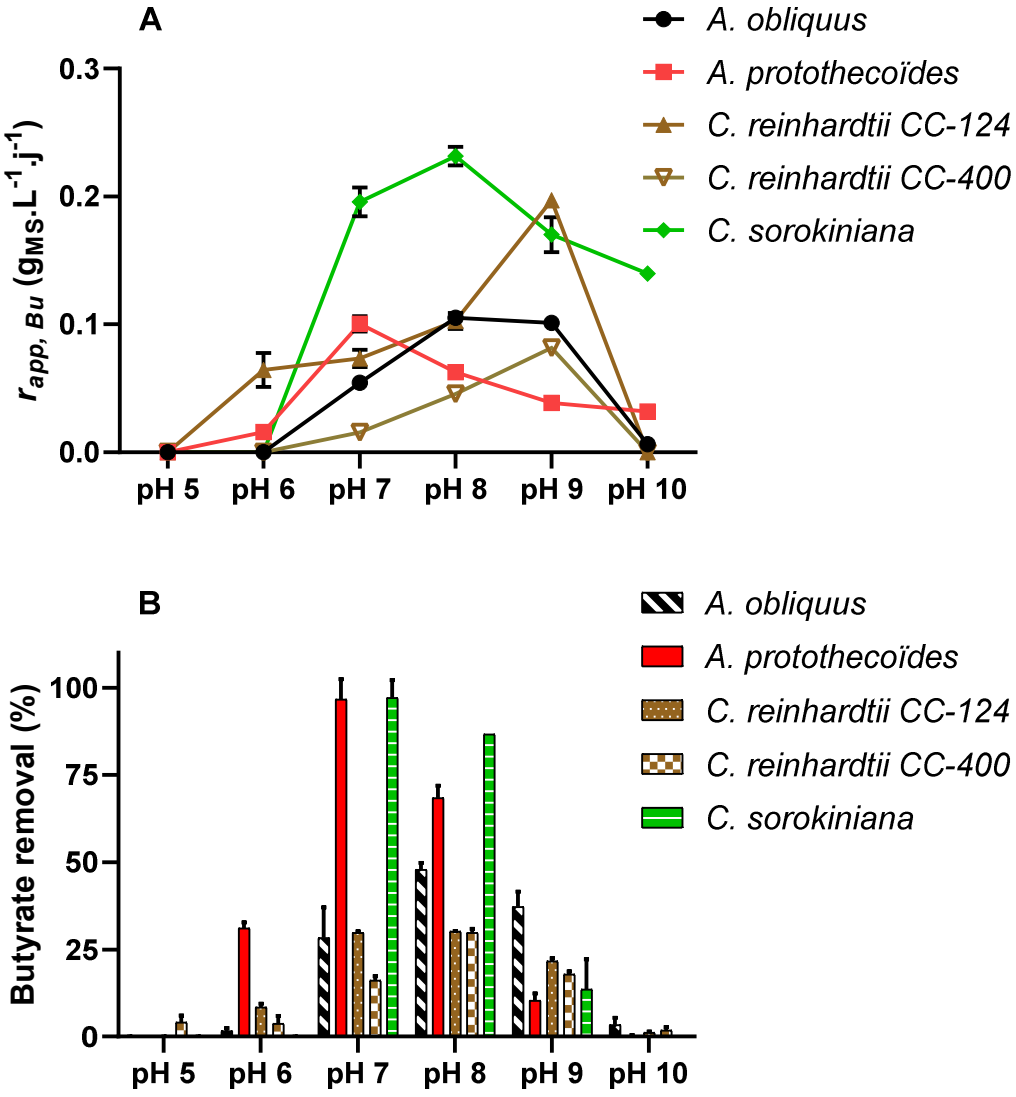

Fig. 3: Mixotrophic cultivation on 0.8 $g.L^{-1}$ butyrate and atmospheric CO_2 of *A. obliquus*, *A. protothecoïdes*, *C. reinhardtii* CC-124, *C. reinhardtii* CC-400, and *C. sorokiniana* at various initial pH values. (A) Apparent biomass productivity $r_{app, Bu}$ ($g.L^{-1}.d^{-1}$). (B) Butyrate removal at the end of the experiment. Standard deviations are given for 3 biological replicates.

Strain	Initial pH	Y_M ($g.g^{-1}$)	Y_H ($g.g^{-1}$)
A. obliquus	5	NA	NA
	6	NA	NA
	7	2.84 ± 0.84	0.22 ± 0.03
	8	3.72 ± 0.03	1.03 ± 0.08
	9	4.30 ± 0.48	1.06 ± 0.12
	10	NA	NA
A. protothecoïdes	5	NA	NA
	6	0.767 ± 0.06	-0.06 ± 0.10
	7	1.07 ± 0.07	0.47 ± 0.04
	8	1.11 ± 0.01	0.74 ± 0.02
	9	NA	NA
	10	NA	NA
C. reinhardtii 124	5	NA	NA
	6	NA	NA
	7	2.64 ± 0.22	0.98 ± 0.06
	8	3.28 ± 0.21	1.38 ± 0.21
	9	7.86 ± 0.36	3.32 ± 0.21
	10	NA	NA
C. reinhardtii 400	5	NA	NA
	6	NA	NA
	7	NA	NA
	8	1.87 ± 0.07	0.72 ± 0.7
	9	4.82 ± 0.27	0.55 ± 0.09
	10	NA	NA
C. sorokiniana	5	NA	NA
	6	NA	NA
	7	1.88 ± 0.13	0.91 ± 0.09
	8	2.11 ± 0.06	0.42 ± 0.06
	9	11.66 ± 6.59	0.44 ± 0.03
	10	NA	NA

Table 2: Biomass yields on 0.5 $g_C.L^{-1}$ butyrate. Y_M ($g.g^{-1}$) stands for global mixotrophic yield. Y_H ($g.g^{-1}$) is the estimated yield only due to heterotrophy. Standard deviations are given for 3 biological replicates. When no values are given, there was either no growth observed or no substrate consumed.

Butyrate uptake by *A. protothecoïdes* and *C. sorokiniana* was greatly affected by the initial pH (Fig. 3-B). They were the only strains to completely consume butyrate at

pH 7. However, while *A. protothecoïdes* could consume all acetate at pH 8 and 9, the strain removed only 68.4 ± 3.5 % and 10.3 ± 2.0 % butyrate at pH 8 and pH 9 respectively. In the other strains, the uptake of butyrate was somewhat less impacted by the pH but was lower to begin with, and it was never assimilated completely. Uptake was highest at pH 8 for both *C. reinhardtii* strains (around 30% of initial butyrate) and *A. obliquus* (47.9 ± 1.9 % of initial butyrate). Surprisingly, *A. obliquus* assimilated more butyrate than *C. sorokiniana* and *A. protothecoïdes* at pH 9. No butyrate could be assimilated at pH 10.

Calculated yields varied considerably between strains and pH conditions (Table 2). For instance, *A. obliquus* Y_H was close to 1.0 $g.g^{-1}$ at pH 8 and 9 while the Y_H of *C. sorokiniana* was maximum at pH 7 (0.91 ± 0.09 $g.g^{-1}$) and halved at pH 8 and 9. It appeared thus that physiological differences between the strains for butyrate assimilation are much more pronounced than for acetate. The general trend is that the uptake rate of butyrate is much slower than acetate, as reflected by the fact that Y_M were always very higher than 1. This means that a substantial amount of atmospheric carbon was assimilated during the duration of the experiment.

The growth inhibition observed at pH 6 shows that the detrimental effect of organic acid suggested in the first section is not only pH-dependant but substrate-dependant as well, as growth was observed at pH 6 in presence of acetate (Fig. 1). Increasing the pH by one unit enabled the growth on butyrate, showing that substrate concentration in itself could not explain solely why growth was prevented. Moreover, butyrate was found to be inhibitory at concentrations as low as 0.16 $g.L^{-1}$ [13] for *C. sorokiniana* while *C. vulgaris* ESP-6 could be cultivated on a DF effluent containing 0.3 $g.L^{-1}$ acetate and 0.8 $g.L^{-1}$ butyrate [14]. The strains, even though different, are both *Chlorella* species and thus relatively close. The fact that *C. vulgaris* could grow on

butyrate concentration 4 times higher than *C. sorokiniana* could be attributed to the elevation of pH, as the former study was performed at pH 6.5 while the latter was done at 7.5. Another explanation could be the presence of acetate. In case of a mixture of VFAs, acetate is consumed first by microalgae in a diauxic pattern [13]. Since assimilation of acetate leads to alkalisation of the medium [27] inhibition by butyrate may have been alleviated. However, butyrate assimilation is greatly reduced at pH above 7. This result, as well as the observed decreased productivity on acetate at alkaline pH, further show that pH medium should be tightly controlled during cultivation of microalgae on dark fermentation metabolites.

3.3 Inhibition of C. sorokiniana is due to the undissociated acid form

To dissociate the effect of pH or ROOH concentrations, *C. sorokiniana* was cultivated on various organic acid concentrations at three different supposedly inhibitory pH values: 5.5, 6.0 and 6.5 (Fig. 4, S4 and S5). This range of concentrations and the initial pH were chosen based on preliminary results (data not shown).

When plotting biomass productivity r_{app} versus concentration of organic acid expressed as total acetate concentration (Fig 4A), $r_{app, Ac}$ of *C. sorokiniana* increased from 0.45 ± 0.05 to 2.44 ± 0.11 $g.L^{-1}.d^{-1}$ when total acetate concentration increased from 0.16 to 4.1 $g.L^{-1}$. Lower initial pH permitted lower total acetate concentrations, with growth being inhibited at 0.64 $g.L^{-1}$ acetate at pH 5.5 while growth occurred at the same concentration at pH 6.0. This is explained by the difference in AcOOH concentration at these different pH values. When expressing the biomass productivity as a function of initial AcOOH, an inhibition threshold could be determined (Fig. 4B). At a given initial pH, the productivity increased or remained constant with increasing initial AcOOH

concentrations. However, above 88.5 mg.L^{-1} AcOOH, growth was totally prevented, independently of the pH value.

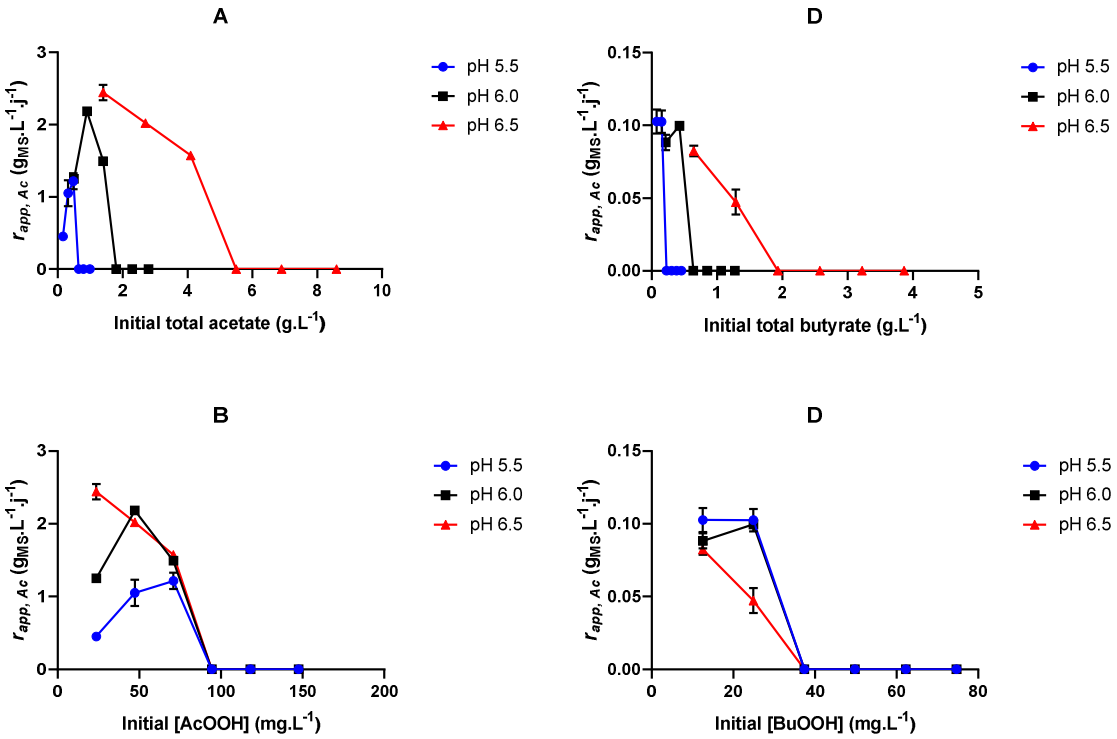

Fig. 4: Inhibition is caused by the undissociated acid form. Mixotrophic growth of *C. sorokiniana* on increasing concentrations of acetate expressed as total acetate (AcOOH + AcOO$^-$) (A) or acetic acid (AcOOH) (B) and increasing concentrations of butyrate expressed as total butyrate (BuOOH + BuOO$^-$) (C) or butyric acid (BuOOH) (D) at pH 5.5 (circles), 6.0 (squares) or 6.5 (triangle). The strain is cultivated under atmospheric CO_2. Standard deviations are given for 3 biological replicates.

As opposed to acetate, high concentrations of total butyrate affected biomass productivity, as it decreased from 0.10 ± 0.008 to 0.05 ± 0.008 g.L^{-1}.d^{-1} with increasing concentrations of total butyrate from 0.08 to 1.3 g.L^{-1} (Fig. 4C). Still, when expressing $r_{app, Bu}$ as a function of BuOOH, an inhibition threshold was observed. Growth was

possible as long as undissociated butyric acid concentration was below 37.4 $mg.L^{-1}$, above which no growth was detected.

These results support the previous observations that no growth could be detected at pH 5 on acetate but at pH 6 on butyrate. At the concentration of total acid used in these experiments (1.1 $g.L^{-1}$ of acetate), concentration of AcOOH was 396 $mg.L^{-1}$ at pH 5.0, far above the threshold. Similarly, at butyrate concentration of 0.8 $g.L^{-1}$, BuOOH concentration was 288 $mg.L^{-1}$ at pH 5 and 42.6 $mg.L^{-1}$ at pH 6. The inhibition threshold on butyrate is 3 times lower than the one of acetate, suggesting a more toxic effect of this organic acid and explaining why initial pH required for butyrate growth is higher. This difference could be attributed to the mechanism of ROOH toxicity: upon entry in the cells, ROOH will dissociate in the cytoplasm where pH is near neutral. According to [32], anion accumulation is the primary toxic effect of organic acids. When present in too high concentrations, cells are unable to efficiently metabolise the acid while also being internally damaged [17]. Acetate can readily be metabolized by the cells via a one-step reaction producing acetyl-CoA. In contrast, butyrate, being a 4-C organic acid, is metabolized via a 5-step pathway producing two reducing equivalents in the form of NADH. One of these steps might be limiting, letting butyrate accumulate inside the cells at higher rate than does acetate, which can induce a lethal effect at lower concentrations. The more toxic effect of longer chain organic acid was also reported in the yeast *Yarrowia lipolytica* where the C_{50} (concentration of acid necessary to lower the growth rate by 50%) decreased with increasing carbon chain length [33].

3.4 Inhibition threshold of other strains

The behaviour of *A. obliquus*, *A. protothecoïdes*, *C. reinhardtii* CC-124 and CC-400 on increasing concentrations of acetic and butyric acid was assessed and compared to *C. sorokiniana* (Fig. 5, S6 and S7). Cultivation was performed at pH 6. Total acetate concentrations ranged from 0.43 to 4.3 $g.L^{-1}$ while total butyrate concentrations ranged from 0.2 to 1.3 $g.L^{-1}$.

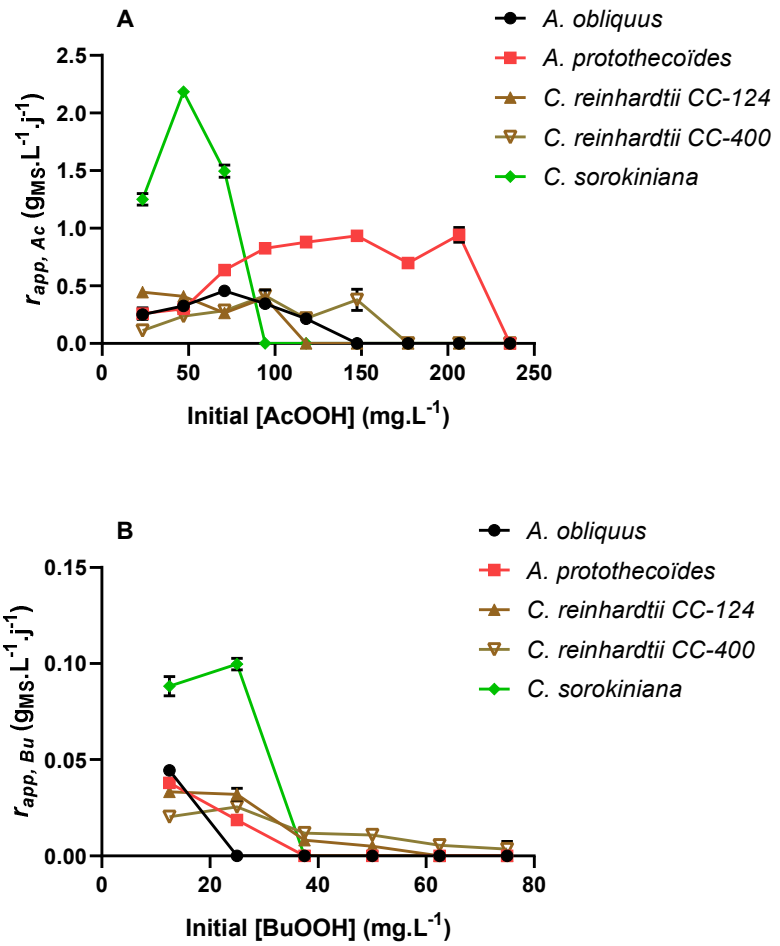

Fig. 5: Mixotrophic growth rates of *A. obliquus*, *A. protothecoïdes*, *C. reinhardtii* CC-124, CC-400, *C. sorokiniana* on increasing concentrations of acetic acid (AcOOH) (A) or butyric acid (BuOOH) (B) at pH 6.0 under atmospheric CO_2. Standard deviations are given for 3 biological replicates.

C. sorokiniana exhibited the highest production rate on acetate (2.18 ± 0.02 $g.L^{-1}.d^{-1}$) (Fig. 5A). Production rate of *A. protothecoïdes* increased from 0.26 ± 0.05 to 0.94 ± 0.06 $g.L^{-1}.d^{-1}$ with increasing acetate concentration. *A. obliquus*, *C. reinhardtii* CC-124 and CC-400 production rates were relatively constant for all the tested acetate levels and remained lower than the other two strains. An inhibition threshold could be determined for each of the five micro-algae strains and ranged from 47 to 207 $mg.L^{-1}$. Resistance to acetic acid was different for all strains, with *A. obliquus* being inhibited above 47.2 $mg.L^{-1}$ of AcOOH while *A. protothecoïdes* could stand an AcOOH concentration 4 times higher. *C. reinhardtii* CC-400 supported a higher initial concentration of acetic acid than *C. reinhardtii* CC-124 (150 $mg.L^{-1}$ vs 100 $mg.L^{-1}$).

As opposed to the behaviour on acetate, the $r_{app, Bu}$ of all strains except *C. sorokiniana* sharply decreased with increasing concentrations of butyrate (Fig 5B). The threshold concentration was 25 $mg.L^{-1}$ of BuOOH for most strains. *A obliquus* was the least resistant strain with growth inhibition at 12.5 $mg.L^{-1}$ of BuOOH. Again, *C. reinhardtii* CC-400 supported an initial concentration of 72.5 $mg.L^{-1}$ BuOOH while *C. reinhardtii* CC-124 was inhibited at 50 $mg.L^{-1}$.

The results presented above could help choosing the appropriate strain and mixotrophic growth conditions in presence of VFAs, notably to allow efficient coupling of DF process with microalgal cultivation. Usually, dark fermentation is operated at an acidic pH around 5.5 [12]. The fermentation of 10 $g.L^{-1}$ glucose would be lead to around 1.7 $g.L^{-1}$ acetate (total) and 3.5 $g.L^{-1}$ butyrate (total) considering that the theoretical equation (5) [34]:

$$4\,C_6H_{12}O_6 + 2\,H_2O \rightarrow 3\,CH_3CH_2CH_2COOH + 2\,CH_3COOH + 8\,CO_2 + 10\,H_2 \quad (5)$$

This leads to AcOOH and BuOOH concentration around 278 $mg.L^{-1}$ and 582 $mg.L^{-1}$ at pH 5.5, far above the estimated threshold of this study. As the ROOH

concentration depends on total VFA concentration and pH value, DF effluent should either be diluted or the pH should be adjusted to a high enough value to minimize ROOH concentrations with regard to the selected (or predominant) strain(s) and its resistance to organic acid and its most favourable growth conditions. Indeed, increasing the pH from 5.0 to 7.0 result in AcOOH and BuOOH concentration of 10.5 $mg.L^{-1}$ and 21.9 $mg.L^{-1}$, enabling microalgae growth. Of course, adjustements should be made according to the VFA concentration in the effluent. The effect of a mixture of substrates however, which may influence the values of threshold concentrations, remains to be further investigated.

It was shown that all strains could assimilate efficiently acetate as long as the pH remained around neutral values. On the other hand, only *C. sorokiniana* and *A. protothecoïdes* could completely consume butyrate although their biomass production rates remained quite low. The three other strains seemed to be able to assimilate butyrate, but their uptake was never complete and thus probably limited by either transport or metabolic capacity. Consequently, these other three strains should not be considered for the coupled process, although their carbon reserve productivity (lipids, carbohydrates) was not assessed in the present study. They may however present an interest for metabolic studies to explore the strain dependence for acetate and butyrate uptake and their associated enzymatic pathways. In fact, *C. reinhardtii* acetate uptake has been widely studied, but its ability to consume butyrate as a single substrate has not, and to the best of our knowledge, it is the first time that butyrate assimilation by *C. reinhardtii* has been evidenced. The improved resistance to organic acids of the cell wall less strain (*C. reinhardtii* CC-400) was contrary to what we expected since VFA and other short chain fatty acids inhibit bacterial growth and induce cell death notably

by damaging cell membranes [35]. As literature is rather scarce on this specific substrate, answering this question would require further studies.

4. Conclusions

Mixotrophic growth on acetate or butyrate as a function of the initial pH was evaluated for several microalgal strains. *C. sorokiniana* was found to be the most productive strain on both substrates with the highest biomass yield. VFAs were found to be inhibitory when the undissociated acid form was too concentrated. A threshold concentration of undissociated acetic or butyric acid below which growth was possible was determined for each strain. Therefore, effluents should be either diluted or the initial pH increased to lower the inhibitory effect of these acids. Besides, studying further the model strain *C. reinhardtii* on butyrate could give more insight on the metabolic bottlenecks limiting butyrate assimilation.

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Statement of informed consent, human and animal rights

No conflicts, informed consent, or human or animal rights are applicable to this study.

Author contributions

All author contributed to the work either in the design of experiment, collecting or analysing data. All authors participated to the drafting of the article or to its revising. All authors approved the final version of the manuscript submitted.

References

[1] Y. Chisti, Algae production comparrison, Biotechnol. Adv. 25 (2007) 294–306. https://doi.org/10.1016/j.biotechadv.2007.02.001.

[2] K.W. Chew, J.Y. Yap, P.L. Show, N.H. Suan, J.C. Juan, T.C. Ling, D.J. Lee, J.S. Chang, Microalgae biorefinery: High value products perspectives, Bioresour. Technol. 229 (2017) 53–62. https://doi.org/10.1016/j.biortech.2017.01.006.

[3] L. Brennan, P. Owende, Biofuels from microalgae-A review of technologies for production, processing, and extractions of biofuels and co-products, Renew. Sustain. Energy Rev. 14 (2010) 557–577. https://doi.org/10.1016/j.rser.2009.10.009.

[4] O. Perez-Garcia, F.M.E. Escalante, L.E. de-Bashan, Y. Bashan, Heterotrophic cultures of microalgae: Metabolism and potential products, Water Res. 45 (2011) 11–36. https://doi.org/10.1016/j.watres.2010.08.037.

[5] J. Zhan, J. Rong, Q. Wang, Mixotrophic cultivation, a preferable microalgae cultivation mode for biomass/bioenergy production, and bioremediation, advances and prospect, Int. J. Hydrogen Energy. 42 (2017) 8505–8517. https://doi.org/10.1016/j.ijhydene.2016.12.021.

[6] T. Li, Y. Zheng, L. Yu, S. Chen, Mixotrophic cultivation of a Chlorella sorokiniana strain for enhanced biomass and lipid production, Biomass and Bioenergy. 66 (2014) 204–213. https://doi.org/10.1016/j.biombioe.2014.04.010.

[7] Y. Liang, Producing liquid transportation fuels from heterotrophic microalgae, Appl. Energy. 104 (2013) 860–868. https://doi.org/10.1016/j.apenergy.2012.10.067.

[8] J. Lowrey, M.S. Brooks, P.J. Mcginn, Heterotrophic and mixotrophic cultivation of microalgae for biodiesel production in agricultural wastewaters and associated challenges — a critical review, (2015) 1485–1498. https://doi.org/10.1007/s10811-014-0459-3.

[9] V. Turon, E. Trably, E. Fouilland, J.P. Steyer, Potentialities of dark fermentation effluents as substrates for microalgae growth: A review, Process Biochem. 51 (2016) 1843–1854. https://doi.org/10.1016/j.procbio.2016.03.018.

[10] A. Chalima, L. Oliver, L. Fern, A. Karnaouri, T. Dietrich, E. Topakas, Utilization of Volatile Fatty Acids from Microalgae for the Production of High Added Value

Compounds, (n.d.) 1–17. https://doi.org/10.3390/fermentation3040054.

[11] R. Moscoviz, E. Trably, N. Bernet, H. Carrère, The environmental biorefinery: State-of-the-art on the production of hydrogen and value-added biomolecules in mixed-culture fermentation, Green Chem. 20 (2018) 3159–3179. https://doi.org/10.1039/c8gc00572a.

[12] A. Ghimire, L. Frunzo, F. Pirozzi, E. Trably, R. Escudie, P.N.L. Lens, G. Esposito, A review on dark fermentative biohydrogen production from organic biomass: Process parameters and use of by-products, Appl. Energy. 144 (2015) 73–95. https://doi.org/10.1016/j.apenergy.2015.01.045.

[13] V. Turon, C. Baroukh, E. Trably, E. Latrille, E. Fouilland, J.P. Steyer, Use of fermentative metabolites for heterotrophic microalgae growth: Yields and kinetics, Bioresour. Technol. 175 (2015) 342–349. https://doi.org/10.1016/j.biortech.2014.10.114.

[14] C.H. Liu, C.Y. Chang, Q. Liao, X. Zhu, C.F. Liao, J.S. Chang, Biohydrogen production by a novel integration of dark fermentation and mixotrophic microalgae cultivation, in: Int. J. Hydrogen Energy, Elsevier Ltd, 2013: pp. 15807–15814. https://doi.org/10.1016/j.ijhydene.2013.05.104.

[15] Q. Fei, R. Fu, L. Shang, C.J. Brigham, H.N. Chang, Lipid production by microalgae Chlorella protothecoides with volatile fatty acids (VFAs) as carbon sources in heterotrophic cultivation and its economic assessment, Bioprocess Biosyst. Eng. 38 (2015) 691–700. https://doi.org/10.1007/s00449-014-1308-0.

[16] S.C. Ricke, Perspectives on the use of organic acids and short chain fatty acids as antimicrobials, Poult Sci. 82 (2003) 632–639. https://doi.org/10.1093/ps/82.4.632.

[17] A.P. Desbois, V.J. Smith, Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential, Appl. Microbiol. Biotechnol. 85 (2010) 1629–1642. https://doi.org/10.1007/s00253-009-2355-3.

[18] Z. Zuo, Y. Zhu, Y. Bai, Y. Wang, Plant Physiology and Biochemistry Acetic acid-induced programmed cell death and release of volatile organic compounds in Chlamydomonas reinhardtii, Plant Physiol. Biochem. 51 (2012) 175–184. https://doi.org/10.1016/j.plaphy.2011.11.003.

[19] M.J. Griffiths, C. Garcin, R.P. van Hille, S.T.L. Harrison, Interference by pigment in the estimation of microalgal biomass concentration by optical density, J. Microbiol. Methods. 85 (2011) 119–123. https://doi.org/10.1016/j.mimet.2011.02.005.

[20] H. Endo, H. Sansawa, K. Nakajima, Studies on Chlorella regularis, heterotrophic fast-growing strain II. Mixotrophic growth in relation to light intensity and acetate concentration :, Plant Cell Physiol. 18 (1977) 199–205. http://ci.nii.ac.jp/naid/110003717232/en/ (accessed September 24, 2019).

[21] V. Turon, E. Trably, E. Fouilland, J.-P. Steyer, Growth of Chlorella sorokiniana on a mixture of volatile fatty acids: the effects of light and temperature, Bioresour. Technol. 198 (2015) 852–860. https://doi.org/10.1016/j.biortech.2015.10.001.

[22] P.B. Heifetz, B. Förster, C.B. Osmond, L.J. Giles, J.E. Boynton, Effects of Acetate on Facultative Autotrophy inChlamydomonas reinhardtii Assessed by Photosynthetic Measurements and Stable Isotope Analyses, Plant Physiol. 122 (2000) 1439 LP – 1446. http://www.plantphysiol.org/content/122/4/1439.abstract.

[23] H. Qiao, G. Wang, K. Liu, W. Gu, Short-term effects of acetate and microaerobic conditions on photosynthesis and respiration in chlorella sorokiniana gxnn 01 (chlorophyta), J. Phycol. 48 (2012) 992–1001. https://doi.org/10.1111/j.1529-8817.2012.01189.x.

[24] M. Casal, C. Leão, Utilization of short-chain monocarboxylic acids by the yeast Torulaspora delbrueckii: Specificity of the transport systems and their regulation, BBA - Mol. Cell Res. 1267 (1995) 122–130. https://doi.org/10.1016/0167-4889(95)00067-3.

[25] E. Jolkver, D. Emer, S. Ballan, R. Kra, B.J. Eikmanns, K. Marin, Identification and Characterization of a Bacterial Transport System for the Uptake of Pyruvate , Propionate , and Acetate in Corynebacterium glutamicum , 191 (2009) 940–948. https://doi.org/10.1128/JB.01155-08.

[26] J.H. Hwang, A.N. Kabra, J.R. Kim, B.H. Jeon, Photoheterotrophic microalgal hydrogen production using acetate- and butyrate-rich wastewater effluent, Energy. 78 (2014) 887–894. https://doi.org/10.1016/j.energy.2014.10.086.

[27] H.U. Cho, Y. Mo, Y. Choi, X. Xu, D. Yun, J. Moon, Bioresource Technology Effects of pH control and concentration on microbial oil production from Chlorella vulgaris cultivated in the effluent of a low-cost organic waste fermentation system producing volatile fatty acids, 184 (2015) 245–250.

[28] V. Kumar, M. Muthuraj, B. Palabhanvi, A.K. Ghoshal, D. Das, High cell density lipid rich cultivation of a novel microalgal isolate Chlorella sorokiniana FC6 IITG in a single-stage fed-batch mode under mixotrophic condition, Bioresour. Technol. 170 (2014) 115–124. https://doi.org/10.1016/j.biortech.2014.07.066.

[29] K. Kumar, C.N. Dasgupta, D. Das, Cell growth kinetics of Chlorella sorokiniana and nutritional values of its biomass, Bioresour. Technol. 167 (2014) 358–366. https://doi.org/10.1016/j.biortech.2014.05.118.

[30] H.-Y. Ren, B.-F. Liu, C. Ma, L. Zhao, N.-Q. Ren, A new lipid-rich microalga Scenedesmus sp. strain R-16 isolated using Nile red staining: effects of carbon and nitrogen sources and initial pH on the biomass and lipid production., Biotechnol. Biofuels. 6 (2013) 143. https://doi.org/10.1186/1754-6834-6-143.

[31] C.E. De Farias Silva, E. Sforza, A. Bertucco, Effects of pH and Carbon Source on Synechococcus PCC 7002 Cultivation: Biomass and Carbohydrate Production with Different Strategies for pH Control, Appl. Biochem. Biotechnol. 181 (2017) 682–698. https://doi.org/10.1007/s12010-016-2241-2.

[32] J.B. Russell, Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling, J. Appl. Bacteriol. 73 (1992) 363–370. https://doi.org/10.1111/j.1365-2672.1992.tb04990.x.

[33] G. Rodrigues, C. Pais, The Influence of Acetic and Other Weak Carboxylic Acids on Growth and Cellular Death of the Yeast Yarrowia lipolytica, Food

Technol. Biotechnol. 38 (2000) 27–32.

[34] F.R. Hawkes, I. Hussy, G. Kyazze, R. Dinsdale, D.L. Hawkes, Continuous dark fermentative hydrogen production by mesophilic microflora: Principles and progress, Int. J. Hydrogen Energy. 32 (2007) 172–184. https://doi.org/10.1016/j.ijhydene.2006.08.014.

[35] L.A. Royce, P. Liu, M.J. Stebbins, B.C. Hanson, L.R. Jarboe, The damaging effects of short chain fatty acids on Escherichia coli membranes, Appl. Microbiol. Biotechnol. 97 (2013) 8317–8327. https://doi.org/10.1007/s00253-013-5113-5.

Supplementary Information

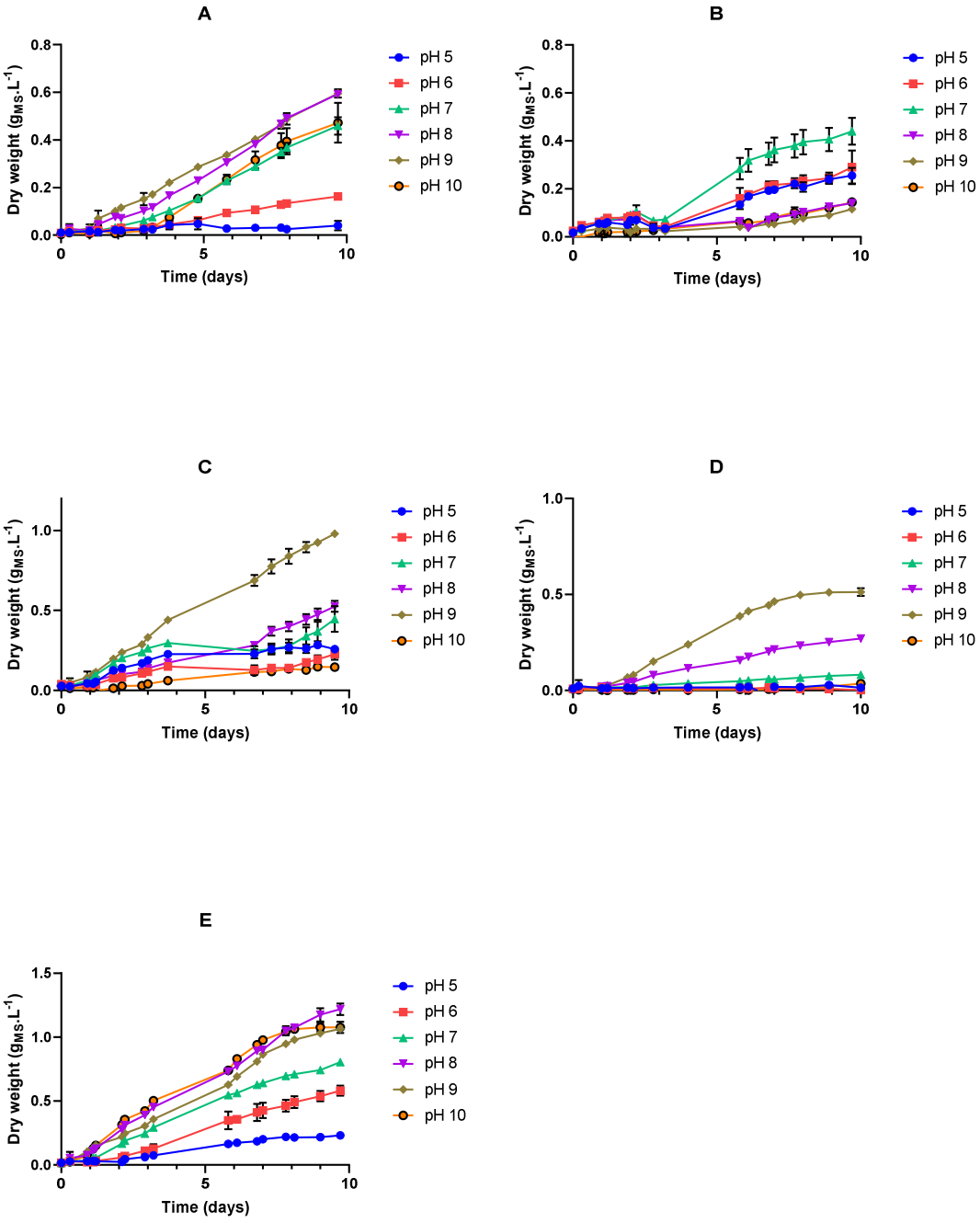

Fig. S1: Growth curves of (A) *A. obliquus*, (B) *A. prototheco*ïdes, (C) *C. reinhardtii* CC-124, (D) *C. reinhardtii* CC-400 and (E) *C. sorokiniana* in autotrophic conditions at various initial pH in presence of atmospheric CO_2.

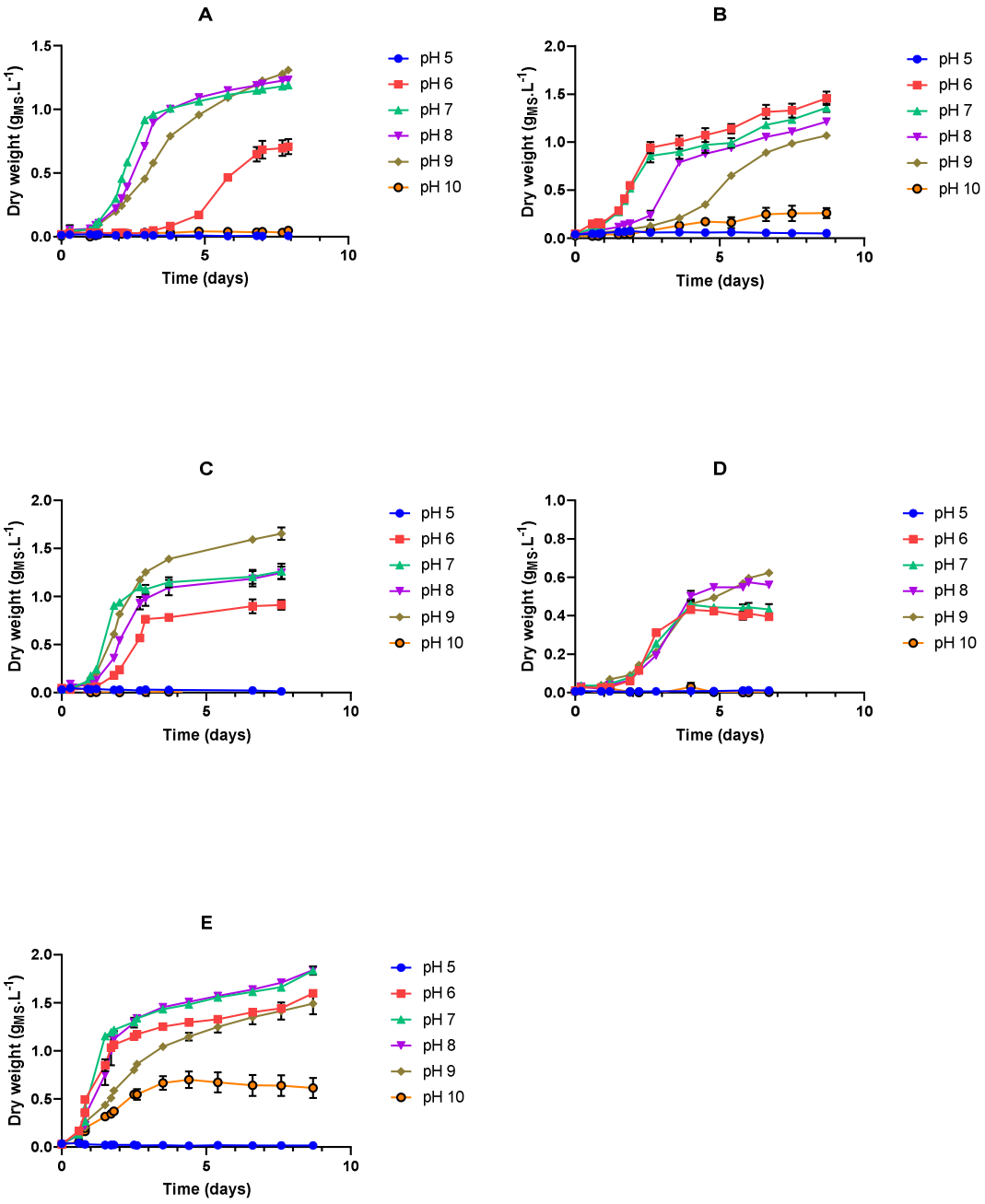

Fig. S2: Growth curves of (A) *A. obliquus*, (B) *A. prototheco*ï*des*, (C) *C. reinhardtii* CC-124, (D) *C. reinhardtii* CC-400 and (E) *C. sorokiniana* in mixotrophic conditions at various initial pH in presence of 1.1 $g.L^{-1}$ acetate.

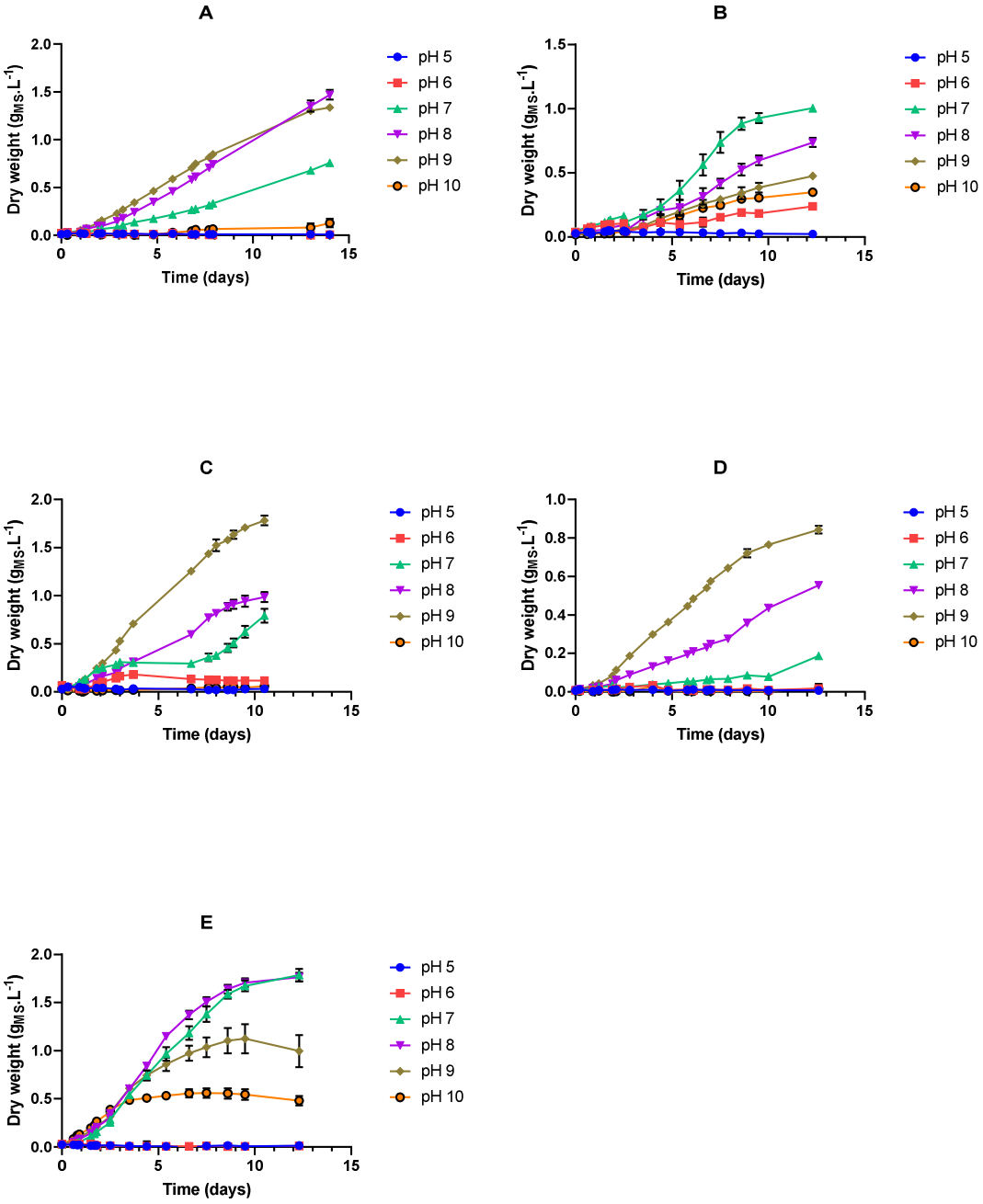

Fig. S3: Growth curves of (A) *A. obliquus*, (B) *A. protothecoïdes*, (C) *C. reinhardtii* CC-124, (D) *C. reinhardtii* CC-400 and (E) *C. sorokiniana* in mixotrophic conditions at various initial pH in presence of 0.8 $g.L^{-1}$ butyrate.

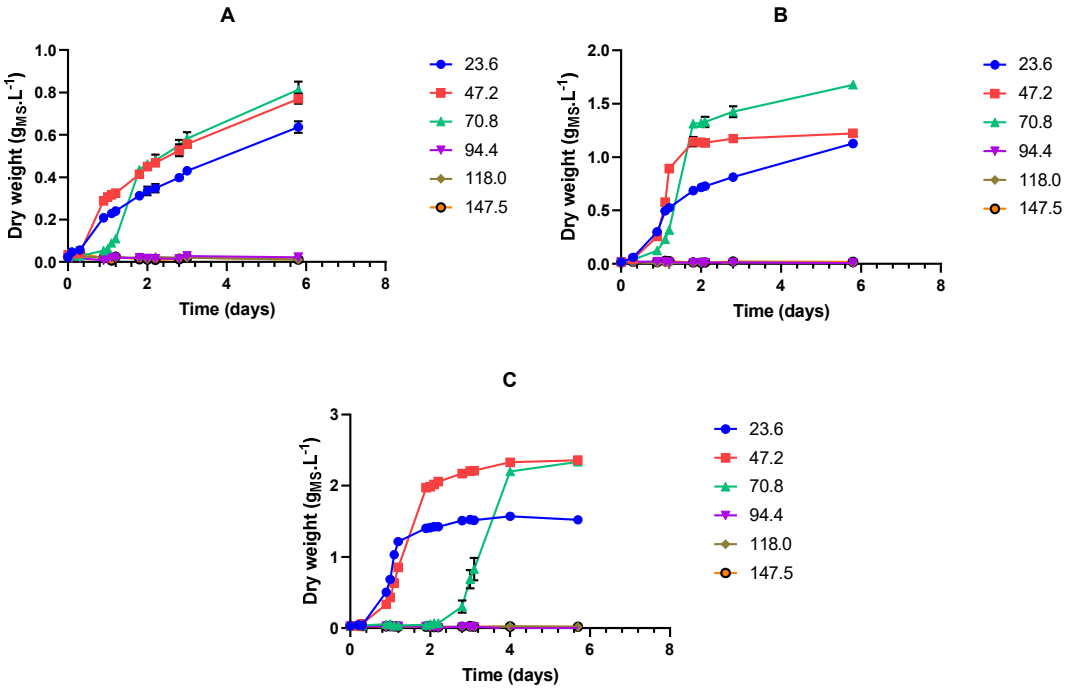

Fig. S4: Growth curves of *C. sorokiniana* at various initial [AcOOH] (expressed in mg.L^{-1}) at initial pH (A) 5.5, (B) 6.0, (C) 6.5 in mixotrophic conditions.

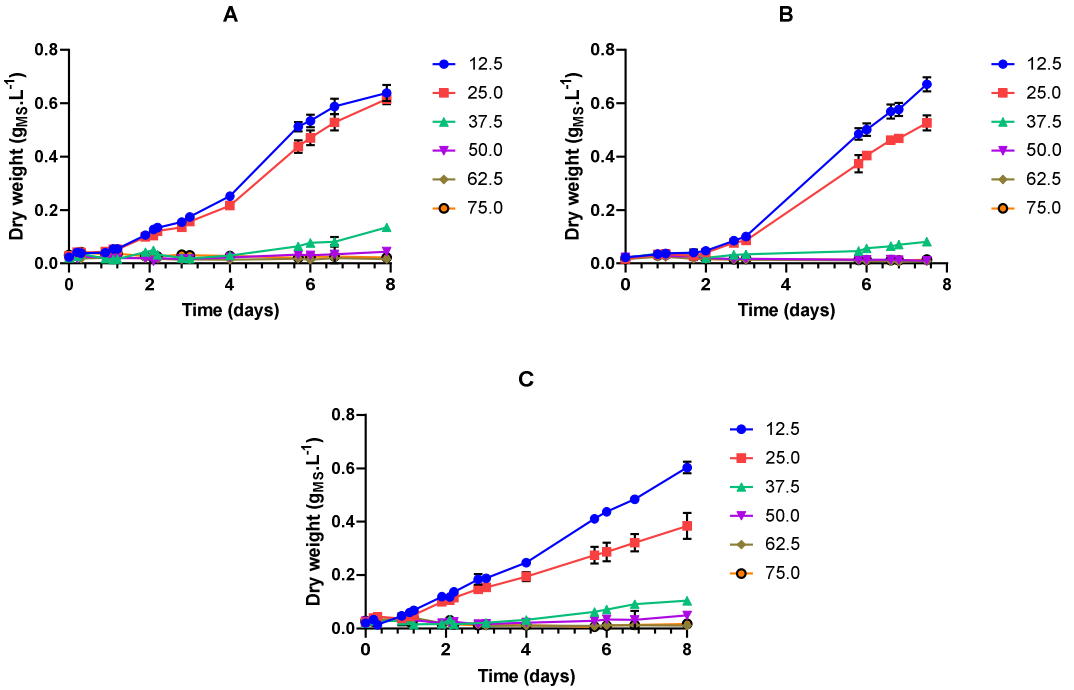

Fig. S5: Growth curves of *C. sorokiniana* at various initial [BuOOH] (expressed in mg.L^{-1}) at initial pH (A) 5.5, (B) 6.0, (C) 6.5 in mixotrophic conditions.

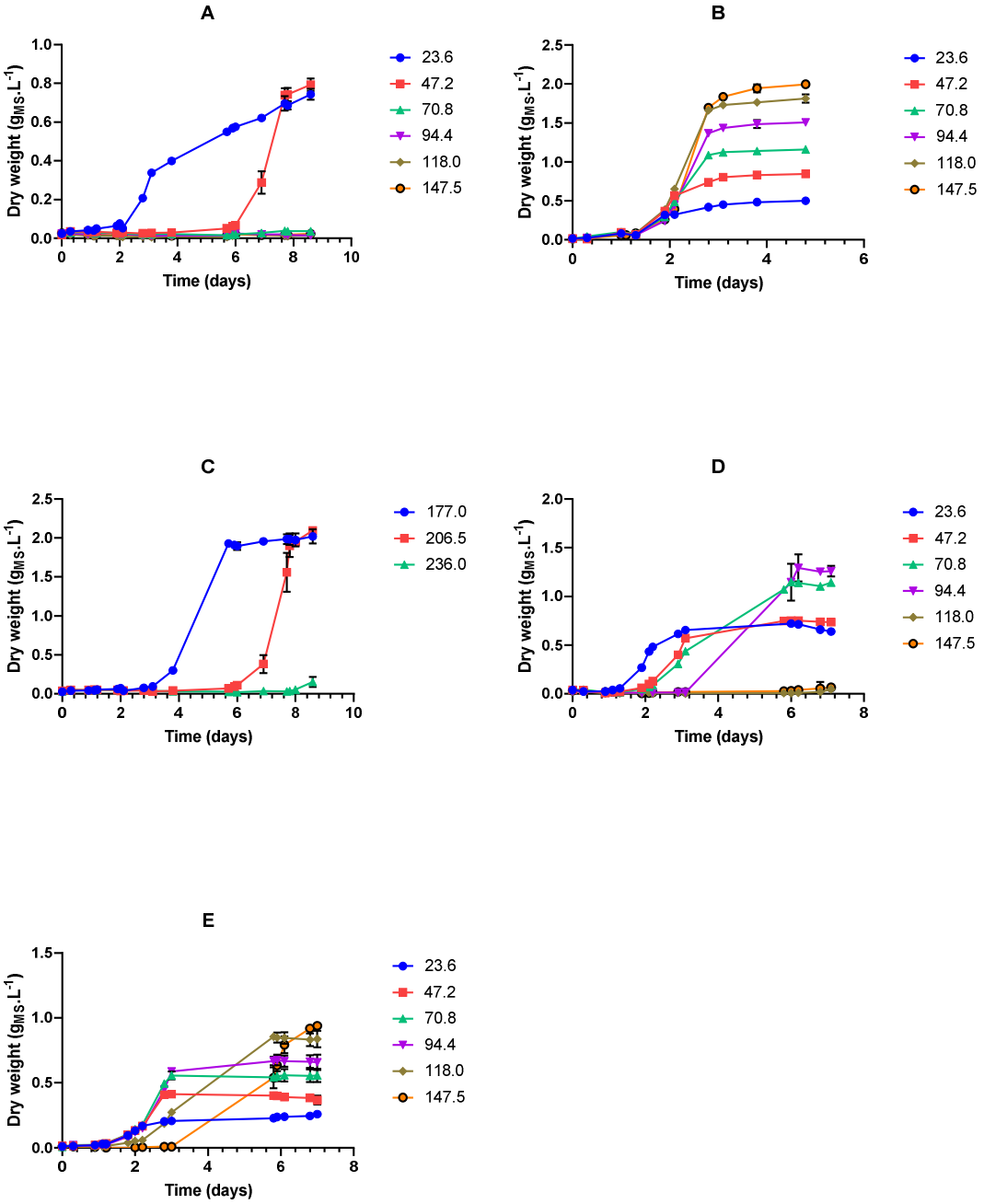

Fig. S6: Growth curves of (A) *A. obliquus*, (B) and (C) *A. protothecoïdes*, (D) *C. reinhardtii* CC-124 and (E) *C. reinhardtii* CC-400 at various initial [AcOOH] (expressed in $mg.L^{-1}$) at initial pH 6.0 in mixotrophic conditions.

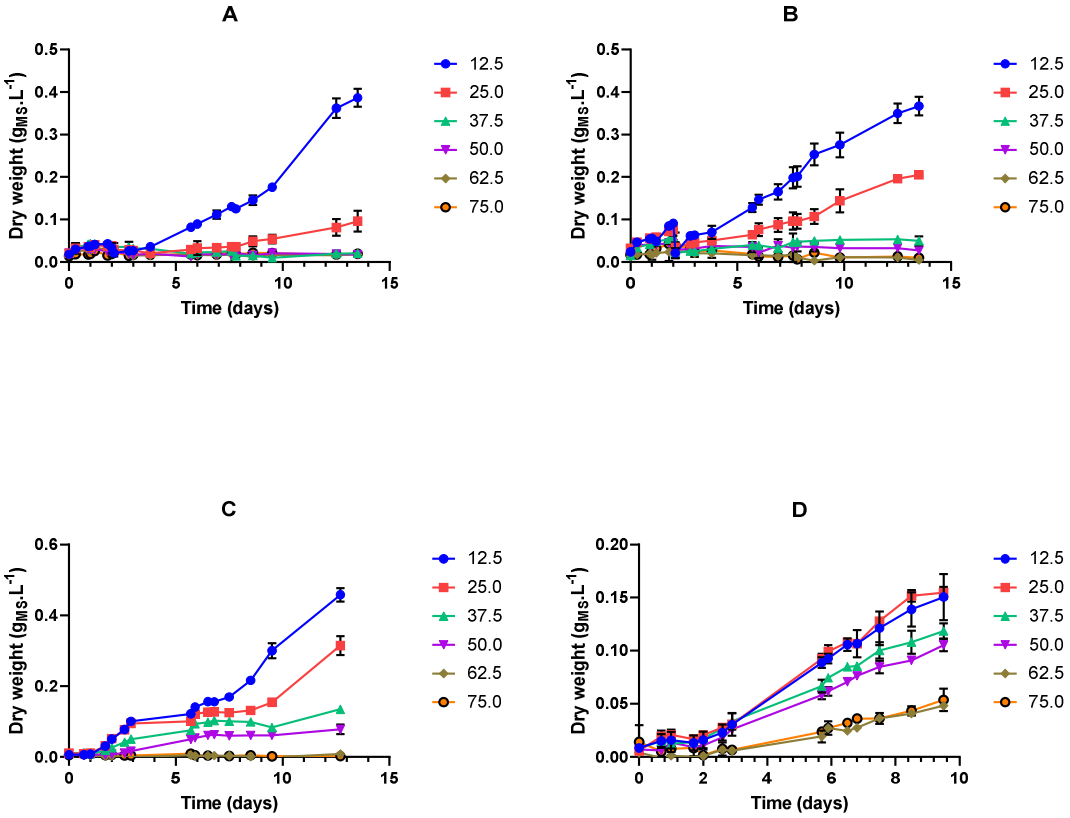

Fig. S7: Growth curves of (A) *A. obliquus*, (B) and (C) *A. protothecoïdes*, (D) *C. reinhardtii* CC-124 and (E) *C. reinhardtii* CC-400 at various initial [BuOOH] (expressed in mg.L^{-1}) at initial pH 6.0 in mixotrophic conditions.