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Microalgae screening for heterotrophic and mixotrophic growth on butyrate

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Abstract :

Volatile fatty acids (VFAs) produced by fermentative bacteria can be used instead of high value carbohydrates like glucose to reduce the costs associated to mixotrophic microalgal cultivation. This process is however limited by a poor assimilation of butyrate and a low tolerance of microalgae to high VFA concentrations. In this study, 10 microalgae strains, isolated from local natural environments or selected from culture collections were screened for their ability to grow on either acetate or butyrate. The non-photosynthetic Chlorophyte Polytomella sp. was found to be the fastest growing strain on both VFAs, exhibiting a growth rate of 4.0 d−1 on acetate and 2.5 d−1 on butyrate. The tolerance of the different strains to concentrated VFA was further assessed using increasing acid concentrations (1.5–60 g·L−1 for acetate and 1–40 g·L−1 for butyrate). Polytomella sp. appeared as the most resistant, being able to grow up to 38 g·L−1 on acetic acid and to 18 g·L−1 on butyric acid with no inhibitory effect. Finally, biomass yield and productivity, as well as lipid and carbohydrate yields were assessed on a synthetic VFAs mixture (0.8 g·L−1 acetate and 1.2 g·L−1 butyrate) for the most promising strains. Polytomella sp. and Euglena gracilis exhibited the highest carbohydrate yield (respectively 0.65 and 0.58 g·g−1) while lipid yield was maximum for Chlorella sorokiniana (0.42 g·g−1). This work provides new insights in the trophic metabolism of microalgae of highly divergent lineages using VFAs as substrates.

Highlights

► Butyrate but not the acetate in dark fermentation effluents limits microalgae cultivation on effluents. ► Ten algal species from peatbogs or culture collections were tested for growth on butyrate and acetate. ► Only the heterotrophic alga *Polytomella* sp. exhibited high growth rates and tolerance to both acids. ► Four strains were further cultivated on a synthetic dark fermentation effluent. ► Lipids were highest for *Chlorella sorokiniana* and sugars for *Polytomella* sp. and *Euglena gracilis*.

Keywords : microalgae, heterotrophy, mixotrophy, volatile fatty acids, lipids, carbohydrates

1. Introduction

Dark fermentation (DF) is an anaerobic biological process allowing concomitant production of energy and treatment of various organic waste sources such as food or crop wastes. During DF, anaerobic bacteria convert complex organic matter into hydrogen and a mixture of end-product metabolites. These metabolites can vary according to fermentation

conditions and substrate nature, but are on average mainly composed of volatile fatty acids (VFAs) particularly acetate and butyrate [1]. These VFAs can serve as low-cost organic substrates for the growth of various micro-organisms either non photosynthetic such as yeast and thraustochytrids or photosynthetic such as microalgae [2,3].

Butyrate was identified as a key factor limiting the potential of such a coupling, particularly in the case of microalgae [4,5] due to inhibitory effects [6] or inability to grow on more than 1.25 $g.L^{-1}$ of butyrate without light [4]. Several attempts have been made to increase the consumption of butyrate by acting on operational parameters such as light and temperature [7], higher acetate content in the medium [6] or pH adjustment [8]. However, even if butyrate inhibition can be alleviated using these strategies, butyrate uptake rates remain about 10-fold lower as compared to acetate [9]. So far, most research published on the coupling of DF and microalgae cultivation has focused on lipid production using strains such as *Chlorella* or *Scenedesmus* species obtained from collections [5,6,9–11]. However, strains isolated from organic rich environments may be better adapted to the consumption of butyrate. Indeed, collection strains are usually maintained on designed media, leading to an adaptation to laboratory conditions. As a result, these strains may exhibit lower tolerance or lower growth on alternative substrates compared to environmental isolate species [12].

If photosynthetic Chlorophytes seem limited in their ability to consume DF metabolites, purely heterotrophic species could be more adapted to grow on organic acids since they rely entirely on organic carbon for growth. As an illustration, Chalima et al., [13] tested the growth of the heterotrophic marine dinoflagellate *Crypthecodinium cohnii* on various VFAs (C2-C4). The strain could grow on acetic, propionic and butyric acids at 30 $g.L^{-1}$, 10 $g.L^{-1}$ and 15 $g.L^{-1}$, respectively. Moreover, since most of the aforementioned studies mostly focused on lipid production, other microalgal species which accumulate polysaccharides have received little attention. Some freshwater strains, such as *Euglena*

gracilis, Ochromonas danica or *Polytomella* sp. possess indeed several traits that make them potential candidates to be cultivated on DF effluents. For example, *E. gracilis* is a photosynthetic protist which accumulates up to 80% of its weight as a valuable polysaccharide resembling starch (paramylon) and that can grow in various nutritional modes such as photo-autotrophy, heterotrophy or mixotrophy [14]. *O. danica*, a chrysophyte (golden-brown microalgae), can grow under mixotrophic condition on organic acids and can also prey on bacteria [15]. Since it is undesirable to sterilize DF effluents due to operational costs, these effluents will contain bacteria that will likely outcompete microalgae for butyrate [16]. These bacteria could be eaten by *O. danica* and therefore increase final algal biomass. Finally, *Polytomella sp*. is a colourless Chlorophyte alga closely related to the model organism *Chlamydomonas reinhardtii*, which has lost its photosynthetic properties [17,18]. This alga grows purely heterotrophically and is able to consume various organic acids and alcohols while accumulating large amount of starch [19].

The objective of this study was to identify microalgal strains that could tolerate high concentrations of butyrate and consume it more efficiently than the most commonly studied species (*i.e.* Chlorophytes such as *Chlorella* sp. or *Scenedesmus* sp.). To this end, several microalgal strains were isolated from different local natural environments using butyrate as selective factor. In parallel, the pure strains *Euglena gracilis, Ochromonas danica, Polytomella* sp. and *Polytomella magna* were selected as alternative candidates for their mixo- or heterotrophic capacities. The growth of all these strains on acetate and butyrate as well as their tolerance to VFAs were assessed and compared to that of *C. sorokiniana*. Finally, the biomass, lipid and carbohydrate production potentialities of the candidate strains were evaluated on a synthetic DF effluent.

2. Materials and methods

2.1. Cultivation media and conditions

Microalgae were maintained and precultured on synthetic medium HEPES-Acetate-Phosphate (HAP), which is based on Tris-Acetate-Phosphate (TAP) medium [20] where HEPES buffer replaces Tris. When butyrate was used instead of acetate, the medium is referred to as HBP (HEPES-Butyrate-Phosphate). Buffer concentration was set to 20 mM for maintenance and precultures. To evaluate the growth characteristics, acetate or butyrate were added individually as sodium salts at a concentration of 1.25 $g.L^{-1}$ or 0.9 $g.L^{-1}$ respectively to set carbon concentration to 0.5 $g_C.L^{-1}$ for both substrates. Beijerincks solution (https://utex.org/products/beijerincks-solution) was added at 25 $mL.L^{-1}$ leading to concentration of 7.5 mM NH_4^+, 0.6 mM $MgSO_4$ and 0.3 mM $CaCl_2$. Potassium phosphate (K_2HPO_4) was added at 1 mM and Hutner's trace element solution [21] at 1 $mL.L^{-1}$. Medium pH was adjusted to 7.0 by addition of NaOH prior to sterilization at 121°C for 20 min. After cooling, 100 $\mu L.L^{-1}$ of a vitamin mix (50 mM vitamin B1, 1 mM biotin and 1 mM cyanocobalamin), sterilized by filtration through 0.2 µm filter, was added into the medium. To obtain solid medium, agar was added before sterilization (15 $g.L^{-1}$). All chemicals were purchased from Sigma Aldrich (Merck).

2.2. Microalgal strains

Chlorella sorokiniana SAG211-8k, *Euglena gracilis* SAG 1224-5/15, *Ochromonas danica* SAG933-7, *Polytomella* sp. Pringsheim SAG 198.80 and *Polytomella* magna SAG 63-9 were purchased from the Culture Collection of Algae in Göttingen (Sammlung von Algenkulturen Göttingen [SAG]; http://www.epsag.uni-goettingen.de). *Polytomella* strains were maintained in liquid HAP cultures at 25°C without light control, while the photosynthetic strains were maintained on HAP agar plates under constant illumination (100

± 10 µmol.m^{-2}.s^{-1}). Five other strains were obtained through isolation as described in section 2.3.

Algae were inoculated in liquid medium prior to growth studies. The algal suspensions were centrifuged (735 g, 10 min), the supernatant discarded and the biomass dispersed in sterile phosphate buffer saline (PBS) medium. In each experiment, the initial microalgal concentration was set to an optical density (OD_{750}) of 0.05. All cultivations were carried out in triplicates, under constant illumination using cool-white fluorescent lamps (100 ± 10 µmol.m^{-2}.s^{-1}) at 25°C under agitation of 120 rpm (2.5 cm orbit). Both *Polytomella* strains were cultivated without shaking and light control. Strain screening and undissociated acid tolerance determination were carried out in microplates as described in [8,22]. Cultivations to evaluate the biomass production potential on synthetic DF effluent (composition described in section 2.5) were carried out in 500 mL Erlenmeyer flasks, filled with 200 mL medium.

2.3. Sampling and isolation procedure of strains from environmental habitats

Three natural peat bogs were sampled in February 2020 in the Occitanie region, France. Two of them were acidic (pH 6), named hereinafter A1 (43°38’47.33’’N/2°34’30.02’’E) and A2 (44°14’45.69’’N/2°56’2.53’’E) and one of them was alkaline (pH 8), named hereinafter B (44°27’8.51’’N/2°0’27.73’’E). Samples were taken from both the water surface (Code WS) and from the first 30-cm of the peat (Code P). Besides peat bogs, two aquatic ecosystems rich in organic matter were sampled, namely a wastewater treatment plant (code WWTP) and a microalgal raceway (code RW) fed with synthetic wastewater. Samples were stored at 4°C until processing (maximum a day after sampling).

Environmental aqueous samples (15 mL) were directly streaked onto HBP agar plates and incubated at 25°C under constant illumination (100 ± 10 µmol.m^{-2}.s^{-1}). After about 10-15 days, green colonies developed. They were picked and streaked on fresh HBP plates. In the

case of peat samples, 100 g of peat were mixed for 5 min with 500 mL isotonic water, and prefiltered on coffee filters to remove large solids. Microorganisms in the filtrate were collected via a second filtration step on a 0.7 µM GF/F Whatman® filter. The filters were subsequently used to inoculate two flasks filled with HAP medium (40 mL), either with 100 $mg.L^{-1}$ ampicillin, in order to prevent excessive growth of bacteria, or without to allow any antibiotic sensitive microalgae to grow. Flasks were then placed under constant illumination (100 $\mu mol.m^{-2}.s^{-1}$) at 25°C and shaking (130 rpm). After 10 days, these enriched cultures were streaked onto HBP agar plates, incubated at 25°C under constant illumination. Colonies were picked based on colour (green) and streaked until purification. All algal strains were maintained on HAP agar plates.

2.4 Molecular identification and phylogenetic analysis of isolates

Microalgal strains were first identified by 18S rRNA sequencing. Total DNA was first extracted from growing cells using the DNeasy PowerWater Kit (Qiagen). The 18S rRNA (400 bp) amplicon was amplified by PCR using the primers 5'-CTTTCCCTACACGACGCTCTTCCATCTGCGGTAATTCCAGCTCCAA-3' (541F) and 5'-GCAGTTCAGACGTGTGCTCTTCCGATCTTTGGCAAATGCTTTCGC-3' (952R). PCR products were purified and sequences by Illumina MiSeq at Genotoul Get-Plage (get.genotoul.fr). After cleaning, sequenced were sorted by OTU with a similarity of 97%. This first identification only allowed identification at the genus level.

To identify strains at the species level, further identification was done. A rapid extraction of total cell DNA from the algal colonies growing on solid media was performed according to Newman et al. [23] with slight modifications. Briefly, cells were collected from plates and resuspended in a 1.5 ml microfuge tube containing 0.2 mL TEN buffer (150 mM

NaCl, 10 mM Na-EDTA, 10 mM Tris-HCI, pH 8.0). Cell lysis was done in 0.4 mL of SDS-EB buffer (2% SDS, 400 mM NaCl, 50 mM EDTA, 100 mM Tris-HCl pH 8) containing RNase A at a final concentration of 40 µg.µL^{-1} and incubated at 37°C for 30 min. DNA was extracted with phenol/chloroform/isoamyl alcohol in a ratio 25:24:1 (v/v/v) and then with chloroform/isoamyl alcohol (24:1 v/v). Total DNA was precipitated by addition of isopropyl alcohol, collected by centrifugation and washed with cold 70% ethanol, air dried and resuspended in TE buffer.

The nuclear sequence spanning the genes for 18S ribosomal RNA-ITS1-5.8S rRNA-ITS2-28S rDNA (500 first bases of 28S) region was amplified by PCR using *Pfu* DNA polymerase (Promega) and the pair of primers EAF3 (TCGACAATCTGGTTCACCTAC)/ITS055R (GTCCTTGGTCCGTGTTTCAAGACGGG) [24]. The PCR products were purified on a QIAquick Gel Extraction Kit (Qiagen) and sequenced directly at Eurofins Genomics (https://eurofinsgenomics.eu) with universal and specific primers. Sequences were submitted to Genbank and accession numbers are provided in Table 1.

A phylogenetic tree was constructed with the MEGA-X program [25] using whole 18S rRNA gene sequences. The MUSCLE module was used to align the DNA sequences using standard parameters. Only the consensus sequence was used, removing highly divergent ends. A Maximum Likelihood tree was produced from this alignment using standard parameters.

2.5. Undissociated acid inhibition thresholds

The tolerance threshold to undissociated acid concentration was determined according to [8]. Briefly, several media were prepared as described in section 2.1, with modifications in pH, buffer and substrate concentration. For each medium, the pH was set at 6.5 and buffered

with 100 mM MES buffer. Increasing total concentrations of either acetate or butyrate were added to the media to obtain the desired range of acetic or butyric acid respectively, determined by the Henderson-Hasselbalch equation (1).

$$[RH] = \frac{C_t}{1+10^{(pH-pKa)}} \quad (1)$$

With [RH] the concentration of either acetic or butyric acid ($g.L^{-1}$), C_t the total concentration of acetate or butyrate ($g.L^{-1}$) and *pKa* the dissociation constant of each acid (close to 4.8 for both acetate and butyrate).

2.6. Biomass production potential

Biomass production potential of candidate strains was evaluated by cultivating them on synthetic DF medium in Erlenmeyer flasks. DF medium was prepared as described in section 2.1., with modifications in buffer and substrate concentration. VFA concentration was set to 1.0 $g_C.L^{-1}$ by mixing 0.33 $g_C.L^{-1}$ (0.8 $g.L^{-1}$) acetate and 0.67 $g_C L^{-1}$ (1,2 $g.L^{-1}$) butyrate, resulting in an acetate:butyrate carbon mass ratio of 1:2. This ratio is representative of an average DF effluent as reported [1]. Buffer concentration was set to 100 mM and pH was set to 7.0. Molar C:N:P ratio was set to 106:16:1 by adjusting the amount of ammonium and phosphate salts ($N\text{-}NH_4$ = 225 $mg.L^{-1}$ and $P\text{-}PO_4$ = 75 $mg.L^{-1}$). Media were sterilized by autoclave (121°C, 20 min).

2.7. Biomass measurement

Biomass growth in microplates was determined via the measurement of the optical density at 750 nm (OD_{750}) by an Infinite Nanoquant M200 (TecanR©) spectrophotometer. Biomass production in flasks was determined by dry weight determination (DW) after

filtration of 2 – 20 mL of samples (depending on biomass concentration) over a dried Whatman© GF/C glass microfiber filter (1.2 µm) and overnight drying at 105°C.

2.8. Volatile fatty acid measurements

Acetate and butyrate concentrations in the growth media were quantified by gas chromatography. Cultures were sampled at indicated growth times, and centrifuged at 15,000 rpm for 10 min. Supernatants were collected, filtered through a 0.45 µm filter 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 $g.L^{-1}$). VFAs were analyzed on 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).

2.9. Lipids and sugars measurements

Samples from fresh cultures were immediately centrifuged, the supernatant was discarded and the pellet stored at -20°C until use. Prior to analysis, the pellets were thawed, resuspended in distilled water and transferred to a 10 mL glass tubes for either lipid or sugar measurement.

Total lipids were measured by the phosphovanillin method [26]. Phosphovanillin reagent was freshly prepared before analysis by dissolving 0.6 g vanillin in 10 ml ethanol, 90 ml deionized water and 400 ml of H_3PO_4 (85%). The resulting reagent was stored in the dark until use. First, 2 mL of H_2SO_4 (98%) were added in the tubes containing microalgae samples. The tubes were heated 10 min at 100°C. After cooling on ice, the reaction was initiated by addition of 5 mL of phospho-vanillin reagent prior incubation for 15 min at 37°C. Tubes were

periodically shaken by inversion and were then left in the dark at room temperature for 45 min for color development. After cooling, absorbance of suspensions was measured at 530 nm with an Aqualytic® spectrometer and compared to distilled water. Calibration curves were obtained using canola oil.

Total sugars were measured by the anthrone method [27]. Anthrone reagent was prepared by dissolving 200 mg of anthrone in 100 mL of H_2SO_4 (98%). Two mL of anthrone reagent were added in the tubes containing microalgae. Tubes were cooled down on ice and then incubated at 100°C for 10 min. After cooling, absorbance of suspensions was measured at 625 nm with an Aqualytic® spectrometer and compared to distilled water. Calibration curves were obtained using glucose solution.

3. Results and discussion

3.1. Isolation and identification of microalgae strains

The first strategy followed to isolate environmental microalgae able to use butyrate as carbon source was to explore natural ecosystems featuring characteristics similar to anaerobic fermenters. Therefore, samples were taken from different wetlands (peatbogs), which are organic rich ecosystems flooded by water either seasonally or permanently. The high water table limits gas exchanges and makes the ecosystem largely anoxic. Organic matter is thus degraded through anaerobic bioprocesses leading to fermentative by-products such as acetate, butyrate or hydrogen [28]. In addition, aqueous samples were taken from artificial ecosystems: WWTP activated sludge and biomass from a microalgae raceway fed with synthetic wastewater. Although mostly aerobic, these open ecosystems contain high amount of organic matter. It was expected that microalgae isolated from these environments would exhibit the ability to compete for organic carbon to some extent with bacteria (and thus high growth rate) and tolerance to organic carbon. From all five environmental samples, green

algal colonies developed on butyrate-containing plates. Colonies were first identified by metabarcoding (MiSeq). The OTU could not allow identification to the species level but allowed genus discrimination. It could be concluded that each isolate contained a single genus and that all five algae belong to the Chlorophyte division. Thus, a total of five different algal strains could be obtained as pure culture, after enrichment on a butyrate containing medium (Table 1).

Table 1: Summary of the isolated species and their identification. Isolates were sampled from different peatbogs water surface or peats, a local wastewater treatment plant (WWTP) sludge and a local microalgal raceway. Identification was done by sequencing the nuclear ribosomal DNA operon and BLAST searches.

Strain code	Sampling source	Closest strain	Sequence alignment
Chlamydomonas asymmetrica A2WS (2993 bp) (ON077054)	Peatbog water surface	*Chlamydomonas asymmetrica* CCAP 11/41 (2557 bp) FR865583	2524/2557
Chlorella sorokiniana A1WS (3070 bp) (ON077055)	Peatbog water surface	*Chlorella sorokiniana* NIES:2173 (2487 bp)	G1826 T (ITS1)
Chlorella sorokiniana BP (3070 bp) (ON077056)	Peatbog peat	*Chlorella sorokiniana* SAG 211-8k (2461 bp) FMZ05834	Identical to the lab strain
Desmodesmus armatus WWTP (2955 bp) (ON077057)	WWTP sludge	*Desmodesmus armatus* GM4k (2591 bp) AB17138	A deletion T 1803 ITS1
Desmodesmus armatus RW (2955 bp) (ON077058)	Microalgal raceway		

However, the 400 bp amplicon did not allow precise identification of each species. The rDNA operon has emerged as a tool for species delimitation, and hence, identification [29]. To identify at the molecular level all isolated algal species, the nuclear sequence of the rDNA operon (spanning the 18S rDNA, ITS1, 5.8S, ITS2 and the 28S rDNA regions) was thus amplified and sequenced. From the peat bogs were isolated two algae closely related to *Chlorella sorokiniana* (Trebouxiophyceae) SAG 211-8k strain, used routinely in the laboratory. The rDNA sequence of the isolate *C. sorokiniana* BP was identical to this reference strain while the isolate *C. sorokiniana* A1WS differs in one single base located in the ITS1 (Table 1). The third algal species isolated from peat bogs was identified as *Chlamydomonas asymmetrica* (Chlorophyceae). From the artificial ecosystems (WWTP sludge and raceway) were isolated two algae identified as *Desmodesmus armatus*. These two species differ from the *D. armatus* strain GM4k SAG in one nucleotide deletion in the ITS1 region. Due to the very high sequence identity of the isolated strains with published strains over the whole length of the rDNA operon including the highly variable ITS regions, species identification was highly confident (BLAST e-value=0).

Overall, very little diversity was obtained throughout the isolation process since all isolated algae belong to only three species of Chlorophytes. Yet, other species such as desmidiae and diatoms were detected during microscope observation of the environmental samples and greater diversity in peat bog samples was already reported elsewhere in the literature [30]. It is thus possible that the media HAP or HBP used for the enrichment from the environmental samples are relatively selective for Chlorophytes. For example, using acetate containing media, similar results were obtained by other authors who isolated four *C. sorokiniana* strains from a local wetland (Lake Massaciuccoli located in Tuscany, Italy) [30,31]. Implementing HAP/HBP media with silica might have allowed the emergence of diatoms. Besides, the other major nutritive elements, N and P, were added in the same form

that the one found in DFEs, respectively NH_4^+ and PO_4^{3-}, consequently adding an extra selection pressure. C*hlorella* and *Desmodesmus* are reported as "lab weeds" due to their ability to grow over a wide range of cultivation conditions [32]. These strains are thus often isolated from various ecosystems and with various selection conditions [31,33,34]. It would be undeniably very interesting to use a comprehensive isolation procedure in order to obtain a maximum of species capable of growing or tolerating butyrate. Nevertheless, in the context of microalgae cultivation on DFE effluent, some specific constraints such as the one evoked above (chemical form of the nutrients, absence of certain element) should still be considered. Therefore, other strains obtained via an extensive screening, which would be extremely efficient at consuming butyrate in specific growth condition, may not be adapted to grow on the more adverse and variable conditions of a DFE.

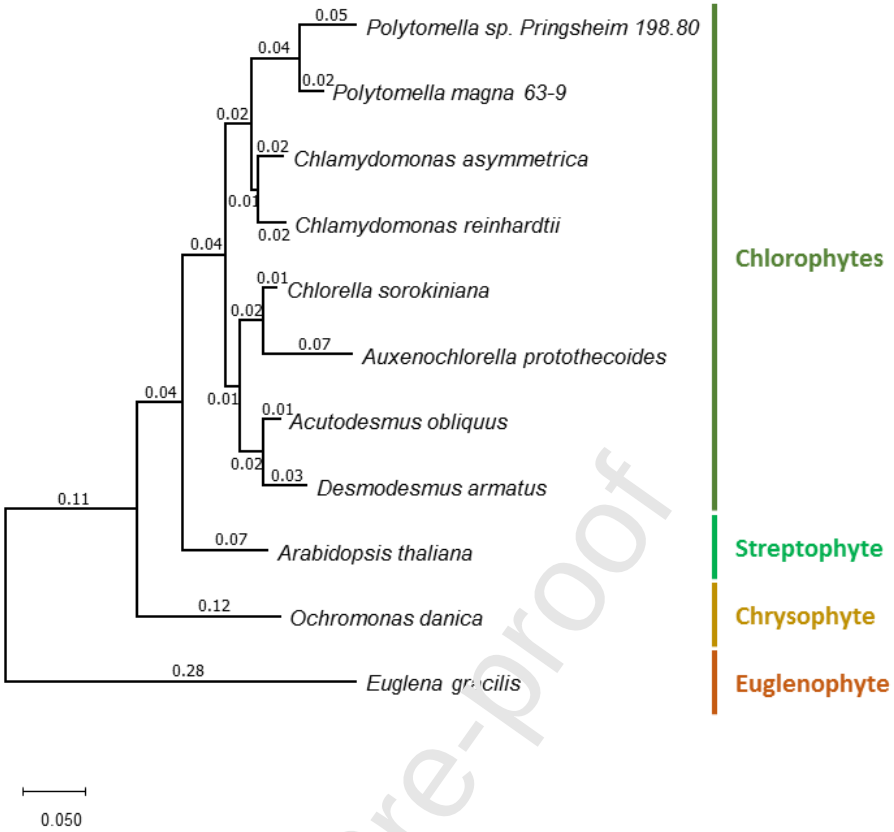

Figure 1: Maximum Likelihood tree on the investigated species. Since isolate sequences are nearly identical to reported sequences and will not show on the tree, only the reported sequences were included in the tree. Accession numbers of the sequences used were: *Polytomella* sp., FR750564.1; *P. magna*, KP299175.1; *C. asymmetrica*, FR865607.1; *C. reinhardtii*, FR865575.1; *C. sorokiniana*, LK021940.1; *A. protothecoides*, LN610701.1; *A. obliquus*, AB917100.1; *D. armatus*, AB917138.1; *A. thaliana*, X16077.1; *O. danica*, JQ281514.1; *E. gracilis*, AJ532426.1.

As can be seen on the Maximum Likelihood tree (Fig. 1), all isolated species were closely related to each other and to other model strains such as *Acutodesmus obliquus*,

Auxenochlorella protothecoïdes or *Chlamydomonas reinhardtii.* These model strains were previously investigated for their ability to grow on various VFAs and shown to poorly assimilate butyrate [6,8]. Therefore, we also included other species, namely *Euglena gracilis*, *Ochromonas danica*, *Polytomella sp.* and *Polytomella magna* in our screening. Both *Polytomella* strains are non-photosynthetic Chlorophytes closely to *C. reinhardtii* which rely on heterotrophic growth [18]. *E. gracilis* and *O. danica* belong to the very distinct Euglenophyte and Chrysophyte divisions, respectively. *E. gracilis* and *O. danica* have been shown earlier to grow on various organic compounds in heterotrophy [35,36]. Microscopy images of all strains are given in the supplemental figure.

3.2. Polytomella sp. stands out as the fastest butyrate consuming strain

The growth of the environmental strains as well as the four selected strains obtained from the SAG collection was compared to that of our reference strain *C. sorokiniana* SAG 211-8k in the light. From the growth curves on each separate substrate (not shown), growth and substrate consumption rates on either acetate (Fig. 2) or butyrate (Fig. 3) were determined.

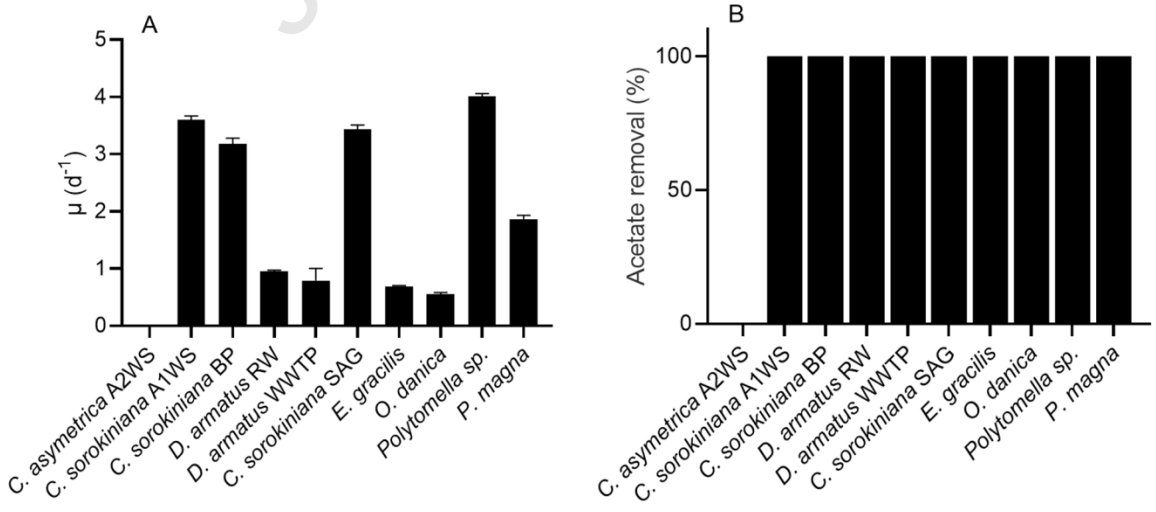

Figure 2: Strain screening on acetate. Growth rates (μ, d^{-1}) (A) were calculated from the exponential phase while acetate removal (%) (B) was calculated at the end point of cultivation. Strains were cultivated in microplates on 0.5 $g_C.L^{-1}$ acetate at pH 7 under constant illumination and 100 rpm agitation for a maximum of 3 days, except for *Polytomella* strains. All data are given as mean and standard deviation of three biological replicates.

Each isolated strain except for *Chlamydomonas asymmetrica* A2WS could grow on acetate and reach stationary phase in less than 3 days. The fact that this strain could not grow in liquid media could indicate that it was likely isolated due to its robustness and tolerance to adverse conditions (presence of bacteria and butyrate). Its growth on agar plates may have been favoured by the presence of bacteria, which could consume the VFA and increase local CO_2 concentration but was not due to direct VFA consumption. The other strains exhibited growth rates between 0.84 ± 0.04 to 3.60 ± 0.07 d^{-1}, averaging at 2.0 d^{-1} (Figure 2A). The strain *C. sorokiniana* SAG 211-8k exhibited a growth rate of 3.60 ± 0.07 d^{-1} on acetate, a value in accordance with previous work with this strain [22]. The isolated strains *C. sorokiniana* A1WS and BP show the same growth rate as the reference strain. The growth rate on acetate of the *D. armatus* strains was significantly lower (factor 3-4) than that of the *C. sorokiniana* strains. Similarly, the growth rate of *E. gracilis* and *O. danica* was respectively 5.0- and 6.2-fold lower than that of *C. sorokiniana*. In contrast, the heterotrophic chlorophyte *Polytomella* sp. exhibited a growth rate of 4.01 ± 0.05 d^{-1} on acetate, significantly higher than any other strain. However, *Polytomella magna* growth rate was around 2-fold lower than that of *Polytomella* sp.

Acetate was always entirely consumed in 3 days (Fig. 2B). Qualitative consumption of acetate by *E. gracilis, O. danica* and *Polytomella* species has been reported by several other authors but few quantitative data are available. Some authors reported for *E. gracilis* a growth rate of 1.08 d^{-1} on glucose and 0.6 d^{-1} on ethanol, comparable to the 0.6 d^{-1} here on acetate

[37]. Similarly, a growth rate between 0.45 and 0.75 d^{-1} was determined for *O. danica* on glucose and acetate, in line with our results [35].

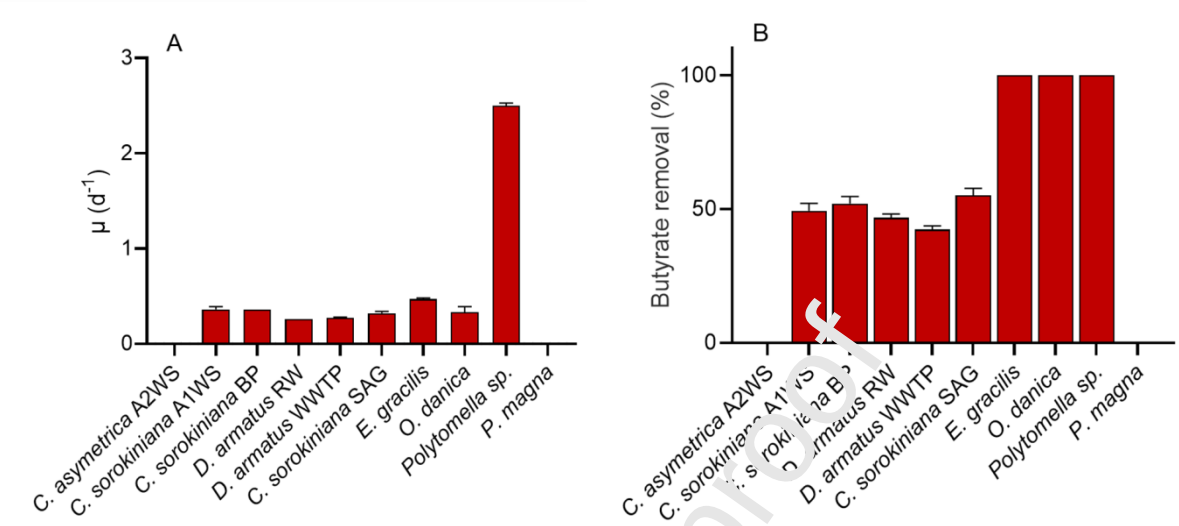

Figure 3: Strain screening on butyrate. Growth rates (μ, d^{-1}) (A) were calculated from exponential phase while butyrate removal (%) (B) was calculated at the end point of cultivation. Strains were cultivated in microplates on 0.5 $g_C.L^{-1}$ butyrate at pH 7 under constant illumination and 100 rpm agitation for a maximum of 7 days, except for *Polytomella* strains. All data are given as mean and standard deviation of three biological replicates.

On the other hand, use of butyrate as substrate always induced a reduction in growth rate compared to acetate (Fig. 3A). As observed for acetate, no growth could be detected on butyrate for *C. asymetria* A2WS. Growth rate of *C. sorokiniana*, either the isolates or the lab strain, was reduced by 10-fold, in line with previous results [22]. Growth rates of *E. gracilis*, *O. danica* and *Polytomella* sp. were however only reduced by 32%, 40% and 37% respectively. It should still be highlighted that *Polytomella* sp. exhibited a much higher growth rate than any other strain on butyrate, topping at 2.5 d^{-1}. Furthermore, these three strains always consumed butyrate entirely within 7 days, with complete butyrate consumption in less than 2.5 days by *Polytomella* sp. In comparison, all other photosynthetic strains

consumed around 50% of butyrate in 7 days (Fig. 3B). Interestingly, *Polytomella magna* did not grow at all in presence of butyrate, indicating that butyrate consumption within the *Polytomella* genus is species specific. Of note, *P. magna* remains evolutionary closer to the *Chlamydomonas*-like photosynthetic ancestor of these colorless species [17], and has retained some metabolic capacities such as β-carotene synthesis in contrast to other *Polytomella* species [38]. Where its closest green relative *C. reinhardtii* can use only acetate efficiently [39], *Polytomella* sp. has evolved towards a heterotrophic lifestyle with the capacity to use a variety of organic substrates, notably C2-C5 VFAs, alcohols, pyruvate and citric acid cycle intermediates [40,41]. Although very little is known on the pathways involved in the assimilation of butyrate or other organic substrates in *Polytomella* sp., major differences in metabolic enzymes have been described especially on the level of the plastid [42,43] and mitochondria [44–47] that likely reflect adaptation to heterotrophy and which may guide further studies on this topic.

3.3. Polytomella sp. stands out as the most tolerant strain to undissociated acid

The tolerance to undissociated acid concentration (RH) of the strains able to grow in the initial screening was then assessed since this was shown as a determining factor in growth inhibition on VFAs [8]. For this, the same protocol as described in previous study was followed [8]. Briefly, all strains were cultivated on increasing concentrations of acetic (AcH) or butyric (BuH) acid at a fixed pH and growth rates were monitored. This protocol enabled to determine for each strain an RH concentration threshold above which growth was completely inhibited (Fig. 4 and table 2).

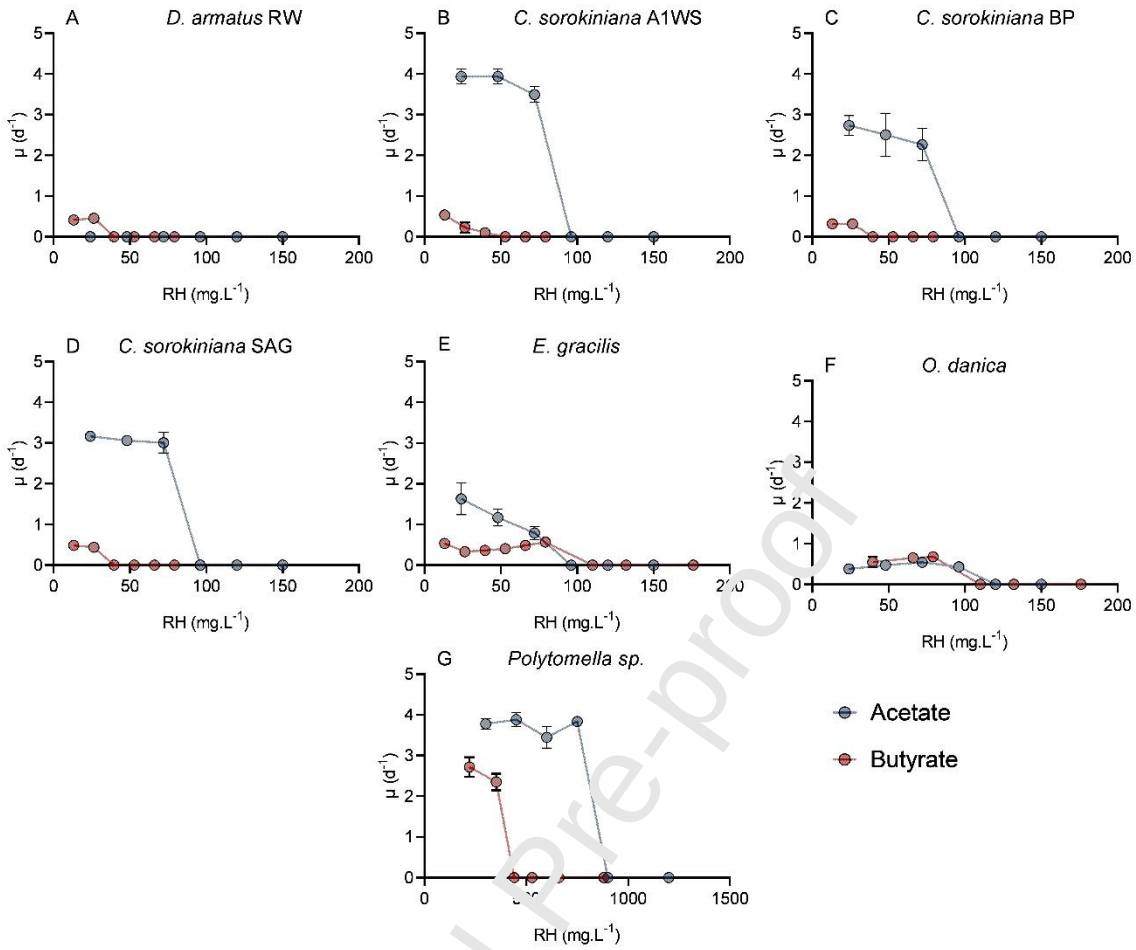

Figure 4: Determination of the undissociated acid tolerance of each strain. The figures represent growth rates (μ, d^{-1}) of (A) *D. armatus* RW, (B) *C. sorokinana* A1WS, (C) *C. sorokiniana* BP, (D) *C. sorokiniana* SAG, (D) *E. gracilis*, (E) *O. danica* and (F) *Polytomella* sp. cultivated on increasing undissociated acid concentration (RH, $mg.L^{-1}$) of acetate (blue) or butyrate (red). Growth rates were calculated from exponential phase and RH concentration using the Henderson-Hasselbalch equation. Strains were cultivated in microplates under constant illumination for a maximum of 7 days. All data are given as mean and standard deviation of three biological replicates.

The isolated strains, *C. sorokiniana* A1WS and BP presented a very similar behaviour to the lab strain *C. sorokiniana* SAG 211-8k (Fig. 4A, B, D). These three strains were

completely inhibited above 72 mg.L^{-1} AcH (12 g.L^{-1} total acetate at pH 7) and 26 mg.L^{-1} BuH (4 g.L^{-1} butyrate at pH 7) (Table 2). The isolate *D. armatus* RW showed a lower tolerance to the acids, being completely inhibited below 26 mg.L^{-1} AcH (4 g.L^{-1} acetate at pH 7) (Fig. 4C). Thus, in line with the fact that isolated strains did not exhibit growth rates much higher than those of the lab strain, the isolates did not exhibit any particular resistance to AcH or BuH compared to the lab strain *C. sorokiniana*. The isolation strategy did thus not allow isolating a strain more resistant to the acids. The medium pH during the isolation process was set between 7 and 8, in order to effectively minimize RH concentrations. This choice was based on the hypothesis that the tolerance of a specific strain to RH concentration is not necessarily correlated to fast substrate consumption. Thus, raising the pH ensured growth of any microalgae strain in the starting inoculum, even at a low cell density. Therefore, although this strategy does select for fast growth as competitive parameter, it did not result in the selection of any strain showing higher tolerance to RH.

Table 2: Maximum undissociated acid (RH) concentration for which microalgal growth is not entirely inhibited as determined in Fig. 4.

Strain	RH threshold (mg.L^{-1})	
	Ac	Bu
D. armatus RW	0	26
C. sorokiniana A1WS	72	39
C. sorokiniana BP	72	26
C. sorokiniana SAG	72	26
E. gracilis	72	79
O. danica	96	79
Polytomella sp.	750	352

On the other hand, inhibition threshold of *E. gracilis, O. danica* and *Polytomella sp.* exhibited distinct tolerance characteristics compared to *C. sorokiniana* (Fig. 4D-G). *E. gracilis* presented a similar tolerance to AcH as *C. sorokiniana* (72 $mg.L^{-1}$ AcH) while *O. danica* presented a slightly higher tolerance, up to 96 $mg.L^{-1}$ AcH (Fig. 4D-F). However, both strains tolerated a concentration of BuH 3-fold higher than that of *C. sorokiniana* (79 $mg.L^{-1}$ BuH) (Fig. 4D-F) (Table 2). An acetate concentration above 1.0 $g.L^{-1}$ has been reported to trigger the cell death of *O. danica* [35]. Similarly, when formulating a maintenance medium for *O. danica*, acetate was found inhibitory to its growth [48]. The knowledge of the inhibition threshold allows interpreting these differences. In their studies, pH was set to 5.0. At this pH, the AcH is 387 $mg.L^{-1}$ for 1 $g.L^{-1}$ acetate, much higher than the determined threshold of 79 $mg.L^{-1}$ of the present study. In the same way, optimal growth of *E. gracilis* has been reported to occur at acidic pH, between 3 and 5 [37] but acetate addition at this pH was found to completely inhibit cell growth [49]. Considering the inhibition threshold of *E. gracilis* around 75 $mg.L^{-1}$, the minimum pH tolerated by the cells for 1.0 $g.L^{-1}$ total acetate would be 6. Therefore, optimum pH for growth does not necessarily coincide with the minimum pH necessary to avoid intolerance to RHs. The knowledge of this threshold value is thus essential to determine both the adequate growth pH and total acid concentration to avoid inhibition. Lastly, *Polytomella sp.* presented exceptional resistance to both acids (Fig. 4G, Table 2). The strain indeed conserved a stable growth rate around 3.8 d^{-1} up until 750 $mg.L^{-1}$ AcH, which corresponds to 38 $g.L^{-1}$ of total acetate at pH 6.5. Similarly, it tolerates BuH concentrations of 350 $mg.L^{-1}$ (18 $g.L^{-1}$ total butyrate), which is three times higher than those tolerated by *E. gracilis* or *O. danica* and about ten times higher than those tolerated by *C. sorokiniana*. The tolerance of *Polytomella* sp. to RH concentrations up to 10 times higher than the other strains is a remarkable property of the strain.

To our knowledge, the organisms most tolerant to RH are fermentative bacteria or yeast, which can tolerate AcH concentration as high as 9 $g.L^{-1}$. These organisms have evolved various tolerance mechanisms to withstand the deleterious effects of the acids, such as a higher expression of ATP-H^+ pumps [50,51] or by letting their internal pH decrease [52]. The yeast *S. cerevisiae* can also adapt membrane morphology to reduce acid diffusion during extended stress period [53]. *Polytomella* sp. has been firstly isolated from moderately acidic organic rich environments with high fermentative bacterial activity [54]. It is thus likely that the strain developed similar tolerance mechanisms to the fermentative metabolites such as acetate and butyrate. The higher adaptation to VFAs (and probably other organic acids) may be related to its acidophilic nature. At lower pH the organic acids are more present in the RH form and liable to diffuse into the cell [52,55]. This will exert a higher selective pressure to acquire tolerance mechanisms such as found in yeast, creating favourable conditions to subsequently develop heterotrophic capacities. Their newfound heterotrophic capacities, or pre-existing ones as the case may be, will serve them as competitive advantage since they can occupy a larger (acidic) pH range before organic acids become toxic to them. Among other processes, acidophilic species can grow at low pH by being able to maintain their internal pH close to neutrality due to a higher expression of ATP-H^+ pumps, which in turn has been shown to be related to a higher tolerance to RH [50,56]. However, our results indicate that the property of a strain to tolerate acidic pH is not necessarily linked to its ability to withstand RH. This was also shown for another algal species, *Dunaliella acidophila*, which can grow at pH levels close to 1. Its photosynthetic activity was still reduced by 50% by AcH concentrations as low as 0.033 $mg.L^{-1}$ [56]. However, *D. acidophila* is purely autotrophic and cannot assimilate acetate. It is possible that lower tolerance to RH is linked to the photosynthetic activity of a strain, which could partially explain the difference between *E. gracilis*, *O. danica* and *Polytomella* sp. Since no photosynthetic species are known that are as

tolerant to RH as *Polytomella* sp., and this alga is the only one that is non-photosynthetic, it should be further investigated whether photosynthetic activity is less compatible with high RH tolerance. If that happens to be the case, it may be proposed that the *Chlamydomonas*-like photosynthetic ancestor of *Polytomella* could only acquire more heterotrophic capacities after losing photosynthesis, instead of before.

3.4. Growth of candidate strains on synthetic DF effluent

Given the results in terms of growth rates and tolerance to butyrate, *E. gracilis*, *O. danica* and *Polytomella* sp. were chosen for further studies to evaluate their biomass production potentialities on synthetic DF effluent and to compare them with *C. sorokiniana* chosen as the reference alga. These four strains were cultivated in 500 mL Erlenmeyer flasks on a synthetic mixture of VFAs composed of acetate and butyrate, at a carbon ratio of 1:2 representing an average DF effluent in terms of VFAs. For each strain, biomass growth, VFA consumption and lipids and sugars productions were determined.

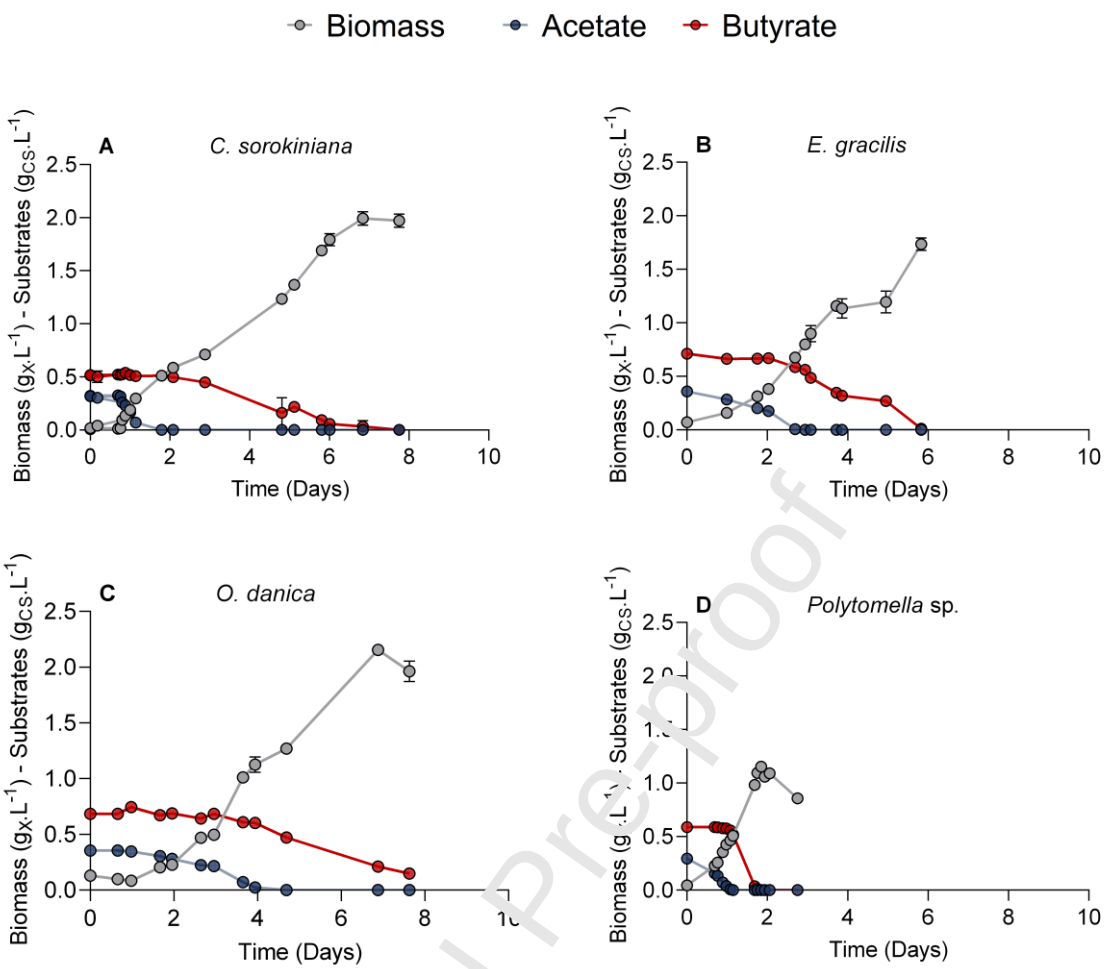

Figure 5: Growth of candidate strains on synthetic DFE. The figures represent biomass dry weight ($g_X.L^{-1}$, grey circles) and substrate concentration ($g_{CS}.L^{-1}$, acetate in blue circles and butyrate in red circles) evolution as a function of time (days) in (A) *C. sorokiniana* SAG, (B) *E. gracilis*, (C) *O. danica* and (D) *Polytomella* sp. cultures. Strains were cultivated in flasks in synthetic medium (0.3 $g_{CS}.L^{-1}$ acetate (Ac) and 0.7 $g_{CS}.L^{-1}$ butyrate (Bu); pH 7) under constant illumination and shaking. *Polytomella* species were cultivated without light control and shaking. All data are given as mean and standard deviation of three biological replicates.

Each strain could grow on the synthetic DF effluent, and growth curves and substrate consumption of each strain are provided in Figure 5. For each strain, two growth phases could be distinguished, attributable to the substrate assimilated. Acetate was always consumed first, followed by butyrate. This phenomenon, known as diauxic growth or carbon catabolite

repression, was already been reported for *C. sorokiniana* and *Auxenochlorella protothecoïdes* growing on acetate and butyrate mixtures [9,22]. The results further highlight this effect and confirm that it occurs for other microalgal strains. However, it should be noted that *O. danica* did consume a fraction of butyrate (12.5 ± 1.6%) before complete acetate exhaustion. As a comparison, *C. sorokiniana*, *E. gracilis* et *Polytomella* sp. respectively only consumed 2.0 ± 4.6, 5.7 ± 4.5 and 1.9 ± 1.6 % initial butyrate, without significant difference, before complete acetate exhaustion. The half inhibitory constant of *C. sorokiniana* associated to diauxic growth on the acetate and butyrate mixture has been determined in a previous study [9]. They found a value of 2.10^{-10} $g_C.L^{-1}$, meaning that the consumption of butyrate for this strain is inhibited for extremely low acetate concentration. The fact that *O. danica* started to consume butyrate slightly before complete acetate exhaustion indicates that its half inhibitory constant is higher than that of *C. sorokiniana*. This suggests differences at the metabolic level in the responses to the presence of both substrates between the various strains.

Considering this diauxic effect, each growth phase was considered individually to calculate growth rates and yields (Table 3). During the acetate phase, *C. sorokiniana* presented the highest growth rate (3.34 ± 0.17 d^{-1}) followed by *Polytomella* sp. (2.21 ± 0.04 d^{-1}). These two strains consumed acetate within only two days. On the other hand, acetate was exhausted in almost four days by *O. danica*, making it the slowest acetate consuming strain. After acetate exhaustion, butyrate started to be consumed. Growth rate on this substrate was always slower than on acetate. *Polytomella* sp. was the fastest butyrate consuming strain, exhibiting a growth rate of 1.20 ± 0.04 d^{-1} during this phase. It should be noted here that the growth rates obtained in this experiment for *Polytomella* sp., in presence of both substrates, are around 2-fold lower than those obtained with the separate substrates (section 3.2). One explanation is that butyrate would inhibit growth on acetate. However, butyrate concentration was here only 1.2 $g.L^{-1}$, corresponding to 7.5 $mg.L^{-1}$ BuH at pH 7.0. BuH concentration was

therefore around 50-times lower than the inhibitory threshold determined in section 3.3. Furthermore, growth rates obtained for other strains were similar between the two experiments. For *Polytomella* sp., the most probable explanation lies in the different cultivation vessel. The screening was indeed carried out in microplates while the biomass production potential was carried out in Erlenmeyer flasks. Although it was confirmed that growth rates between microplates and shake flasks were similar for *C. sorokiniana* [22], no such validation was done for *Polytomella* sp. *Polytomella* cells are generally cultured without any shaking since the cells are relatively fragile (absence of cell wall) and shaking can inhibit growth [57]. During growth, *Polytomella* cells swim along a water column between the bottom of the vessel and the medium surface in order to oxygenate themselves [19]. These movements will depend on the depth of the vessel. Since medium volume is much lower in microplates, it is possible that cells could oxygenate faster in this vessel, thus exhibiting a higher growth rate. All of this considered, *Polytomella* sp. remained the fastest strain on the synthetic effluent since substrate was entirely consumed after two days against a minimum of six days for other strains. Additional gentle shaking or aeration system might further speed up organic consumption and should be considered for further studies with this strain.

Table 3: Growth characteristics of candidate strains on synthetic DFE (0.3 $g_C.L^{-1}$ acetate (Ac) and 0.7 $g_C.L^{-1}$ butyrate (Bu); pH 7). Biomass yields ($Y_{X/S}$, $g_X.g_{CS}^{-1}$) and growth rates (μ, d^{-1}) were calculated for each substrate growth phase. Biomass productivities (Q_X, $g_X.L^{-1}.d^{-1}$) were calculated considering the whole cultivation duration. Values are given as mean and standard deviation for three biological replicates.

Strain	$Y_{X/S}$ ($g_X.g_{CS}^{-1}$)		μ (d^{-1})		Q_X ($g_X.L^{-1}.d^{-1}$)
	Ac	Bu	Ac	Bu	Global
C. sorokiniana SAG	1.58 ± 0.16	3.03 ± 0.6	3.34 ± 0.17	0.30 ± 0.01	0.64 ± 0.10
E. gracilis	1.71 ± 0.06	1.66 ± 0.08	0.85 ± 0.01	0.38 ± 0.09	0.30 ± 0.15
O. danica	3.40 ± 0.33	3.40 ± 0.54	0.81 ± 0.04	0.28 ± 0.03	0.18 ± 0.01
Polytomella sp.	1.46 ± 0.12	1.24 ± 0.03	2.20 ± 0.18	1.28 ± 0.01	0.62 ± 0.04

At the end of the acetate phase, *O. danica* exhibited the highest biomass yield (3.40 ± 0.33 $g_X.g_{CS}^{-1}$). By using the intracellular carbon content of *O. danica* (2.02 pmol per cell growing in mixotrophy) [15] and the dry biomass quantity as a function of cell number (between 3.7 and $5.5.10^{-11}$ $g.cell^{-1}$) [35], it was possible to estimate the C content of *O. danica* per biomass dry weight at an average of 57.9%. The carbon biomass yield on substrate of *O. danica* would thus be as high as 1.96 $g_{CX}.g_{CS}^{-1}$. This would mean that a large amount of CO_2 was photosynthetically fixed by the cells during growth.

For the three other strains, biomass yields were between 1.46 and 1.71 $g_X.g_{CS}^{-1}$ without significant difference (Table 3). By estimating the biomass carbon content to be of 53%, close to that estimated for *C. reinhardtii* [58], a carbon yield on substrate was calculated to be of 0.77 $g_{CX}.g_{CS}^{-1}$ for *Polytomella* sp. Unlike the other strains, *Polytomella* sp. is a pure heterotroph strain unable to perform photosynthesis. Therefore, the amount of biomass produced can only originate from organic carbon assimilation. No quantitative data are available for the growth of *Polytomella* sp. and this value can thus be only compared to other strains grown in heterotrophy. This value is similar to that obtained for the close relative *C. reinhardtii* in heterotrophy on acetate by [58]. By using the same carbon content estimation for *C. sorokiniana*, the carbon biomass yield on substrate for this strain was also calculated to

be 0.76 $g_{CX}.g_{CS}^{-1}$ (Table 3). Since the strain is photosynthetic and can thus photosynthetically fix CO_2, a higher biomass yield was expected. This could mean that either *C. sorokiniana* grew only in heterotrophy on acetate, without fixing CO_2 or that the heterotrophic yield of *Polytomella* sp. is higher than that of *C. sorokiniana* on acetate. This latter hypothesis would be consistent with previous results [9,59], who reported for *C. sorokiniana* a biomass yield of 0.4 $g_{CX}.g_{CS}^{-1}$ and 0.5 $g_{CX}.g_{CS}^{-1}$ respectively on acetate in heterotrophy. Here, the use of mixotrophic condition increased the biomass yield of *C. sorokiniana* to 0.76 $g_{CX}.g_{CS}^{-1}$ by allowing the strain to photosynthetically fix atmospheric CO_2.

In the case of *E. gracilis*, a biomass yield of 1.71 $g_X.g_{CS}^{-1}$ was obtained on acetate. No yield on acetate was reported so far due to the inhibitory effects at low pH discussed previously. However, some data are available on ethanol and lactic acid. Fujita et al., [36] reported for *E. gracilis* in mixotrophy a value of 0.77 $g_X.g_{CS}^{-1}$ on ethanol but a value of 1.33 $g_X.g_{CS}^{-1}$ on lactic acid, which is in the same order of magnitude of what was obtained here [36]. This would mean that VFAs, such as acetate, butyrate or lactate are better substrates for *E. gracilis*, as long as inhibitory concentrations are avoided.

At the end of the butyrate phase, *Polytomella* sp. presented a yield significantly lower than obtained on acetate (1.24 ± 0.03 $g_X.g_{CS}^{-1}$ against 1.46 ± 0.12 $g_X.g_{CS}^{-1}$ on acetate). This indicates that although the strain can quickly consume butyrate, its assimilation requires more energy than acetate since carbon is diverted from biomass production. On the other hand, the biomass yield of *E. gracilis* and *O. danica* did not change significantly compared to the acetate phase (respectively 1.66 ± 0.08 $g_X.g_{CS}^{-1}$ and 3.39 ± 0.54 $g_X.g_{CS}^{-1}$) while the yield of *C. sorokiniana* drastically increased up to 3.0 ± 0.6 $g_X.g_{CS}^{-1}$. As these strains can perform photosynthesis, it is probable that light could be used as an extra energy source to uptake and metabolize butyrate. The carbon biomass yield of *C. sorokiniana* on butyrate, 1.6 $g_{CX}.g_{CS}^{-1}$, indicates that a much higher fraction of CO_2 is photosynthetically fixed by the strain during

butyrate consumption compared to growth on acetate. This is due to the low growth rate on butyrate, only slightly higher than the autotrophic growth rate at atmospheric CO_2 levels [22].

3.5. Production of lipids and carbohydrates by the candidate strains

As lipids and carbohydrates production is the main incentive for microalgal cultivation on waste streams, the concentration of these two products were measured during growth (Figure 6). The corresponding productivities and yields are reported in table 4. Microalgal lipids and sugars were respectively quantified by the phosphovanilin and the anthrone method, successfully used previously for different microalgal strains, including chlorophytes [26,60,61] or traustochytrids [62], with little deviation compared to standard gravimetric protocols. Some factors may interfere with the accuracy of these colorimetric approaches, such as the type and concentration of pigments, the type of lipid or carbohydrate analyzed or the standard used to establish the calibration curves [61]. Although results may be validated using gravimetric methods, these approaches are generally considered appropriate as a first estimate of the relative amount of each storage compound between strains.

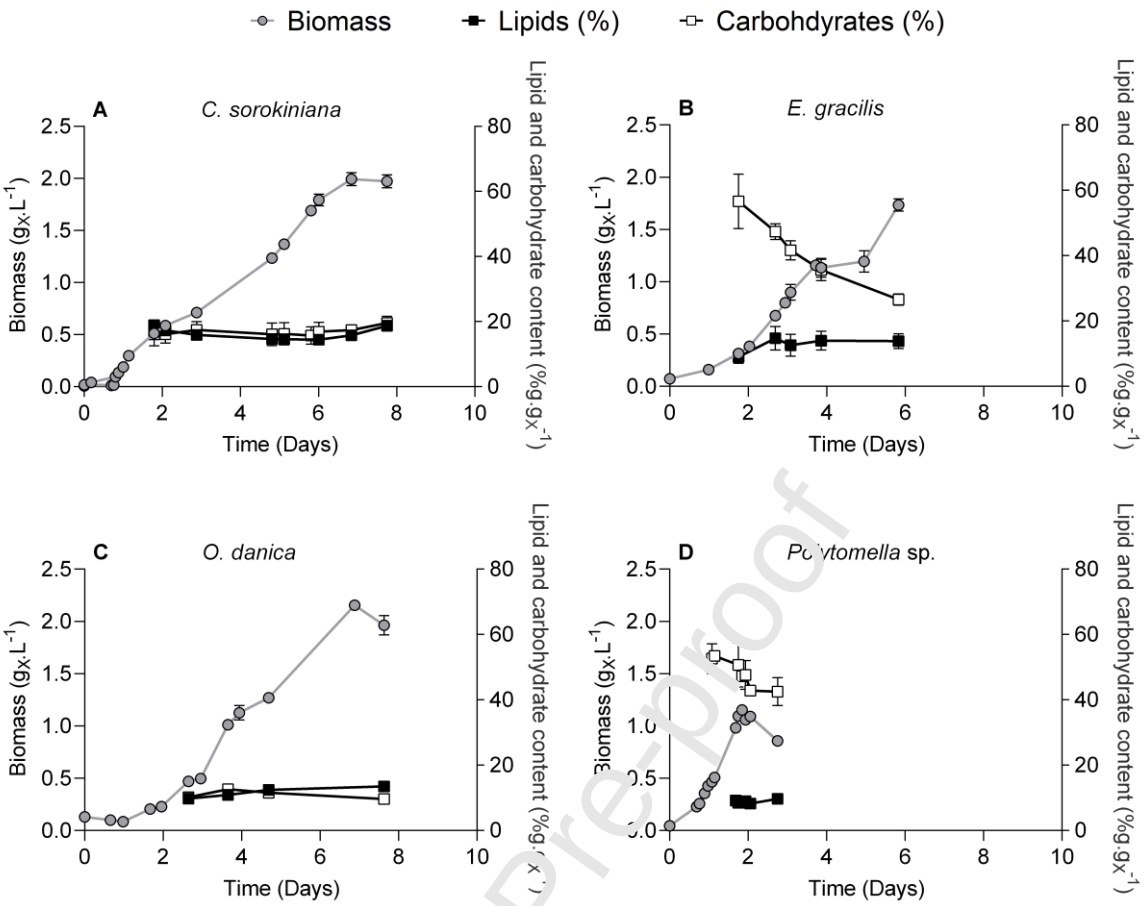

Figure 6: Carbohydrates and lipids production by candidate strains on synthetic DFE. The figures represent biomass dry weight ($g_X.L^{-1}$, grey circles) and product content in each strain ($\%g.g_X^{-1}$, carbohydrates in white squares and lipids in black squares) evolution as a function of time (days) in (A) *C. sorokiniana* SAG, (B) *E. gracilis*, (C) *O. danica* and (D) *Polytomella* sp. cultures. Strains were cultivated in flasks in synthetic medium (0.3 $g_{CS}.L^{-1}$ acetate (Ac) and 0.7 $g_{CS}.L^{-1}$ butyrate (Bu); pH 7) under constant illumination and shaking. All data are given as mean and standard deviation of three biological replicates. Lack of data points in early phase indicates a product concentration below quantification limit due to lack of sufficient biomass.

The highest carbohydrates content was found for *Polytomella* sp. and *E. gracilis* (52.6 ± 4.5% and 53.5 ± 11.2% respectively) at the end of the acetate phase. This result was

expected since both strains are known for accumulating starch for the former [19] and paramylon for the latter [63]. However, both strains differed in their accumulation dynamics. The carbohydrates content of *Polytomella* sp. remained stable as long as organic substrate was present in the medium. Its content did decrease progressively down to 35.7 ± 3.9% once organic carbon had been completely exhausted. This is because *Polytomella* sp. can only rely on organic carbon for growth: in its absence, the cells consume stored carbon (*i.e.* starch) to form immobile cysts and assure long term survival. Despite its lower biomass yield compared to other strains, the combination of the highest growth rate on both acetate and butyrate as well as the highest carbohydrate yield made *Polytomella* sp. the most productive strain for carbohydrates (Table 4).

Table 4: Lipid and carbohydrates production of candidate strains on synthetic DFE (0.3 $g_C.L^{-1}$ acetate (Ac) and 0.7 $g_C.L^{-1}$ butyrate (Bu); pH 7). Product (either carbohydrates or lipids) yields ($Y_{P/S}$, $mg.g_{CS}^{-1}$) and product productivities (Q_P, $mg.L^{-1}.d^{-1}$) were calculated considering the whole cultivation duration. Values are given as mean and standard deviation of three biological replicates.

Strain	$Y_{P/S}$ ($mg.g_{CS}^{-1}$)		Q_P ($mg.L^{-1}.d^{-1}$)	
	Carbohydrates	Lipids	Carbohydrates	Lipids
C. sorokiniana SAG	395.7 ± 49.7	420.7 ± 21.2	50.6 ± 5.6	45.9 ± 0.3
E. gracilis	575.5 ± 145.1	181.2 ± 3.9	78.9 ± 2.7	33.8 ± 1.5
O. danica	226.8 ± 38.0	315.1 ± 5.1	28.4 ± 5.7	43.8 ± 2.12
Polytomella sp.	648.7 ± 66.0	109.8 ± 1.7	309.6 ± 24.5	53.2 ± 1.2

On the other hand, the carbohydrates content of *E. gracilis* significantly declined from 47.3 ± 2.4% after acetate exhaustion down to 26.6 ± 1.7% at the end of the butyrate phase. A paramylon productivity of 80.1 $mg.L^{-1}.d^{-1}$ has been reported on 2 $g.L^{-1}$ acetate in line with the global productivity that was obtained here [64]. However, they found that paramylon content of *E. gracilis* could reach up to 70.8% on acetate (without any limitation). The same authors also showed that paramylon content was greatly affected by the carbon sources but did not investigate the effect of butyrate. These results thus complement their findings and indicate that butyrate does not enhance paramylon content as much as acetate. A possible explanation could be that full butyrate metabolization demands more energy to the cells compared to acetate. Indeed, butyrate is more reduced than acetate and thus its conversion to acetyl-CoA requires more metabolic steps contrary to acetate which can directly fuel the tricarboxylic acid or the glyoxylate cycle [65]. In *E. gracilis*, this cycle is directly linked to paramylon synthesis [66]. In the case of butyrate, some more carbon might be diverted toward energy production and not biomass production as has been discussed for *Polytomella* sp.

Lipids and carbohydrates content of both *C. sorokiniana* and *O. danica* remained stable during growth (Figure 6). The carbohydrate and lipid content of *O. danica* remained lower than other strains (respectively 13.5 ± 1.0% and 9.6 ± 1.4%) throughout the whole culture. This lipid content might seem low in regards to previously reported values. For example, intracellular concentration could reach up 79% when cultivating *O. danica* on waste grease [67]. This value was however obtained under nitrogen (N) limitation and in late growth phase. Microalgae tend to not accumulate lipids or carbohydrates when mineral nutrients such as N or phosphorus (P) are not limiting [68]. In the present work, focus was on biomass growth and VFA removal potential of the candidate strains. As such, N and P concentrations were adjusted according to the Redfield ratio 106:16:1, which was found in a previous study to promote biomass growth of *C. sorokiniana* [22]. Carbon was thus the limiting nutrient

which probably explains the low carbohydrate and lipid contents of *O. danica.* This alga has also been shown to excrete free fatty acids when growing mixotrophically growth during early and mid-growth phase [69]. It is thus possible that a part of organic carbon was diverted toward fatty acids excretion instead of lipid accumulation. Since the SPV method employed to measure lipids cannot identify free fatty acids, this should be investigated in further studies.

Lipid productivities were in the same order of magnitude between all strains despite disparities in lipid content and yield (Table 4). For example, *Polytomella* sp. was the most lipid productive strain thanks to its high growth rate, but it however presented the lowest lipid content of all strains (8.4 ± 0.5%). Due to its high biomass production, *O. danica* was second to *C. sorokiniana* in terms of lipid yield. Nevertheless, *C. sorokiniana* should remain the most interesting strains in terms of lipid production since it had the highest lipid content (around 18%) of all strains, which would be favourable for further extraction. The use of an N-limitation strategy should nonetheless change this result by triggering lipid accumulation by *O. danica.* Still, in the case of dark fermentation effluent treatment, the main objective would remain maximum organic carbon removal, which can only be achieved without N-limitation [22]. Such a strategy could thus only be applied in a post-treatment stage, as has been proposed by [70].

4. Conclusions

A main goal of this study was to identify microalgal strains able to grow on butyrate as fast as on acetate (and ideally simultaneously). This ability was not found to be widespread among the investigated green Chlorophytes. In addition, there was no apparent difference between the natural and the lab strains, indicating that butyrate metabolism did not seem to evolve significantly under different environmental conditions. As such, only three strains,

namely *E. gracilis, O. danica* and *Polytomella* sp. were found to share this characteristic. Among them, *Polytomella* sp. stands out due to its growth properties and tolerance to butyrate. As a close relative of *C. reinhardtii*, it has been used as a model strain to understand the evolutionary events that lead the species from an autotrophic/mixotrophic to a heterotrophic mode of nutrition. The unique physiology of *Polytomella* sp. on acetate and butyrate emphasizes the interest of this strain to identify both fast butyrate assimilation and high tolerance mechanisms actually present in *Polytomella* sp. and absent in photosynthetic strains such as *C. reinhardtii* or *C. sorokiniana*.

The second objective of this work was to evaluate the biomass production potential of these strains on a synthetic effluent. It was found that the heterotrophic alga *Polytomella* sp. and the photosynthetic species *E. gracilis* accumulated high levels of carbohydrates during growth. This trait is advantageous since it would allow the conversion of VFAs into carbohydrates in continuous culture mode without limitation strategy needed. It was also found that the cellular composition of both *C. sorokiniana* and *O. danica* remained constant during growth and was more oriented toward proteins production. Overall, this work provides new growth kinetic data of various microalgal strains using VFAs as substrates, which could be further used to design various dark fermentation effluent valorisation strategies.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CRediT Author contributions

JL: conceptualization, investigation, data acquisition, data curation, formal analysis and writing of the original draft. **PJ**: conceptualization, investigation, and writing. **AA** and **AB**: investigation, data curation, validation, review and editing of the original draft. **JPS** and **ET**: supervision, funding acquisition, validation, review and editing of the original draft. **RVL**: conceptualization, funding acquisition, supervision, validation, review and editing of the original draft. All authors approved the final version of the manuscript.

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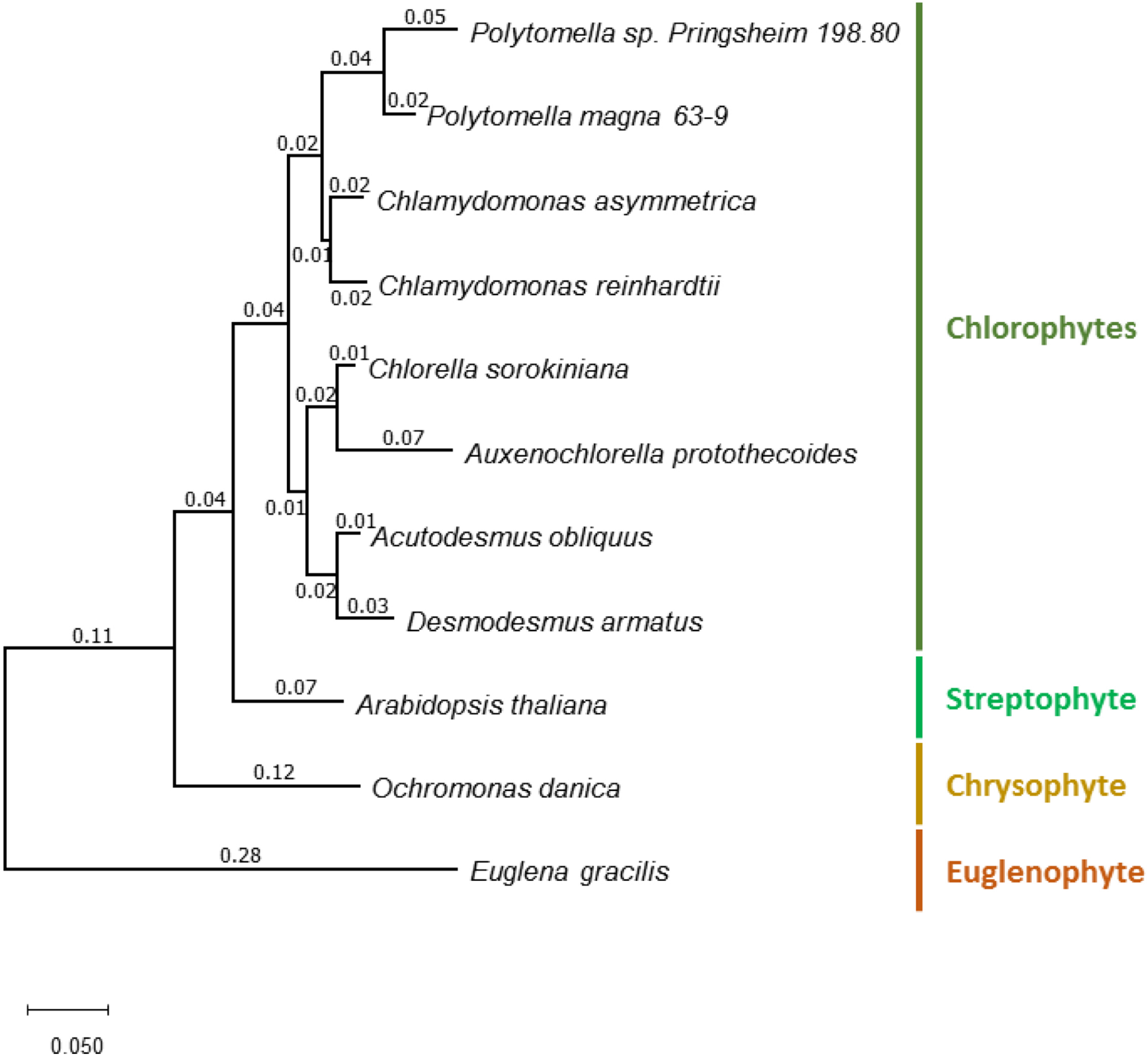
0.05
Polytomella sp. Pringsheim 198.80
0.04
0.02
Polytomella magna 63-9
0.02
0.02
Chlamydomonas asymmetrica
0.01
0.02
Chlamydomonas reinhardtii
0.04
0.01
Chlorella sorokiniana
0.02
0.07
Auxenochlorella protothecoides
0.04
0.01
0.01
Acutodesmus obliquus
0.02
0.03
Desmodesmus armatus
0.11
0.07
Arabidopsis thaliana
0.12
Ochromonas danica
0.28
Euglena gracilis
Chlorophytes
Streptophyte
Chrysophyte
Euglenophyte
0.050

Figure 1

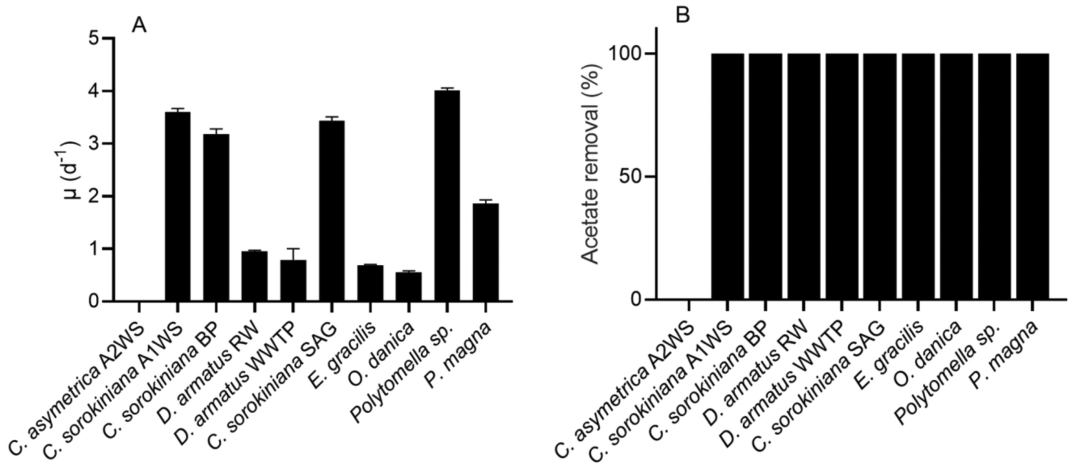
A
μ (d-1)
5
4
3
2
1
0
C. asymetrica A2WS
C. sorokiniana A1WS
C. sorokiniana BP
D. armatus RW
D. armatus WWTP
C. sorokiniana SAG
E. gracilis
O. danica
Polytomella sp.
P. magna
B
Acetate removal (%)
100
50
0
C. asymetrica A2WS
C. sorokiniana A1WS
C. sorokiniana BP
D. armatus RW
D. armatus WWTP
C. sorokiniana SAG
E. gracilis
O. danica
Polytomella sp.
P. magna

Figure 2

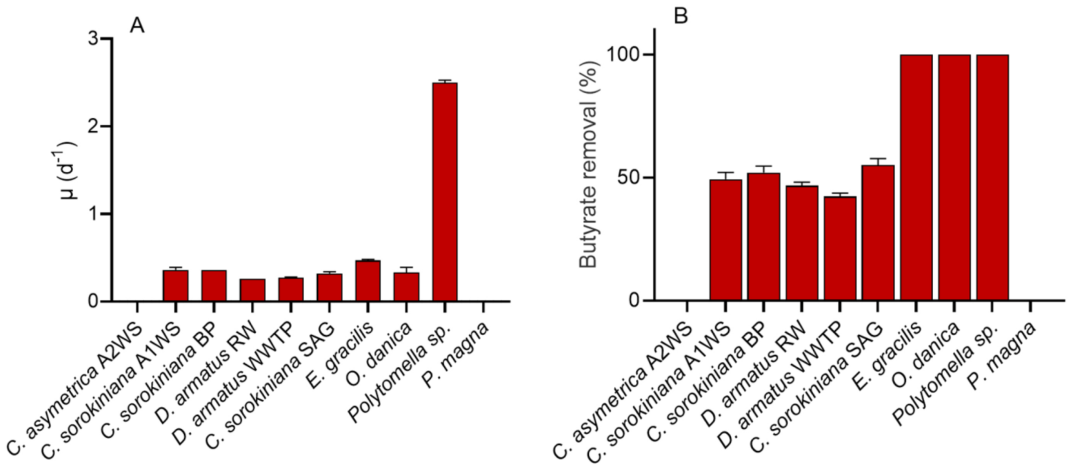
A
μ (d-1)
3
2
1
0
C. asymetrica A2WS
C. sorokiniana A1WS
C. sorokiniana BP
D. armatus RW
D. armatus WWTP
C. sorokiniana SAG
E. gracilis
O. danica
Polytomella sp.
P. magna
B
Butyrate removal (%)
100
50
0
C. asymetrica A2WS
C. sorokiniana A1WS
C. sorokiniana BP
D. armatus RW
D. armatus WWTP
C. sorokiniana SAG
E. gracilis
O. danica
Polytomella sp.
P. magna

Figure 3

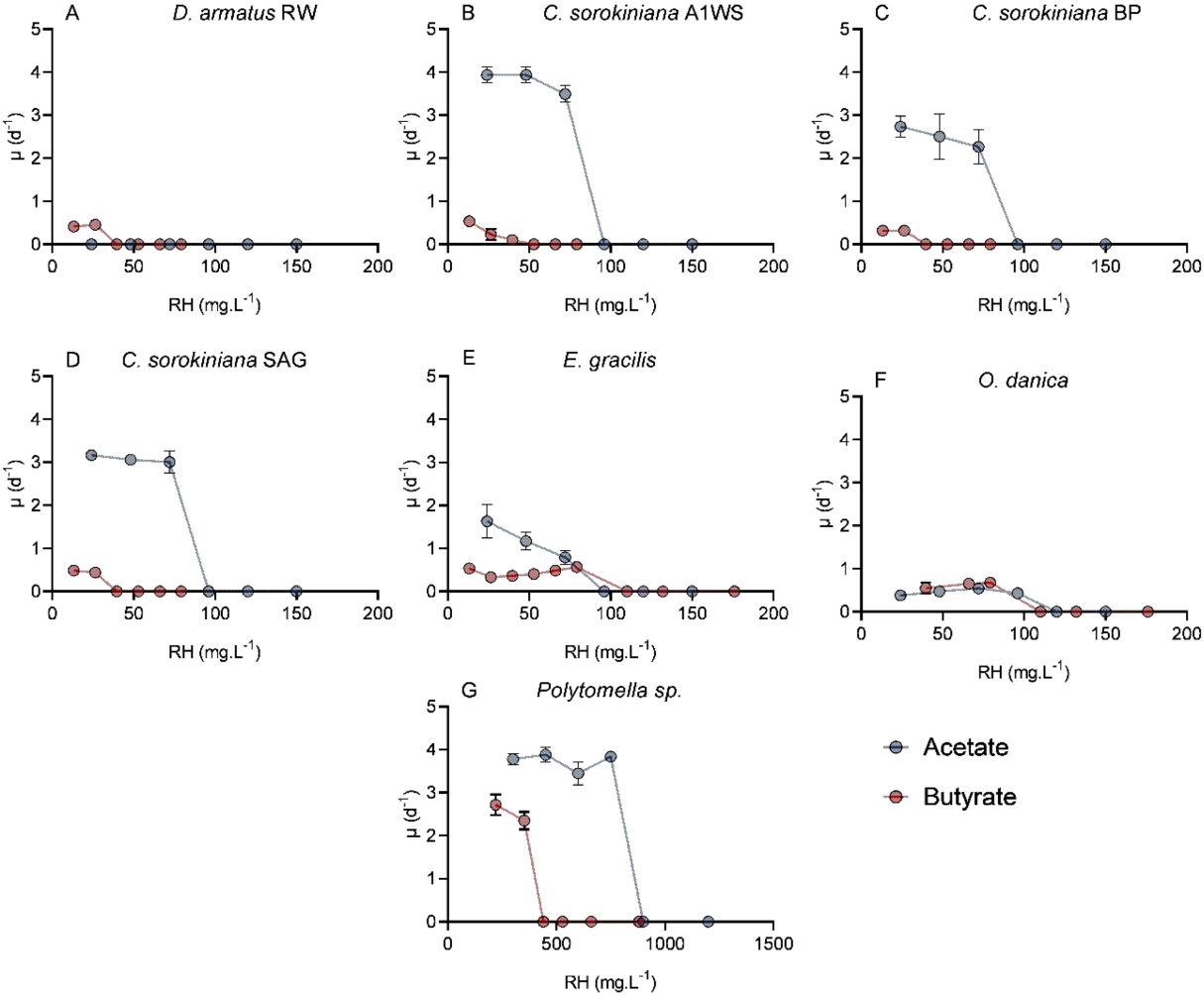
A
D. armatus RW
B
C. sorokiniana A1WS
C
C. sorokiniana BP
D
C. sorokiniana SAG
E
E. gracilis
F
O. danica
G
Polytomella sp.
μ (d-1)
RH (mg.L-1)
Acetate
Butyrate

Figure 4

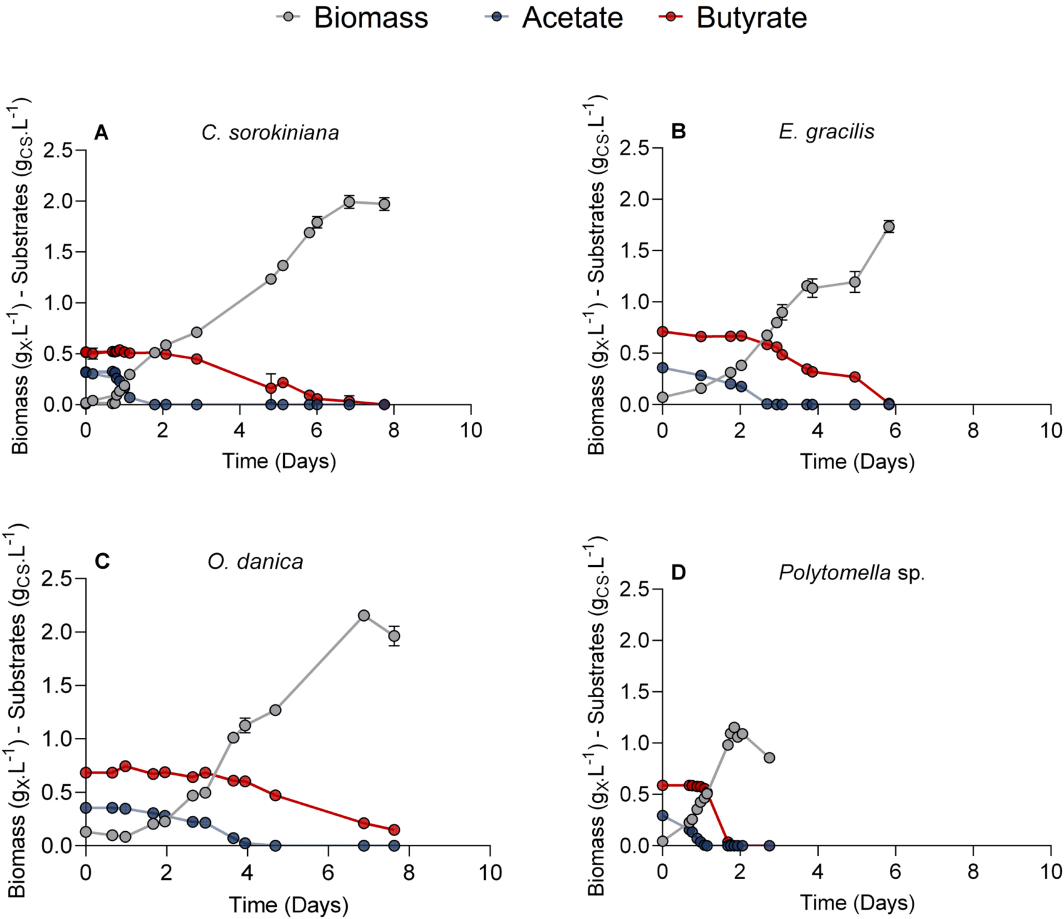
Biomass
Acetate
Butyrate
A
C. sorokiniana
B
E. gracilis
C
O. danica
D
Polytomella sp.
Biomass ($g_X.L^{-1}$) - Substrates ($g_{CS}.L^{-1}$)
Time (Days)
0.0
0.5
1.0
1.5
2.0
2.5
0
2
4
6
8
10

Figure 5

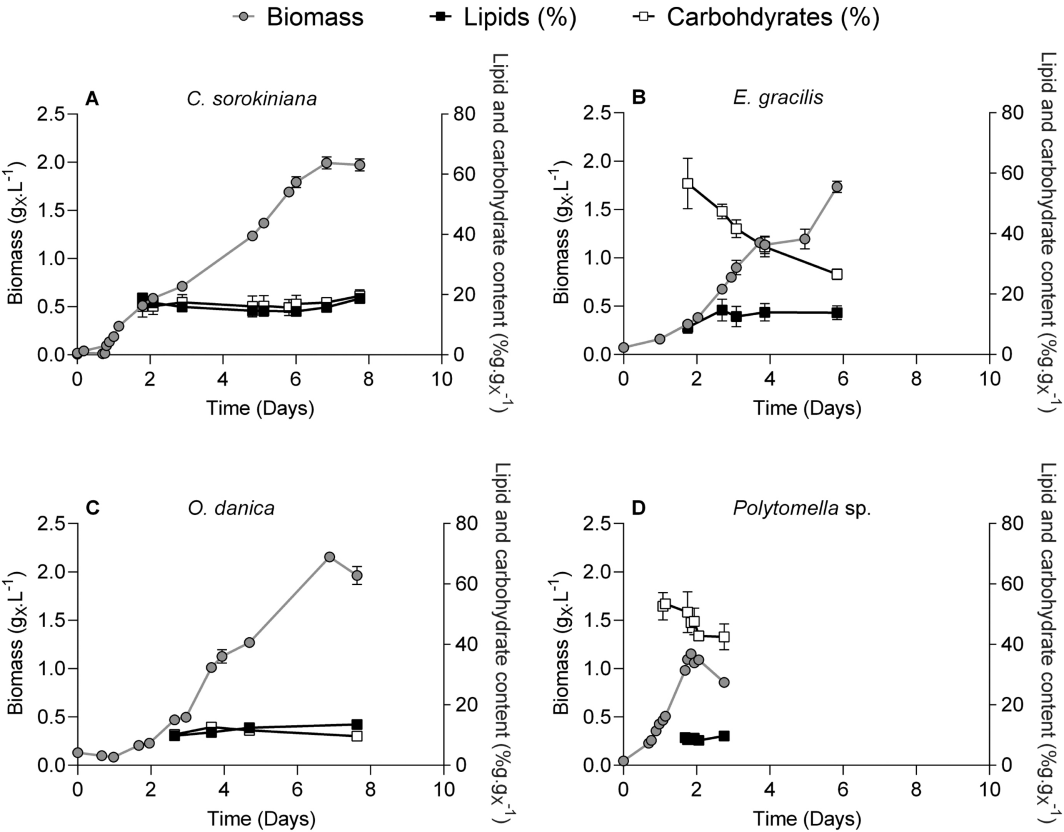
Biomass
Lipids (%)
Carbohdyrates (%)
A
C. sorokiniana
B
E. gracilis
C
O. danica
D
Polytomella sp.
Biomass (gX.L-1)
Lipid and carbohydrate content (%g.gx-1)
Time (Days)

Figure 6