

Raw dark fermentation effluent to support heterotrophic microalgae growth: microalgae successfully outcompete bacteria for acetate

V. Turon, Eric Trably, A. Fayet, E. Fouilland, J.-P. Steyer

► To cite this version:

V. Turon, Eric Trably, A. Fayet, E. Fouilland, J.-P. Steyer. Raw dark fermentation effluent to support heterotrophic microalgae growth: microalgae successfully outcompete bacteria for acetate. Algal Research - Biomass, Biofuels and Bioproducts, 2015, 12, pp.119 - 125. 10.1016/j.algal.2015.08.011 . hal-01921255

HAL Id: hal-01921255 https://hal.umontpellier.fr/hal-01921255

Submitted on 7 Aug 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 Raw dark fermentation effluent to support heterotrophic microalgae

2 growth: microalgae successfully outcompete bacteria for acetate

3 V. TURON^a, E. TRABLY^{a,*}, A. FAYET^a, E. FOUILLAND^b and J-P. STEYER^a

^a INRA, UR 0050, Laboratoire de Biotechnologie de l'Environnement, Avenue des Etangs, 11100
5 Narbonne, France.

^b Marine Biodiversity, Exploitation and Conservation -UMR 9190, SMEL, 2 Rue des Chantiers, 34200
Sète, France.

8 *Corresponding author: <u>eric.trably@supagro.inra.fr</u>; +33 468 425 172.

9 Abstract

10 Coupling dark fermentation (DF), which produces hydrogen from diverse effluents or solid 11 waste, and heterotrophic cultivation of microalgae, which produces lipids, carbohydrates and 12 proteins, is a promising and innovative solution for developing sustainable biorefineries. The 13 use of a raw DF effluent, containing acetate and butyrate, to support the heterotrophic growth 14 of Chlorella sorokiniana was investigated. All the acetate in sterilized and unsterilized DF 15 effluent was exhausted in less than three days of heterotrophic cultivation, whereas butyrate was not used by the microalgae. The microalgae biomass reached 0.33 g L^{-1} with a carbon 16 17 yield on acetate of 55%. The algal yield was higher than previously reported for synthetic DF 18 effluent. It was concluded that compounds other than volatile fatty acids were present in the 19 DF effluent and these could be consumed by the microalgae. After the acetate had been 20 exhausted, butyrate was consumed by facultative and strict aerobic bacteria originating from 21 the DF effluent. The concentration of the bacterial community increased during the 22 experiment but did not have any significant impact on heterotrophic microalgae growth. A 23 high microalgal biomass yield was achieved without requiring the DF effluent to be sterilized.

1 Keywords

2 Biohydrogen, Heterotrophic cultivation, Dark fermentation, Volatile Fatty Acids (VFAs),

3 Bacterial diversity, Chlorella sorokiniana

4 **1** Introduction

5 Over the past decade, increasing attention has been paid to the growth of microalgae in 6 heterotrophic conditions, i.e. in the dark using organic carbon sources, due to (i) high growth 7 rates, biomass densities and lipid yields achieved, (ii) the possibility of using non-arable land, 8 (iii) high volumetric production and (iv) the use of existing technologies, such as microbial 9 fermenters [1]. Heterotrophic microalgae can be cultivated to produce either low-added value 10 molecules such as lipids for biofuels, or high-added value molecules such as the omega 3 fatty 11 acids, DHA and EPA, for human nutrition [2]. However, owing to the high cost of the most 12 common substrate, glucose, using heterotrophic microalgae is only currently economically 13 competitive for human nutrition [3].

14 In recent years, coupling bacterial dark fermentation (DF), to produce hydrogen, and 15 heterotrophic cultivation of microalgae, to produce lipids, has been suggested as being a very 16 promising sustainable approach for producing gaseous and liquid biofuels [4]. DF is a simple 17 process that can convert a wide range of solid waste and effluents into hydrogen, a high-18 energy gas [5]. During DF, anaerobic bacteria break down complex carbon compounds from 19 the organic matter contained in waste (e.g., food waste or agricultural waste) and wastewater 20 (e.g., wastewater from agriculture, the paper industry or the sugar industry) into simple 21 organic acids [6]. Acetic and butyric acids are the two main end products of DF and can be 22 further used as low cost carbon sources to sustain the growth of heterotrophic microalgae [7]. 23 The main advantage of DF is that organic carbon compounds from complex waste that are not 24 directly available to microalgae degradation are simplified into low molecular weight volatile

1	fatty acids (VFAs) [8]. DF effluents also contain substantial amounts of nitrogen and
2	phosphorus that are required to sustain the heterotrophic growth of microalgae. Cho et al
3	pointed out that DF effluent can be regarded as a good medium for growing heterotrophic
4	microalgae in a biorefinery [9].
5	Recent studies investigating microalgae growth on a synthetic DF effluent medium showed
6	very promising results. When grown heterotrophically on a mixture of acetate and butyrate,
7	Chlorella protothecoides reached a carbon yield, (g carbon of biomass per g carbon of VFAs)
8	of 34% and a lipid content of 48% of cellular dry weight (CDW) [3]. Turon et al. [10]
9	reported that Chlorella sorokiniana could grow heterotrophically on acetate with a growth
10	rate of 2.23 d^{-1} and a carbon yield of 42% and on butyrate with a much lower growth rate of
11	0.16 d^{-1} and a carbon yield of 56%. Recent studies showed that heterotrophic microalgae
12	growth was possible using sterilized DF effluents. For example, heterotrophic Scenedesmus
13	sp. produced lipids up to 41% of CDW using acetate from sterilized DF effluent containing
14	ethanol but no butyrate [7]. Chlorella vulgaris was grown in heterotrophic conditions on
15	diluted DF effluent containing acetate and butyrate, converting VFAs efficiently into
16	carbohydrates (51% CDW) [11]. Furthermore, Chlorella sp. was recently reported to produce
17	lipids up to 26% of CDW under mixotrophic conditions with raw DF effluent as a medium
18	[12]. For both raw and synthetic effluents, butyrate concentration has been identified as a key
19	factor driving the effective coupling of DF and heterotrophic cultivation of microalgae under
20	heterotrophic or mixotrophic conditions. Although acetate can be efficiently converted into
21	lipids, butyrate uptake by microalgae is much slower and can reduce the microalgae growth
22	when both VFAs are present. This problem can be solved either by increasing the initial
23	microalgae biomass or by increasing the initial acetate:butyrate ratio [11,13].
24	To couple DF and heterotrophic cultivation of microalgae efficiently, the cost of effluent

25 sterilization has to be reduced. According to Park *et al*, sterilization of the medium accounts

1 for more than a quarter of the investment cost of the process when coupling DF and 2 oleaginous yeast cultivation [4]. The importance of being able to use an unsterilized medium 3 to support microalgae growth was also emphasized by Ramos Tercero et al. [14]. Bacterial 4 contamination is one of the main challenges to be faced for upscaling heterotrophic 5 cultivation [15]. During heterotrophic cultivation, the competition between microalgae and 6 bacteria is usually found to be unfavorable to microalgae, limiting the availability of carbon, 7 nitrogen, phosphate and oxygen [16,17]. Nevertheless, these authors suggested that some 8 conditions may be favorable to microalgae growth, such as a low initial bacterial density and 9 high initial nutrient loads. 10 This work set out to determine, for the first time, the role and importance of the bacterial 11 community present in raw DF effluent on microalgae growth for coupling DF and microalgal 12 heterotrophic cultivation. Chlorella sorokiniana was used as a model for heterotrophic 13 microalgae because of its high growth rate on acetate and its ability to produce high amounts 14 of lipids up to 61.5% of its CDW [10,18]. The dynamics of biomass growth and carbon yield

of *C. sorokiniana* were evaluated using sterilized and unsterilized, raw DF effluent and the
biomass and diversity of bacterial community originating from the DF effluent were evaluated

17 for the unsterilized DF effluent.

18 2 Materials and methods

19 **2.1 Dark fermentation test batches**

20 Five identical test batches of "DF effluent" were produced simultaneously in 600 mL glass

- 21 bottles with a working volume of 200 mL. No culture medium was added or removed during
- the fermentation. The culture medium consisted of 100 mM of 2-(*N*-morpholino)
- ethanesulfonic acid (MES) buffer, 5 g L^{-1} of glucose and 5 mL L^{-1} of a micronutrient solution.
- 24 The composition of the micronutrient solution is fully described by Pierra et al. [19]. The

medium was supplemented with 1 mL L^{-1} of F/2 medium vitamin solution (CCAP, 1 2 http://www.ccap.ac.uk/). The flasks were inoculated with 1 mL of heat-treated (15 min at 3 90 °C) anaerobic sludge from an anaerobic digester treating waste from a sugar processing 4 plant (Marseille, France). The initial substrate to biomass ratio S:X was 40, S representing the initial chemical oxygen demand (COD) of the substrate (in gCOD L^{-1}) and X representing the 5 6 initial inoculum (in g of total volatile solids L⁻¹). To ensure anaerobic conditions, the flasks 7 were sealed and flushed with nitrogen gas as described elsewhere [19]. The pH was adjusted 8 to 6 and the bottles were incubated at 37 °C until the glucose was completely exhausted. At 9 the end of the growth phase, i.e. glucose exhaustion and hydrogen accumulation, the five 10 anaerobic cultures batches were mixed to produce the "DF effluent". The pH of the DF 11 effluent was increased to 6.5 with 1 M NaOH. VFAs, and the ammonium and phosphate 12 concentrations were measured. Half of the DF effluent was centrifuged three times at 15,000 13 rpm for 15 min. A fraction of the supernatant was sterilized using Acrodisc® PF syringe filter 14 with 0.8/0.2 µm pores (PALL). The sterilized and unsterilized DF effluent samples were then 15 stored at 4 °C until the start of the experiment.

16 **2.2** Axenic microalgae strain and preparation of the microalgae stock culture

17 Chlorella sorokiniana (CCAP 211/8K) was cultivated axenically in 500 mL Erlenmeyer

18 flasks with a working volume of 200 mL. A modified BG11 medium was used as described

19 by Turon et al. [10]. Sodium bicarbonate (10 mM), ammonium chloride (5 mM) and

20 dipotassium phosphate (0.31 mM) were used as inorganic carbon (C), nitrogen (N) and

21 phosphorus (P) sources, respectively. All the components of the medium as well as the flasks

- 22 were sterilized by autoclaving at 121 °C for 20 min before use. The flasks were incubated
- 23 under autotrophic conditions (light intensity of 100 μ mol photons m⁻² s⁻¹) at 25 °C for 7 days.

1 2.3 Heterotrophic microalgae growth on dark fermentation effluent

2 A fixed volume of either the sterilized or the unsterilized DF effluent (36 mL) was placed in 3 sterile 125 mL black Erlenmeyer flasks sealed with a cotton wool plug. Four mL of microalgae culture, 0.2 g L^{-1} , were added to each flask. The flasks were then incubated on a 4 5 rotary shaker (150 rpm) at 25 °C for 10 days in complete darkness. A 1 mL sample of the 6 culture was taken every day to measure the optical density (OD), VFA concentration, 7 microbial concentration and diversity. The experiment was carried out in triplicate. During the 8 whole of the experiment, the microalgae cultures in the sterilized DF effluent were checked 9 for other living organisms every day by DAPI counterstaining and contrast phase microscopy.

10 2.4 Microbial analysis

11 2.4.1 Microalgae biomass measurement

The microalgae growth was quantified on sterilized DF effluent by measuring the OD at 800 nm (OD₈₀₀) as described by Turon *et al* [10]. The relationship between the cellular dry weight (CDW) and OD₈₀₀ was determined for a wide range of CDW (0 – 1.4 g L⁻¹) by filtering 15 mL of algal samples onto pre-weighed GF/F Whatman® filters that were then dried overnight at 105 °C, giving the following calibration function.

17 CDW (g
$$L^{-1}$$
) = 1.24*OD₈₀₀ (R² = 0.95).

For calculating the biomass yield, the carbon content was estimated at 50% of microalgaebiomass [20].

Because of the presence of small suspended solids in the unsterilized DF effluent, optical density measurement was not used for monitoring the algal biomass. As the chlorophyll content of microalgae during heterotrophic cultivation can change, this is not suitable for monitoring the microalgae growth accurately [21]. Therefore, the dynamics of microalgae

- 1 biomass growth were monitored by amplification of 18S rDNA gene copies, in cultures
- 2 carried out using both sterilized and unsterilized DF effluent.

3 2.4.2 DNA extraction and purification

4 700 μL of the culture sample was centrifuged at 10,000 rpm for 10 min and genomic DNA
5 was extracted using the PROMEGA Wizard® Genomic DNA Kit and then purified using the
6 QIAamp DNA Mini Kit (Qiagen).

7 2.4.3 Quantitative PCR for microalgae (18S rDNA gene)

8 The microalgae biomass was quantified using quantitative PCR (qPCR) and specific primers 9 for Chlorophyta 18S rDNA INT-4F (5' TGGTGAAGTGTTCGGATTGG 3') and INT-5R (5' 10 ARGTG GGAGG GTTTA ATGAA 3') as described by Hoshina et al [22]. The quantitative 11 amplification reaction was carried out with 5 μ L of DNA sample, 12.5 μ L of Universal 12 SYBR® Green Supermix (Biorad) (composed of polymerase, dNTPs and SYBR® Green 13 dye), 1 μ L of forward primer INT-4F, 1 μ L of reverse primer INT-5R and 5.5 μ L of H₂O, for a total volume of 25 μ L. The PCR was run in a 100 TouchTM thermal cycler equipped with a 14 CFX96TM Real-Time System (Bio-rad). There was an initial incubation of 3 min at 95 °C 15 16 followed by 40 cycles of denaturation-amplification (10 s at 95 °C and 30 s at 56 °C). Data 17 analysis was carried out with the Bio-rad CFX Manager software, version 3.0. A linear 18 standard curve was obtained from each assay by amplification of eight 10-fold dilutions of a 19 reference plasmid to count the number of 18S rDNA copies accurately. The reference plasmid 20 (pEX-A2 plasmid, Eurofins MWG Operon) was created using the *Chlorella sorokiniana* 18S 21 rDNA sequence published in the European nucleotide archive under the sequence number 22 X62441.2 (ENA, http://www.ebi.ac.uk/ena). The PCR product from the reference plasmid and 23 Chlorella sorokiniana DNA extracts corresponded to a 77 bp sequence (from 1666 to 1742 24 NT):

25 TGGTGAAGTGTTCGGATTGGCGACCGGGGGGGGGGGGCGGTCTCCGGCCGCCGA

1 GAAGTTCATTAAACCCTCCCACCT (the bold letters correspond to the qPCR primer 2 targets). The cycle threshold (CT), i.e. the number of cycles at which the reaction became 3 exponential, was used to quantify the number of gene copies. A linear standard curve was 4 obtained by plotting the CTs of the dilutions of the reference plasmid against the logarithms 5 of the known number of gene copies of the different dilutions of the reference plasmid. The 6 CT of each sample was then compared to the linear standard curve and the number of gene 7 copies per sample was determined. The total number of 18S rDNA gene copies per sample 8 was finally expressed as the logarithm of the number of target copies per mL of culture 9 sample. For SYBR® Green assays, the specificity of the PCR products was checked by a 10 melting curve analysis using the dissociation protocol from the Bio-rad CFX Manager 11 software. An initial incubation of 3 min at 95 $^{\circ}$ C and 40 cycles of denaturation (10 s at 95 $^{\circ}$ C 12 and 30 s at 56 $^{\circ}$ C) were performed.

13 2.4.4 Quantitative PCR for bacteria (16S rDNA)

14 For quantification of total bacteria, general bacterial primers, BAC338F and BAC805R, and 15 the associated probe, BAC16F, were used as described elsewhere [23]. The quantitative 16 amplification reaction was carried out in a total volume of 12.5 μ L with 2 μ L of sample DNA, 17 $6.5 \,\mu\text{L}$ of Universal probes Supermix (Biorad), $0.5 \,\mu\text{L}$ of each primers and the probe and 2.5 18 μ L of H₂O. The thermal cycler and analysis method were the same as for qPCR of 19 Chlorophyta. An initial incubation of 2 min at 95 °C followed by 40 cycles of denaturation-20 amplification (7 s at 95 °C and 25 s at 56 °C) were performed. The linear standard curve was 21 obtained as described elsewhere [24]. The total number of 16S rDNA gene copies per sample 22 was expressed as the logarithmic value of the number of target copies per mL of culture 23 sample.

1 2.4.5 Sequencing of bacterial 16S rDNA

2	Samples taken on day 0, at day 2.7 and on day 10 (9 samples in total) were used for
3	sequencing. The V4-V5 region of the 16S rDNA gene was amplified over 30 amplification
4	cycles at an annealing temperature of 65 °C, with the forward primer
5	5'-CTTTCCCTACACGACGCTCTTCCGATCTGTGYCAGCMGCCGCGGTA-3' and the
6	reverse primer
7	5'-GGAGTTCAGACGTGTGCTCTTCCGATCTCCCCGYCAATTCMTTTRAGT-3' with
8	their respective linkers. In a second PCR reaction of 12 cycles, an index sequence was added
9	using the primers
10	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC and
11	CAAGCAGAAGACGGCATACGAGAT-index-GTGACTGGAGTTCAGACGTGT. The
12	resulting PCR products were purified and loaded onto the Illumina MiSeq cartridge according
13	to the manufacturer's instructions for sequencing 250 bp reads. Sequencing was carried out at
14	the GeT PlaGe sequencing center of the Genotoul life science network in Toulouse, France
15	(get.genotoul.fr). 595799 forward and reverse sequences were retained after assembly and
16	quality checking using a slightly modified version of the standard operation procedure
17	described by Kozich et al. [25]. Mothur version 1.33.0. SILVA release 102 provided by
18	Schloss et al. [26] was used for alignment and as a taxonomic outline. A 2% dissimilarity
19	threshold was used to define OTUs in Mothur. The known species that were phylogenetically
20	closest to OTUs with a relative abundance of more than 2% at the start of the experiment (day
21	0), at acetate exhaustion (day 2.7) or at the end of the experiment (day 10) were identified
22	using the Basic Local Alignment Search Tool (BLAST) [27]. The sequences identified as C.
23	sorokiniana chloroplast were removed before analysis of bacterial abundance.

1 **2.5** Chemical analysis

2 Acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate and caproate were measured 3 using a gas chromatograph (GC 3900 Varian) equipped with a flame ionization detector. 4 Other non-volatile molecules such as glucose, ethanol, lactate and formate were quantified 5 using HPLC with a refractive index detector and an Aminex HPX-87H column (Biorad®). The column temperature was maintained at 35 °C and the flow rate at 0.4 mL min⁻¹ [28]. 6 7 Biogas production of the DF test batches was monitored daily by measuring the gas pressure 8 in the headspace. The biogas composition $(CO_2, O_2, H_2, N_2 \text{ and } CH_4)$ was measured using a 9 gas chromatograph (Clarus 580, Perkin Elmer) coupled to a thermal conductivity detector 10 (TCD) [28].

11 Ammonium (NH_4^+) and phosphate (PO_4^{3-}) ions were quantified using an ion chromatography 12 system (ICS 3000 Dionex, USA), as described elsewhere [29].

13 **3 Results and discussion**

14 **3.1 Dark fermentation test batches**

15 DF anaerobic test batches were carried out to provide a culture medium for subsequent 16 heterotrophic microalgae growth. The fermentation ended with complete glucose exhaustion, 17 after 6 days of fermentation. The final acetate and butyrate concentrations were 1.09 ± 0.09 g L^{-1} and 1.43 ± 0.07 g L^{-1} , respectively. No other fermentation metabolites, i.e. lactate, ethanol, 18 19 propionate, valerate and caproate, were found. The hydrogen yield, i.e. the molar ratio of hydrogen produced per mol of glucose consumed, was $1.37 \pm 0.18 \text{ mol } H_2 \text{ mol glucose}^{-1}$. The 20 21 hydrogen yield was consistent with previously reported experimental results obtained in 22 similar conditions with mixed cultures [28]. The molar ratio of H_2 per mol of VFAs, the sum of acetate and butyrate produced, was 1.1 ± 0.14 mol H₂ mol VFAs⁻¹. This value is lower than 23

the theoretical value of 2 mol H₂ mol VFAs⁻¹ produced through the acetate pathway (eq (1))
or the butyrate pathway (eq (2)). In general, the H₂ yield can be lowered either by the
consumption of glucose through non-hydrogen-producing pathways, such as ethanol or lactate
pathways, or by the direct consumption of H₂ through homoacetogenesis, i.e. the production
of acetate via CO₂ and H₂ consumption (eq (3)) [28]. Since neither lactate nor ethanol was
found, only homoacetogenesis could have occurred.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$$
 (1)

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$$
(2)

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O \tag{3}$$

7 Three samples of DF effluent were sequenced for microbial community characterization. The 8 closest phylogenetically known sequences of the most representative OTUs are given in Table 9 1. About 53 \pm 1.5 % of initial bacteria were strict anaerobes, with 36.8 \pm 1.3% related to 10 *Clostridium* sp. and $16.2 \pm 0.6\%$ to *Sporolactobacillus* sp. It has been established that bacteria 11 belonging to *Clostridium* genus are responsible for hydrogen fermentation. Sporolactobacillus 12 sp. are known for their ability to degrade glucose to lactate in anaerobic conditions [30]. Since 13 no lactate was found in the medium and *Clostridium tyrobutyricum* was the most abundant 14 species, this suggested that lactate might have been converted into butyrate and hydrogen, as 15 previously reported [31]. C. tyrobutyricum is also known to use the butyrate pathway 16 preferentially during DF. The presence of this species would explain the high butyrate:acetate 17 molar ratio, 1.14, found at the end of DF [32]. 18 The remaining bacteria were either facultative or strict aerobic species. Strict aerobes were 19 previously found in an anaerobic digester, even non-spore producing species [33]. Even 20 though the anaerobic sludge was heat treated before the fermentation, facultative and strict 21 aerobic bacteria may have survived, as reported elsewhere [30]. They in fact play an

- 1 important role in removing residual oxygen in anaerobic systems, which subsequently favors
- 2 the growth of *Clostridium* species and hydrogen production [34].
- 3 **3.2** Microalgae growth on raw fermentation effluent

4 3.2.1 Characterization of the dark fermentation effluent

5 The initial acetate concentrations in the sterilized and unsterilized DF effluent samples were 0.74 ± 0.02 g L⁻¹ and 0.73 ± 0.02 g L⁻¹, respectively. The initial butyrate concentrations in the 6 sterilized and unsterilized DF effluent samples were 1.25 ± 0.06 g L⁻¹ and 1.26 ± 0.03 g L⁻¹, 7 respectively. The acetate: butyrate mass ratio $(g g^{-1})$ was about 0.6 in both cases. These 8 9 concentrations as well as the acetate:butyrate ratio were consistent with previous studies using 10 raw DF effluents to sustain microalgae growth [11,35,36]. Sterilization by microfiltration had 11 no effect on the carbon, nitrogen and phosphorus contents of the effluent. The C:N:P molar 12 ratios in the sterilized and unsterilized DF effluent were 35:6.7:1 and 36:6.5:1, respectively. 13 These ratios were lower than the Redfield C:N:P ratio for phytoplankton cellular composition, 14 i.e. 106:16:1. Here, only the carbon substrate was assumed to be the limiting element for C. 15 sorokiniana growth. 16 Interestingly, other authors used effluents from acidogenic fermentation, instead of effluents from DF, which contained high amounts of VFAs ranging from 5 to 14 g L^{-1} , to sustain 17

18 microalgae growth [12,37]. In acidogenic fermentation, VFA accumulation is targeted rather

- 19 than biohydrogen production and hydrogen conversion to acetate through homoacetogenesis
- 20 is promoted. Nevertheless, the effluents from acidogenic fermentation have to be diluted
- 21 between 8 and 20 fold before use to avoid excess initial VFA concentrations inhibiting

22 microalgae growth [12,37].

1	3.2.2 Microalgae growth on sterilized and unsterilized dark fermentation effluent
2	C. sorokiniana was grown for 10 days on sterilized and unsterilized DF effluent in
3	heterotrophic conditions (Figures 1 and 2, respectively). Microalgae grew during the first 2.7
4	days in both experiments. There was very similar growth in microalgae, expressed as the
5	logarithm of the number of 18S rDNA copies per mL of culture sample reaching 8.13 ± 0.05
6	and $8.20 \pm 0.04 \log(18S \text{ copies mL}^{-1})$ during growth on sterilized and unsterilized DF
7	effluent, respectively (Figure 2). The maximum CDW during heterotrophic cultivation on
8	sterilized DF effluent reached 0.33 ± 0.01 g L ⁻¹ . In both conditions, microalgae concentration
9	did not increase during the last 7 days. Acetate was completely exhausted after 2.7 days in
10	both experiments (Figures 1 and 2). Butyrate was not consumed when axenic C. sorokiniana
11	was grown on sterilized DF effluent (Figure 1). On the other hand, butyrate degradation
12	started after complete acetate exhaustion and ended after 8 to 9 days when C. sorokiniana was
13	grown on unsterilized DF effluent (Figure 2). We concluded that C. sorokiniana grew in both
14	experiments using acetate until it was completely exhausted and that the bacterial community
15	initially present in unsterilized DF effluent was responsible for butyrate degradation. Since
16	very similar microalgae biomass yields were reached during both experiments, C. sorokiniana
17	was probably responsible for the complete exhaustion of acetate, despite the presence of a DF
18	bacterial community. This suggested that C. sorokiniana successfully outcompeted DF
19	bacteria for acetate uptake. The abrupt shift from anaerobic to aerobic culture conditions
20	might have hampered DF bacterial growth and enabled microalgae to degrade acetate without
21	competition with bacteria. It was clear that microalgae heterotrophic growth was favored in a
22	medium supplemented by raw DF effluent.
23	The maximum growth rate, μ_{max} , of the microalgae was assessed during the exponential

24 growth phase and was 1.75 \pm 0.14 $d^{\text{-1}}$ on sterilized DF effluent. This μ_{max} value was consistent

25 with previously reported growth rates of *C. sorokiniana* on acetate under heterotrophic

1 conditions at 25 °C [10]. During growth on sterilized DF effluent, the biomass yield, i.e. the 2 carbon taken from the substrate and incorporated into the biomass, was $55 \pm 4\%$. A similar 3 carbon yield of 52% was also reported with a mixotrophic culture of *Chlorella vulgaris* on 4 raw acidogenic fermentation effluent [9]. Interestingly, this yield is significantly higher than 5 previous values reported on synthetic DF effluents, 42% and 44%, for C. sorokiniana and C. 6 protothecoides, respectively [3,10]. In raw DF effluents, compounds other than VFAs, such as 7 amino acids and proteins, are available for microalgae growth [8]. The uptake of such 8 compounds by microalgae for their own growth could explain the higher yield on acetate 9 found in this study.

- 10 **3.3** Bacterial growth and diversity during microalgae cultivation on raw
- 11

fermentation effluent

12 Bacterial growth was monitored using quantitative PCR during the experiment on unsterilized 13 DF effluent (Figure 3). The bacterial primers were tested on axenic *C. sorokiniana* samples. 14 DNA amplification was observed, probably because the primers matched chloroplast rDNA 15 sequences. However, due to the high initial load of bacteria, the number of 16S rDNA copies 16 due to microalgae was insignificant compared with the copies due to bacteria (data not 17 shown). Therefore, the results presented in Figure 3 were considered to be the result of 18 bacterial growth. Bacterial growth had two phases. The first growth period occurred during 19 acetate exhaustion from the beginning of the experiment to 2.7 days, during the C. 20 sorokiniana growth phase (Fig. 2). The logarithm of the number of 16S rDNA copies per mL 21 of culture sample started at 9.06 \pm 0.22 and ended at 10.04 \pm 0.22 after 2.7 days. As it was 22 suggested that acetate degradation resulted mainly from microalgae activity, the bacterial 23 community probably used other organic compounds initially present in the raw DF effluent or 24 released by microalgae. The second bacterial growth period started with butyrate degradation, 25 and bacterial biomass reached $11.01 \pm 0.4 \log (16 \text{ srDNA copies mL}^{-1})$ on day 7. During

1 butyrate degradation, the butyrate concentration was probably too high to support microalgae 2 growth, as previously suggested [10,35]. No competition was observed between microalgae 3 and bacteria for butyrate. The bacterial community was responsible for the entire butyrate 4 degradation. Butyrate exhaustion by bacteria could be very useful for the efficient cultivation 5 of microalgae on DF effluent. To produce high densities of microalgae and/or lipids, fed-6 batch cultivation has been suggested, i.e. sequential addition of medium after exhaustion of 7 the substrate or when the growth plateau is reached [38]. For biomass production, this 8 technique avoids growth inhibition due to high initial substrate concentration and substrate 9 concentration is maintained at less than the inhibitory concentration during the process [38]. This strategy has been successfully used to produce high concentrations of lipids, 40 g L^{-1} , by 10 11 heterotrophic cultivation of *C* sorokiniana on glucose [39]. If such operational conditions 12 were applied using sterilized DF effluent, butyrate would accumulate as a result of the 13 inability of C. sorokiniana to consume butyrate rapidly and would eventually lead to growth 14 inhibition [10]. Therefore, butyrate degradation by bacteria may be beneficial for lipid 15 production by heterotrophic microalgae, suggesting that bacteria may have a positive effect, if 16 well managed, for upscaling using unsterilized DF effluent. 17 The bacterial community in the raw effluent was dominated by 13 OTUs each with a relative 18 abundance of more than 2% at the start of the experiment, at acetate exhaustion or at the end 19 of the experiment. As shown in Figure 4, there were significant shifts in the bacterial 20 community during the experiment. The dominant bacterial species shifted from strict 21 anaerobes (Clostridium sp. and Sporolactobacillus sp.) to facultative anaerobes (Paenibacillus 22 sp.) and then to strict aerobes (Stenotrophomonas maltophilia). The dominant bacteria at the 23 beginning of the experiment (over 73% of the total bacteria abundance) accounted for less 24 than 3.5% of the bacteria present at day 2.7 when a species close to *Paenibacillus chibensis*

25 was dominant (over 60% of total bacteria) in two of the replicates (Figure 4). In only one of

1 the three replicates (flask 3), a high abundance (42.5%) of a species close to Lysinibacillus 2 xylanilyticus was observed with a reduction in the abundance of a species close to 3 Paenibacillus chibensis, (36.2%). According to Swezey et al. [40], Paenibacillus chibensis is 4 unable to grow on acetate. The presence of a dominant species closely related to 5 Paenibacillus chibensis (99% identity) suggested that acetate exhaustion by this species was 6 unlikely. Because acetate degradation was very similar in all replicates when unsterilized DF 7 effluent was used (Figure 3), the emergence of a different species closely related to 8 Lynsinibacillus xylaniticus in only one of the replicates, suggested that this species was 9 probably not involved in the exhaustion of acetate. These two observations were consistent 10 with and reinforced our previous suggestion that C. sorokiniana was highly competitive and 11 mainly responsible for acetate exhaustion. 12 At the end of the experiment, after complete butyrate exhaustion in the three replicate flasks, 13 species close to Stenotrophomonas maltophilia and Cupriavidus necator, both strict aerobic 14 species, were largely dominant (Figure 4) [33]. *Cupriavidus necator* is known to assimilate 15 butyrate and then produce polyhydroxyalkanoates [41]. The emergence of these bacterial 16 species during this period, during which no growth of C. sorokiniana was observed, 17 confirmed that strict aerobic bacterial species initially present in the raw DF effluent were 18 responsible for the butyrate uptake.

The results of this study clearly showed that the two main obstacles to industrial microalgae cultivation in heterotrophic conditions, i.e. glucose and medium sterilization costs, could be easily overcome by using unsterilized DF effluent. Firstly, acetate uptake by *C. sorokiniana* was fast and sufficiently efficient to enable microalgae to outcompete bacteria. Secondly, the abrupt change in the operational parameters between DF and heterotrophic cultivation, i.e. from anaerobic culture conditions at 37 °C to aerobic culture conditions at 25 °C, favored

microalgae growth and may be a solution to avoid sterilization. Further research to find means
of reducing butyrate inhibition would have considerable potential.

3 4 Conclusions

- 4 This study showed the feasibility of using unsterilized dark fermentation effluent for
- 5 heterotrophic cultivation of microalgae. Microalgae successfully outcompeted bacteria
- 6 originating from DF effluent for acetate and achieved a carbon yield of 55%. The butyrate
- 7 concentration was too high to support microalgae growth but can be degraded by the aerobic
- 8 bacterial species initially present in the raw DF effluent. The use of a fed-batch mode for
- 9 heterotrophic cultivation of microalgae using raw DF effluent is proposed with the medium
- 10 being added at periods set to allow DF bacteria to exhaust the butyrate to prevent
- 11 accumulation and thus allowing microalgae to grow on acetate and accumulate lipids.
- 12 However, further research is required to assess the potential competitive role of the strict
- 13 aerobic bacterial community usually found with non-axenic cultivated microalgae.

14 **5 References**

- S. Venkata Mohan, M.V. Rohit, P. Chiranjeevi, R. Chandra, B. Navaneeth,
 Heterotrophic Microalgae Cultivation to Synergize Biodiesel Production with Waste
 Remediation: Progress and Perspectives, Bioresour. Technol. in press (2014) 169–178.
 doi:10.1016/j.biortech.2014.10.056.
- [2] G. Markou, E. Nerantzis, Microalgae for high-value compounds and biofuels
 production: a review with focus on cultivation under stress conditions., Biotechnol.
 Adv. 31 (2013) 1532–42. doi:10.1016/j.biotechadv.2013.07.011.
- 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.
 (2014) 691–700. doi:10.1007/s00449-014-1308-0.
- [4] G.W. Park, Q. Fei, K. Jung, H.N. Chang, Y.-C. Kim, N.-J. Kim, et al., Volatile fatty
 acids derived from waste organics provide an economical carbon source for microbial

1 2		lipids/biodiesel production., Biotechnol. J. 9 (2014) 1536–46. doi:10.1002/biot.201400266.				
3 4 5 6	[5]	C. Sambusiti, M. Bellucci, A. Zabaniotou, L. Beneduce, F. Monlau, Algae as promising feedstocks for fermentative biohydrogen production according to a biorefinery approach: A comprehensive review, Renew. Sustain. Energy Rev. 44 (2015) 20–36. doi:10.1016/j.rser.2014.12.013.				
7 8 9	[6]	W.S. Lee, A.S.M. Chua, H.K. Yeoh, G.C. Ngoh, A review of the production and applications of waste-derived volatile fatty acids, Chem. Eng. J. 235 (2014) 83–99. doi:10.1016/j.cej.2013.09.002.				
10 11 12 13	[7]	HY. Ren, BF. Liu, F. Kong, L. Zhao, D. Xing, NQ. Ren, Enhanced energy conversion efficiency from high strength synthetic organic wastewater by sequential dark fermentative hydrogen production and algal lipid accumulation, Bioresour. Technol. 157 (2014) 355–359. doi:10.1016/j.biortech.2014.02.009.				
14 15 16 17	[8]	R.R. Singhania, A.K. Patel, G. Christophe, P. Fontanille, C. Larroche, Biological upgrading of volatile fatty acids, key intermediates for the valorization of biowaste through dark anaerobic fermentation., Bioresour. Technol. (2012). doi:10.1016/j.biortech.2012.12.137.				
18 19 20 21	[9]	H.U. Cho, Y.M. Kim, YN. Choi, X. Xu, D.Y. Shin, J.M. Park, 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., Bioresour. Technol. (2014). doi:10.1016/j.biortech.2014.09.069.				
22 23 24	[10]	V. Turon, C. Baroukh, E. Trably, E. Latrille, E. Fouilland, JP. Steyer, Use of fermentative metabolites for heterotrophic microalgae growth: Yields and kinetics, Bioresour. Technol. 175 (2015) 342–349. doi:10.1016/j.biortech.2014.10.114.				
25 26 27 28	[11]	CH. Liu, CY. Chang, Q. Liao, X. Zhu, CF. Liao, JS. Chang, Biohydrogen production by a novel integration of dark fermentation and mixotrophic microalgae cultivation, Int. J. Hydrogen Energy. 38 (2013) 15807–15814. doi:10.1016/j.ijhydene.2013.05.104.				
29 30 31 32	[12]	B. Hu, W. Zhou, M. Min, Z. Du, P. Chen, X. Ma, et al., Development of an effective acidogenically digested swine manure-based algal system for improved wastewater treatment and biofuel and feed production, Appl. Energy. 107 (2013) 255–263. doi:10.1016/j.apenergy.2013.02.033.				
33 34 35 36	[13]	M. Moon, C.W. Kim, WK. Park, G. Yoo, YE. Choi, JW. Yang, Mixotrophic growth with acetate or volatile fatty acids maximizes growth and lipid production in Chlamydomonas reinhardtii, Algal Res. 2 (2013) 352–357. doi:10.1016/j.algal.2013.09.003.				
37 38 39 40	[14]	E.A. Ramos Tercero, E. Sforza, M. Morandini, A. Bertucco, Cultivation of Chlorella protothecoides with urban wastewater in continuous photobioreactor: biomass productivity and nutrient removal., Appl. Biochem. Biotechnol. 172 (2014) 1470–85. doi:10.1007/s12010-013-0629-9.				

1 N. Rashid, M.S. Ur Rehman, M. Sadiq, T. Mahmood, J.-I. Han, Current status, issues [15] 2 and developments in microalgae derived biodiesel production, Renew. Sustain. Energy 3 Rev. 40 (2014) 760-778. doi:10.1016/j.rser.2014.07.104. 4 Y. Zhang, H. Su, Y. Zhong, C. Zhang, Z. Shen, W. Sang, et al., The effect of bacterial [16] 5 contamination on the heterotrophic cultivation of Chlorella pyrenoidosa in wastewater 6 from the production of soybean products., Water Res. 46 (2012) 5509-16. 7 doi:10.1016/j.watres.2012.07.025. 8 [17] N. Kamjunke, B. Köhler, N. Wannicke, J. Tittel, Algae As Competitors for Glucose 9 With Heterotrophic Bacteria, J. Phycol. 44 (2008) 616-623. doi:10.1111/j.1529-10 8817.2008.00520.x. 11 [18] L. Ramanna, A. Guldhe, I. Rawat, F. Bux, The optimization of biomass and lipid yields 12 of Chlorella sorokiniana when using wastewater supplemented with different nitrogen sources., Bioresour. Technol. 168 (2014) 127-35. doi:10.1016/j.biortech.2014.03.064. 13 14 M. Pierra, E. Trably, J.-J. Godon, N. Bernet, Fermentative hydrogen production under [19] 15 moderate halophilic conditions, Int. J. Hydrogen Energy. (2013) 1-10. 16 doi:10.1016/j.ijhydene.2013.08.035. 17 F. Chen, M.R. Johns, Relationship between substrate inhibition and maintenance [20] 18 energy of Chlamydomonas reinhardtii in heterotrophic culture, J. Appl. Phycol. 8 19 (1996) 15-19. 20 J.N. Rosenberg, N. Kobayashi, A. Barnes, E.A. Noel, M.J. Betenbaugh, G.A. Oyler, [21] 21 Comparative analyses of three Chlorella species in response to light and sugar reveal 22 distinctive lipid accumulation patterns in the Microalga C. sorokiniana., PLoS One. 9 23 (2014) e92460. doi:10.1371/journal.pone.0092460. 24 [22] R. Hoshina, Y. Kato, S. Kamako, N. Imamura, Genetic evidence of "American" and 25 "European" type symbiotic algae of Paramecium bursaria Ehrenberg., Plant Biol. 26 (Stuttg). 7 (2005) 526-32. doi:10.1055/s-2005-865912. 27 Y. Yu, C. Lee, J. Kim, S. Hwang, Group-specific primer and probe sets to detect [23] 28 methanogenic communities using quantitative real-time polymerase chain reaction., 29 Biotechnol. Bioeng. 89 (2005) 670-9. doi:10.1002/bit.20347. 30 A. Abbassi-Guendouz, E. Trably, J. Hamelin, C. Dumas, J.P. Steyer, J.-P. Delgenès, et [24] 31 al., Microbial community signature of high-solid content methanogenic ecosystems., Bioresour. Technol. 133 (2013) 256-62. doi:10.1016/j.biortech.2013.01.121. 32 33 J.J. Kozich, S.L. Westcott, N.T. Baxter, S.K. Highlander, P.D. Schloss, Development [25] 34 of a dual-index sequencing strategy and curation pipeline for analyzing amplicon 35 sequence data on the MiSeq Illumina sequencing platform., Appl. Environ. Microbiol. 36 79 (2013) 5112-20. doi:10.1128/AEM.01043-13. 37 P.D. Schloss, S.L. Westcott, T. Ryabin, J.R. Hall, M. Hartmann, E.B. Hollister, et al., [26] 38 Introducing mothur: open-source, platform-independent, community-supported

1 2		software for describing and comparing microbial communities., Appl. Environ. Microbiol. 75 (2009) 7537–41. doi:10.1128/AEM.01541-09.
3 4	[27]	S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipmanl, Basic Local Alignment Search Tool, (1990).
5 6 7 8	[28]	Y. Rafrafi, E. Trably, J. Hamelin, E. Latrille, I. Meynial-Salles, S. Benomar, et al., Sub-dominant bacteria as keystone species in microbial communities producing bio- hydrogen, Int. J. Hydrogen Energy. 38 (2013) 4975–4985. doi:10.1016/j.ijhydene.2013.02.008.
9 10 11 12	[29]	E. Uggetti, B. Sialve, E. Latrille, JP. Steyer, Anaerobic digestate as substrate for microalgae culture: the role of ammonium concentration on the microalgae productivity., Bioresour. Technol. 152 (2014) 437–43. doi:10.1016/j.biortech.2013.11.036.
13 14 15	[30]	N.M.C. Saady, Homoacetogenesis during hydrogen production by mixed cultures dark fermentation: Unresolved challenge, Int. J. Hydrogen Energy. 38 (2013) 13172–13191. doi:10.1016/j.ijhydene.2013.07.122.
16 17 18	[31]	CW. Wu, LM. Whang, HH. Cheng, KC. Chan, Fermentative biohydrogen production from lactate and acetate., Bioresour. Technol. 113 (2012) 30–6. doi:10.1016/j.biortech.2011.12.130.
19 20 21	[32]	Y. Zhu, ST. Yang, Effect of pH on metabolic pathway shift in fermentation of xylose by Clostridium tyrobutyricum., J. Biotechnol. 110 (2004) 143–57. doi:10.1016/j.jbiotec.2004.02.006.
22 23 24 25	[33]	E.A. Assih, A.S. Ouattara, S. Thierry, J.L. Cayol, M. Labat, H. Macarie, Stenotrophomonas acidaminiphila sp. nov., a strictly aerobic bacterium isolated from an upflow anaerobic sludge blanket (UASB) reactor, Int. J. Syst. Evol. Microbiol. 52 (2002) 559–568.
26 27 28 29	[34]	CH. Hung, YT. Chang, YJ. Chang, Roles of microorganisms other than Clostridium and Enterobacter in anaerobic fermentative biohydrogen production systems - A review, Bioresour. Technol. 102 (2011) 8437–44. doi:10.1016/j.biortech.2011.02.084.
30 31 32	[35]	CH. Liu, CY. Chang, Q. Liao, X. Zhu, JS. Chang, Photoheterotrophic growth of Chlorella vulgaris ESP6 on organic acids from dark hydrogen fermentation effluents., Bioresour. Technol. 145 (2012) 331–336. doi:10.1016/j.biortech.2012.12.111.
33 34 35 36	[36]	S. Venkata Mohan, M. Prathima Devi, Fatty acid rich effluent from acidogenic biohydrogen reactor as substrate for lipid accumulation in heterotrophic microalgae with simultaneous treatment., Bioresour. Technol. 123 (2012) 627–35. doi:10.1016/j.biortech.2012.07.004.
37 38	[37]	B. Hu, M. Min, W. Zhou, Z. Du, M. Mohr, P. Chen, et al., Enhanced mixotrophic growth of microalga Chlorella sp. on pretreated swine manure for simultaneous biofuel

1 2		feedstock production and nutrient removal., Bioresour. Technol. 126C (2012) 71–79. doi:10.1016/j.biortech.2012.09.031.			
3 4 5 6	[38]	F. Bumbak, S. Cook, V. Zachleder, S. Hauser, K. Kovar, Best practices in heterotrophic high-cell-density microalgal processes: achievements, potential and possible limitations., Appl. Microbiol. Biotechnol. 91 (2011) 31–46. doi:10.1007/s00253-011-3311-6.			
7 8 9	[39]	Y. Zheng, T. Li, X. Yu, P.D. Bates, T. Dong, S. Chen, High-density fed-batch culture of a thermotolerant microalga Chlorella sorokiniana for biofuel production, Appl. Energy. 108 (2013) 281–287. doi:10.1016/j.apenergy.2013.02.059.			
10 11 12	[40]	J. Swezey, Emended Description of Paenibacillus amylolyticus and Description of Paenibacillus illinoisensis sp . nov . and Paenibacillus chibensis sp . nov ., (1997) 299–306.			
13 14 15 16	[41]	E. Grousseau, E. Blanchet, S. Déléris, M.G.E. Albuquerque, E. Paul, JL. Uribelarrea, Impact of sustaining a controlled residual growth on polyhydroxybutyrate yield and production kinetics in Cupriavidus necator., Bioresour. Technol. 148 (2013) 30–8. doi:10.1016/j.biortech.2013.08.120.			
17 18 19 20	[42]	N. a Logan, E. De Clerck, L. Lebbe, A. Verhelst, J. Goris, G. Forsyth, et al., Paenibacillus cineris sp. nov. and Paenibacillus cookii sp. nov., from Antarctic volcanic soils and a gelatin-processing plant., Int. J. Syst. Evol. Microbiol. 54 (2004) 1071–6. doi:10.1099/ijs.0.02967-0.			
21 22 23 24	[43]	I. Suominen, C. Spröer, P. Kämpfer, F.A. Rainey, K. Lounatmaa, M. Salkinoja- Salonen, Paenibacillus stellifer sp. nov., a cyclodextrin-producing species isolated from paperboard, Int. J. Syst. Evol. Microbiol. 53 (2003) 1369–1374. doi:10.1099/ijs.0.02277-0.			
25 26 27 28 29	[44]	P. Vandamme, E.R.B. Moore, M. Cnockaert, C. Peeters, L. Svensson-Stadler, K. Houf, et al., Classification of Achromobacter genogroups 2, 5, 7 and 14 as Achromobacter insuavis sp. nov., Achromobacter aegrifaciens sp. nov., Achromobacter anxifer sp. nov. and Achromobacter dolens sp. nov., respectively., Syst. Appl. Microbiol. 36 (2013) 474–82. doi:10.1016/j.syapm.2013.06.005.			
30 31	[45]	N. Boutros, N. Gonullu, Ralstonia pickettii traced in blood culture bottles, J. Clin 40 (2002) 2666–2667. doi:10.1128/JCM.40.7.2666.			
32 33	[46]	B. Schiel-Bengelsdorf, P. Dürre, Pathway engineering and synthetic biology using acetogens., FEBS Lett. 586 (2012) 2191–8. doi:10.1016/j.febslet.2012.04.043.			
34					

1

2 6 Figure captions

3 Figure 1. Heterotrophic growth of *Chlorella sorokiniana* on sterilized dark fermentation

4 effluent

- 5 Biomass growth in g $L^{-1}(\bullet)$, acetate concentration in g $L^{-1}(\bullet)$ and butyrate concentration in g
- 6 $L^{-1}(\diamondsuit)$ during heterotrophic growth on sterilized effluent

7 Figure 2. Heterotrophic growth of *Chlorella sorokiniana* on unsterilized dark

- 8 fermentation effluent
- 9 Logarithm of the number of target copies per mL of culture, using chlorophyta specific
- 10 primers for 18S rDNA, during heterotrophic growth on sterilized () and unsterilized ()
- 11 effluent with the acetate concentration in g $L^{-1}(\blacksquare)$ and butyrate concentration in g $L^{-1}(\diamondsuit)$
- 12 during heterotrophic growth on unsterilized effluent.

13 Figure 3. Bacterial growth on unsterilized dark fermentation effluent

- 14 Logarithm of the number of target copies per mL of culture, using bacterial primers for 16S
- 15 rDNA, during growth unsterile (\bullet) effluent with acetate concentration in g L⁻¹(\blacksquare) and
- 16 butyrate concentration in g $L^{-1}(\diamondsuit)$.

17 Figure 4. Bacterial community diversity during aerobic growth on unsterilized dark

- 18 fermentation effluent
- 19 Taxa with less than 2% of individual relative abundance were grouped under "Others".
- 20

Acknowledgements

This study was funded by the National Institute of Agronomic Research (INRA) and the

University of Montpellier II, France.

Table 1.

Classification, relative abundance (%) and physiological characteristics of bacteria at the end of dark fermentation test batches.

ORDER /species (closest known sequence) ¹	Relative abundance (%) ²	Anaerobic or aerobic metabolism	Specific characteristics	Reference
BACILLALES				
Paenibacillus chibensis	3.17 ± 0.1	Strict aerobe	Spore producer Unable to grow on acetate	[40]
Paenibacillus cookii	8.21 ± 0.73	Facultative anaerobe	Spore producer	[42]
Paenibacillus stellifer	10.66 ± 0.91	Facultative anaerobe	Spore producer Unable to grow on acetate	[43]
Sporolactobacillus laevus	16.18 ± 0.65	Strict anaerobe	Spore producer, lactate producer	[30]
BURKHOLDERIALES				
Achromobacter aegrifaciens	3.12 ± 0.32	Strict aerobe	Acetate and butyrate consumer	[44]
Ralstonia pickettii	9.6 ± 0.36	Facultative anaerobe / Strict aerobe	Can grow under anaerobic conditions	[45]
CLOSTRIDIALES				
Clostridium magnum	8.12 ± 0.65	Strict anaerobe	Spore producer, homoacetogene	[46]
Clostridium tyrobutyricum	28.7 ± 0.66	Strict anaerobe	Spore producer, hydrogen producer	[31]
OTHERS ³	12.23 + 1.65			

¹: Names in bold letters correspond to the bacterial orders identified. Names in italics correspond to the closest genetically known sequences, for all sequences, percentages of identity to reference sequence were greater or equal to 97%.

²: Relative abundances were calculated by dividing the number of sequences for the taxon by the total number of sequences per sample. The values are the mean and standard deviation of the sequences from the three samples.

³: Taxa with less than 2% relative abundance were grouped under "Others".







Figure 4 (2-column fitting image)









Figure 4 black and white Figure 4 (2-column fitting image)



Graphical Abstract



Highlights

- Microalgae can outcompete dark fermentation bacteria for acetate uptake.
- Butyrate is not used by *Chlorella sorokiniana* in heterotrophic conditions.
- Bacteria originating from dark fermentation effluent can degrade butyrate.