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Abstract

Coupling dark fermentation (DF), which produces hydrogen from diverse effluents or solid waste, and heterotrophic cultivation of microalgae, which produces lipids, carbohydrates and proteins, is a promising and innovative solution for developing sustainable biorefineries. The use of a raw DF effluent, containing acetate and butyrate, to support the heterotrophic growth of *Chlorella sorokiniana* was investigated. All the acetate in sterilized and unsterilized DF 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 yield on acetate of 55%. The algal yield was higher than previously reported for synthetic DF effluent. It was concluded that compounds other than volatile fatty acids were present in the DF effluent and these could be consumed by the microalgae. After the acetate had been exhausted, butyrate was consumed by facultative and strict aerobic bacteria originating from the DF effluent. The concentration of the bacterial community increased during the experiment but did not have any significant impact on heterotrophic microalgae growth. A high microalgal biomass yield was achieved without requiring the DF effluent to be sterilized.

Keywords

Biohydrogen, Heterotrophic cultivation, Dark fermentation, Volatile Fatty Acids (VFAs), Bacterial diversity, *Chlorella sorokiniana*

1 Introduction

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

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

fatty acids (VFAs) [8]. DF effluents also contain substantial amounts of nitrogen and phosphorus that are required to sustain the heterotrophic growth of microalgae. Cho *et al* pointed out that DF effluent can be regarded as a good medium for growing heterotrophic microalgae in a biorefinery [9].

Recent studies investigating microalgae growth on a synthetic DF effluent medium showed very promising results. When grown heterotrophically on a mixture of acetate and butyrate, *Chlorella protothecoides* reached a carbon yield, (g carbon of biomass per g carbon of VFAs) of 34% and a lipid content of 48% of cellular dry weight (CDW) [3]. Turon *et al.* [10] reported that *Chlorella sorokiniana* could grow heterotrophically on acetate with a growth rate of 2.23 d^{-1} and a carbon yield of 42% and on butyrate with a much lower growth rate of 0.16 d^{-1} and a carbon yield of 56%. Recent studies showed that heterotrophic microalgae growth was possible using sterilized DF effluents. For example, heterotrophic *Scenedesmus sp.* produced lipids up to 41% of CDW using acetate from sterilized DF effluent containing ethanol but no butyrate [7]. *Chlorella vulgaris* was grown in heterotrophic conditions on diluted DF effluent containing acetate and butyrate, converting VFAs efficiently into carbohydrates (51% CDW) [11]. Furthermore, *Chlorella sp.* was recently reported to produce lipids up to 26% of CDW under mixotrophic conditions with raw DF effluent as a medium [12]. For both raw and synthetic effluents, butyrate concentration has been identified as a key factor driving the effective coupling of DF and heterotrophic cultivation of microalgae under heterotrophic or mixotrophic conditions. Although acetate can be efficiently converted into lipids, butyrate uptake by microalgae is much slower and can reduce the microalgae growth when both VFAs are present. This problem can be solved either by increasing the initial microalgae biomass or by increasing the initial acetate:butyrate ratio [11,13].

To couple DF and heterotrophic cultivation of microalgae efficiently, the cost of effluent sterilization has to be reduced. According to Park *et al*, sterilization of the medium accounts

for more than a quarter of the investment cost of the process when coupling DF and oleaginous yeast cultivation [4]. The importance of being able to use an unsterilized medium to support microalgae growth was also emphasized by Ramos Tercero *et al.* [14]. Bacterial contamination is one of the main challenges to be faced for upscaling heterotrophic cultivation [15]. During heterotrophic cultivation, the competition between microalgae and bacteria is usually found to be unfavorable to microalgae, limiting the availability of carbon, nitrogen, phosphate and oxygen [16,17]. Nevertheless, these authors suggested that some conditions may be favorable to microalgae growth, such as a low initial bacterial density and high initial nutrient loads.

This work set out to determine, for the first time, the role and importance of the bacterial community present in raw DF effluent on microalgae growth for coupling DF and microalgal heterotrophic cultivation. *Chlorella sorokiniana* was used as a model for heterotrophic microalgae because of its high growth rate on acetate and its ability to produce high amounts 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 for the unsterilized DF effluent.

2 Materials and methods

2.1 Dark fermentation test batches

Five identical test batches of “DF effluent” were produced simultaneously in 600 mL glass 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. 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, http://www.ccap.ac.uk/). The flasks were inoculated with 1 mL of heat-treated (15 min at 90 °C) anaerobic sludge from an anaerobic digester treating waste from a sugar processing 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 initial inoculum (in g of total volatile solids L^{-1}). To ensure anaerobic conditions, the flasks were sealed and flushed with nitrogen gas as described elsewhere [19]. The pH was adjusted to 6 and the bottles were incubated at 37 °C until the glucose was completely exhausted. At the end of the growth phase, i.e. glucose exhaustion and hydrogen accumulation, the five anaerobic cultures batches were mixed to produce the "DF effluent". The pH of the DF effluent was increased to 6.5 with 1 M NaOH. VFAs, and the ammonium and phosphate concentrations were measured. Half of the DF effluent was centrifuged three times at 15,000 rpm for 15 min. A fraction of the supernatant was sterilized using Acrodisc® PF syringe filter with 0.8/0.2 µm pores (PALL). The sterilized and unsterilized DF effluent samples were then stored at 4 °C until the start of the experiment.

2.2 Axenic microalgae strain and preparation of the microalgae stock culture

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

2.3 Heterotrophic microalgae growth on dark fermentation effluent

A fixed volume of either the sterilized or the unsterilized DF effluent (36 mL) was placed in 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 rotary shaker (150 rpm) at 25 °C for 10 days in complete darkness. A 1 mL sample of the culture was taken every day to measure the optical density (OD), VFA concentration, microbial concentration and diversity. The experiment was carried out in triplicate. During the whole of the experiment, the microalgae cultures in the sterilized DF effluent were checked for other living organisms every day by DAPI counterstaining and contrast phase microscopy.

2.4 Microbial analysis

2.4.1 Microalgae biomass measurement

The microalgae growth was quantified on sterilized DF effluent by measuring the OD at 800 nm (OD_{800}) as described by Turon *et al* [10]. The relationship between the cellular dry weight (CDW) and OD_{800} 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.

$$CDW\ (g\ L^{-1}) = 1.24 * OD_{800}\ (R^2 = 0.95).$$

For calculating the biomass yield, the carbon content was estimated at 50% of microalgae biomass [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

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

2.4.2 DNA extraction and purification

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

2.4.3 Quantitative PCR for microalgae (18S rDNA gene)

The microalgae biomass was quantified using quantitative PCR (qPCR) and specific primers for *Chlorophyta* 18S rDNA INT-4F (5' TGGTGAAGTGTTCGGATTGG 3') and INT-5R (5' ARGTG GGAGG GTTTA ATGAA 3') as described by Hoshina *et al* [22]. The quantitative amplification reaction was carried out with 5 µL of DNA sample, 12.5 µL of Universal SYBR® Green Supermix (Biorad) (composed of polymerase, dNTPs and SYBR® Green dye), 1 µL of forward primer INT-4F, 1 µL of reverse primer INT-5R and 5.5 µL of H_2O, for a total volume of 25 µL. The PCR was run in a 100 Touch™ thermal cycler equipped with a CFX96™ Real-Time System (Bio-rad). There was an initial incubation of 3 min at 95 °C followed by 40 cycles of denaturation-amplification (10 s at 95 °C and 30 s at 56 °C). Data analysis was carried out with the Bio-rad CFX Manager software, version 3.0. A linear standard curve was obtained from each assay by amplification of eight 10-fold dilutions of a reference plasmid to count the number of 18S rDNA copies accurately. The reference plasmid (pEX-A2 plasmid, Eurofins MWG Operon) was created using the *Chlorella sorokiniana* 18S rDNA sequence published in the European nucleotide archive under the sequence number X62441.2 (ENA, http://www.ebi.ac.uk/ena). The PCR product from the reference plasmid and *Chlorella sorokiniana* DNA extracts corresponded to a 77 bp sequence (from 1666 to 1742 NT):

TGGTGAAGTGTTCGGATTGGCGACCGGGGGCGGTCTCCGCTCTCGGCCGCCGA

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

2.4.4 Quantitative PCR for bacteria (16S rDNA)

For quantification of total bacteria, general bacterial primers, BAC338F and BAC805R, and the associated probe, BAC16F, were used as described elsewhere [23]. The quantitative amplification reaction was carried out in a total volume of 12.5 µL with 2 µL of sample DNA, 6.5 µL of Universal probes Supermix (Biorad), 0.5 µL of each primers and the probe and 2.5 µL of H_2O. The thermal cycler and analysis method were the same as for qPCR of *Chlorophyta*. An initial incubation of 2 min at 95 °C followed by 40 cycles of denaturation-amplification (7 s at 95 °C and 25 s at 56 °C) were performed. The linear standard curve was obtained as described elsewhere [24]. The total number of 16S rDNA gene copies per sample was expressed as the logarithmic value of the number of target copies per mL of culture sample.

2.4.5 Sequencing of bacterial 16S rDNA

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

2.5 Chemical analysis

Acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate and caproate were measured using a gas chromatograph (GC 3900 Varian) equipped with a flame ionization detector. Other non-volatile molecules such as glucose, ethanol, lactate and formate were quantified 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].

Biogas production of the DF test batches was monitored daily by measuring the gas pressure in the headspace. The biogas composition (CO_2, O_2, H_2, N_2 and CH_4) was measured using a gas chromatograph (Clarus 580, Perkin Elmer) coupled to a thermal conductivity detector (TCD) [28].

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

3 Results and discussion

3.1 Dark fermentation test batches

DF anaerobic test batches were carried out to provide a culture medium for subsequent heterotrophic microalgae growth. The fermentation ended with complete glucose exhaustion, 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, 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 ± 0.18 mol H_2 mol $glucose^{-1}$. The hydrogen yield was consistent with previously reported experimental results obtained in 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 H_2 mol $VFAs^{-1}$. This value is lower than

the theoretical value of 2 mol H_2 mol $VFAs^{-1}$ produced through the acetate pathway (eq (1)) or the butyrate pathway (eq (2)). In general, the H_2 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 H_2 through homoacetogenesis, i.e. the production of acetate via CO_2 and H_2 consumption (eq (3)) [28]. Since neither lactate nor ethanol was found, only homoacetogenesis could have occurred.

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

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

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O \quad (3)$$

Three samples of DF effluent were sequenced for microbial community characterization. The closest phylogenetically known sequences of the most representative OTUs are given in Table 1. About 53 ± 1.5 % of initial bacteria were strict anaerobes, with 36.8 ± 1.3% related to *Clostridium* sp. and 16.2 ± 0.6% to *Sporolactobacillus* sp. It has been established that bacteria belonging to *Clostridium* genus are responsible for hydrogen fermentation. *Sporolactobacillus* sp. are known for their ability to degrade glucose to lactate in anaerobic conditions [30]. Since no lactate was found in the medium and *Clostridium tyrobutyricum* was the most abundant species, this suggested that lactate might have been converted into butyrate and hydrogen, as previously reported [31]. *C. tyrobutyricum* is also known to use the butyrate pathway preferentially during DF. The presence of this species would explain the high butyrate:acetate molar ratio, 1.14, found at the end of DF [32].

The remaining bacteria were either facultative or strict aerobic species. Strict aerobes were previously found in an anaerobic digester, even non-spore producing species [33]. Even though the anaerobic sludge was heat treated before the fermentation, facultative and strict aerobic bacteria may have survived, as reported elsewhere [30]. They in fact play an

important role in removing residual oxygen in anaerobic systems, which subsequently favors the growth of *Clostridium* species and hydrogen production [34].

3.2 Microalgae growth on raw fermentation effluent

3.2.1 Characterization of the dark fermentation effluent

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 sterilized and unsterilized DF effluent samples were 1.25 ± 0.06 g L^{-1} and 1.26 ± 0.03 g L^{-1}, respectively. The acetate:butyrate mass ratio (g g^{-1}) was about 0.6 in both cases. These concentrations as well as the acetate:butyrate ratio were consistent with previous studies using raw DF effluents to sustain microalgae growth [11,35,36]. Sterilization by microfiltration had no effect on the carbon, nitrogen and phosphorus contents of the effluent. The C:N:P molar ratios in the sterilized and unsterilized DF effluent were 35:6.7:1 and 36:6.5:1, respectively. These ratios were lower than the Redfield C:N:P ratio for phytoplankton cellular composition, i.e. 106:16:1. Here, only the carbon substrate was assumed to be the limiting element for *C. sorokiniana* growth.

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 microalgae growth [12,37]. In acidogenic fermentation, VFA accumulation is targeted rather than biohydrogen production and hydrogen conversion to acetate through homoacetogenesis is promoted. Nevertheless, the effluents from acidogenic fermentation have to be diluted between 8 and 20 fold before use to avoid excess initial VFA concentrations inhibiting microalgae growth [12,37].

3.2.2 Microalgae growth on sterilized and unsterilized dark fermentation effluent

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

The maximum growth rate, μ_{max}, of the microalgae was assessed during the exponential growth phase and was 1.75 ± 0.14 d^{-1} on sterilized DF effluent. This μ_{max} value was consistent with previously reported growth rates of *C. sorokiniana* on acetate under heterotrophic

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

3.3 Bacterial growth and diversity during microalgae cultivation on raw fermentation effluent

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

butyrate degradation, the butyrate concentration was probably too high to support microalgae growth, as previously suggested [10,35]. No competition was observed between microalgae and bacteria for butyrate. The bacterial community was responsible for the entire butyrate degradation. Butyrate exhaustion by bacteria could be very useful for the efficient cultivation of microalgae on DF effluent. To produce high densities of microalgae and/or lipids, fed-batch cultivation has been suggested, i.e. sequential addition of medium after exhaustion of the substrate or when the growth plateau is reached [38]. For biomass production, this technique avoids growth inhibition due to high initial substrate concentration and substrate 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 heterotrophic cultivation of *C sorokiniana* on glucose [39]. If such operational conditions were applied using sterilized DF effluent, butyrate would accumulate as a result of the inability of *C. sorokiniana* to consume butyrate rapidly and would eventually lead to growth inhibition [10]. Therefore, butyrate degradation by bacteria may be beneficial for lipid production by heterotrophic microalgae, suggesting that bacteria may have a positive effect, if well managed, for upscaling using unsterilized DF effluent.

The bacterial community in the raw effluent was dominated by 13 OTUs each with a relative abundance of more than 2% at the start of the experiment, at acetate exhaustion or at the end of the experiment. As shown in Figure 4, there were significant shifts in the bacterial community during the experiment. The dominant bacterial species shifted from strict anaerobes (*Clostridium sp.* and *Sporolactobacillus sp.*) to facultative anaerobes (*Paenibacillus sp.*) and then to strict aerobes (*Stenotrophomonas maltophilia*). The dominant bacteria at the beginning of the experiment (over 73% of the total bacteria abundance) accounted for less than 3.5% of the bacteria present at day 2.7 when a species close to *Paenibacillus chibensis* was dominant (over 60% of total bacteria) in two of the replicates (Figure 4). In only one of

the three replicates (flask 3), a high abundance (42.5%) of a species close to *Lysinibacillus xylanilyticus* was observed with a reduction in the abundance of a species close to *Paenibacillus chibensis, (*36.2%). According to Swezey *et al*. [40], *Paenibacillus chibensis* is unable to grow on acetate. The presence of a dominant species closely related to *Paenibacillus chibensis* (99% identity) suggested that acetate exhaustion by this species was unlikely. Because acetate degradation was very similar in all replicates when unsterilized DF effluent was used (Figure 3), the emergence of a different species closely related to *Lynsinibacillus xylaniticus* in only one of the replicates, suggested that this species was probably not involved in the exhaustion of acetate. These two observations were consistent with and reinforced our previous suggestion that *C. sorokiniana* was highly competitive and mainly responsible for acetate exhaustion.

At the end of the experiment, after complete butyrate exhaustion in the three replicate flasks, species close to *Stenotrophomonas maltophilia* and *Cupriavidus necator*, both strict aerobic species, were largely dominant (Figure 4) [33]. *Cupriavidus necator* is known to assimilate butyrate and then produce polyhydroxyalkanoates [41]. The emergence of these bacterial species during this period, during which no growth of *C. sorokiniana* was observed, confirmed that strict aerobic bacterial species initially present in the raw DF effluent were 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.

4 Conclusions

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

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6 Figure captions

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

Biomass growth in g L^{-1} (●), acetate concentration in g L^{-1}(■) and butyrate concentration in g L^{-1} (♦) during heterotrophic growth on sterilized effluent

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

Logarithm of the number of target copies per mL of culture, using chlorophyta specific primers for 18S rDNA, during heterotrophic growth on sterilized (●) and unsterilized (●) effluent with the acetate concentration in g L^{-1}(■) and butyrate concentration in g L^{-1} (♦) during heterotrophic growth on unsterilized effluent.

Figure 3. Bacterial growth on unsterilized dark fermentation effluent

Logarithm of the number of target copies per mL of culture, using bacterial primers for 16S rDNA, during growth unsterile (●) effluent with acetate concentration in g L^{-1}(■) and butyrate concentration in g L^{-1} (♦).

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

Taxa with less than 2% of individual relative abundance were grouped under “Others”.

Acknowledgements

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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 1_in_color

Figure 1 (*single column fitting image)*

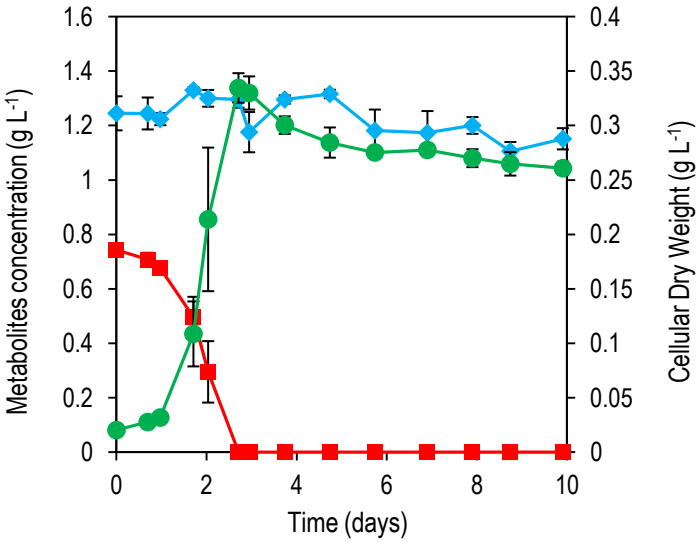

Figure 2_in_color

Figure 2 *(single column fitting image)*

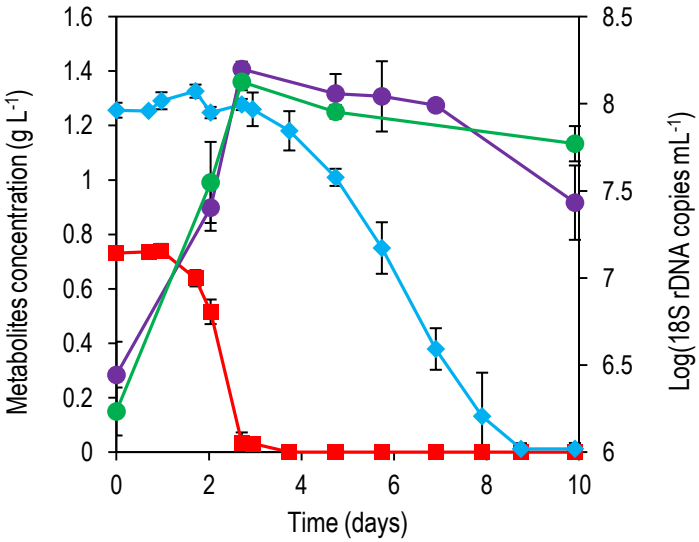

Figure 3_in color

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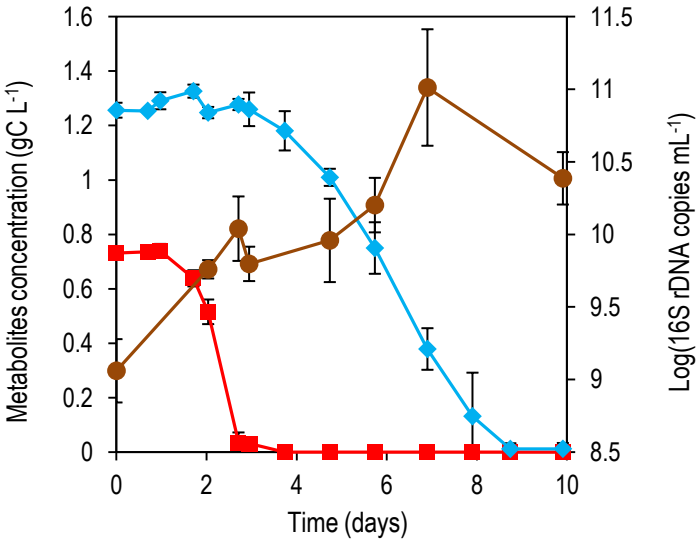

Figure 4_in_color

Figure 4 (2-column fitting image)

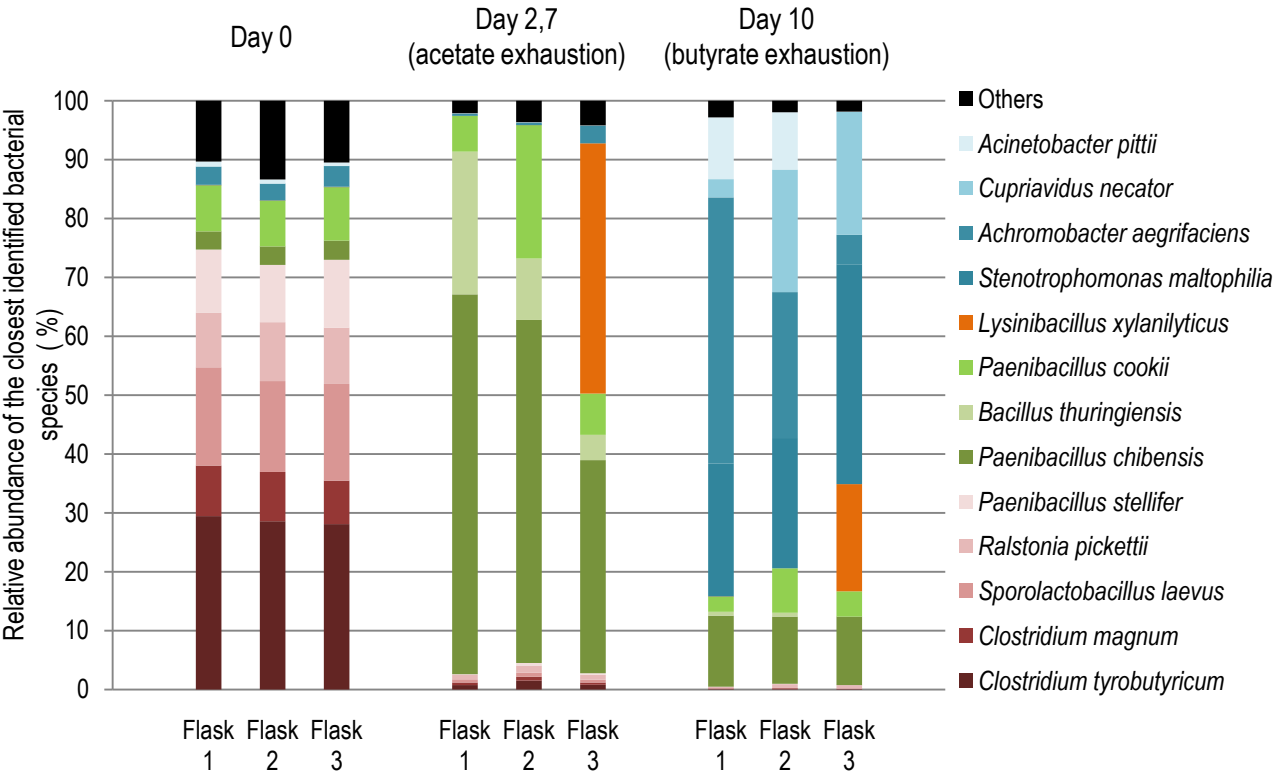

Figure 1_black_and_white

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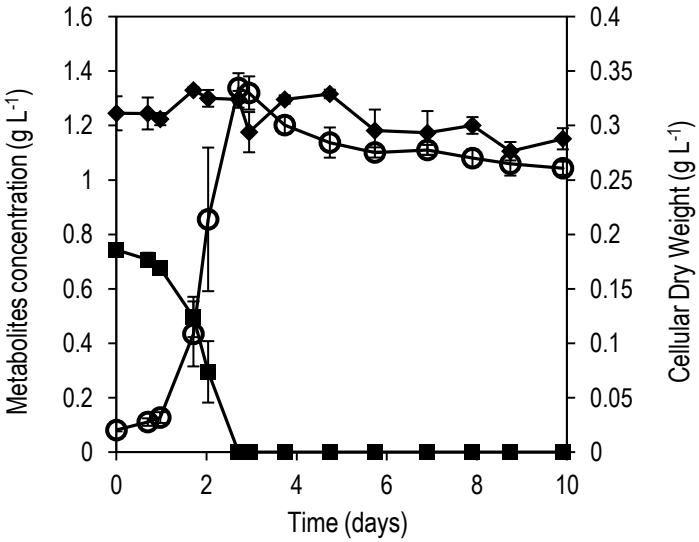

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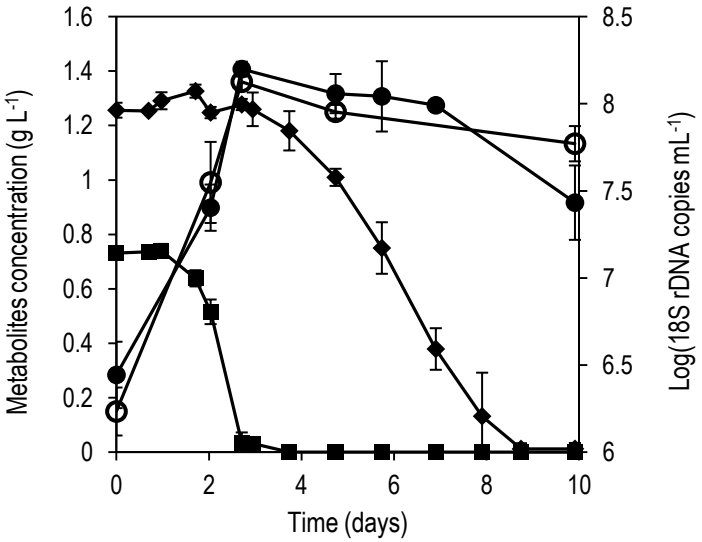

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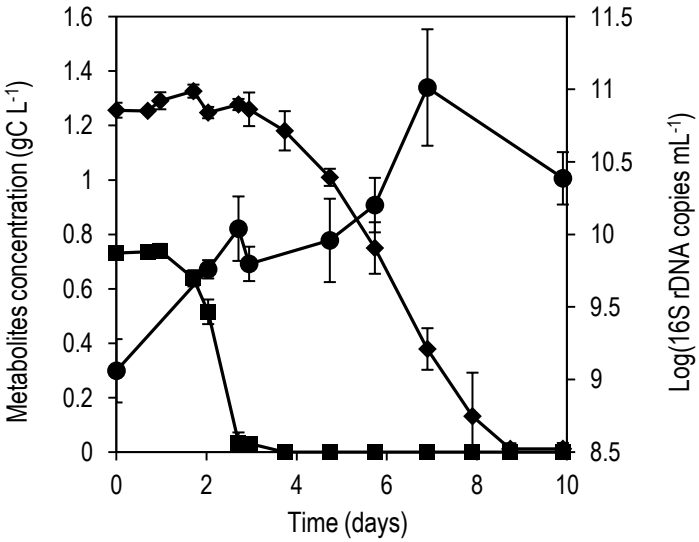

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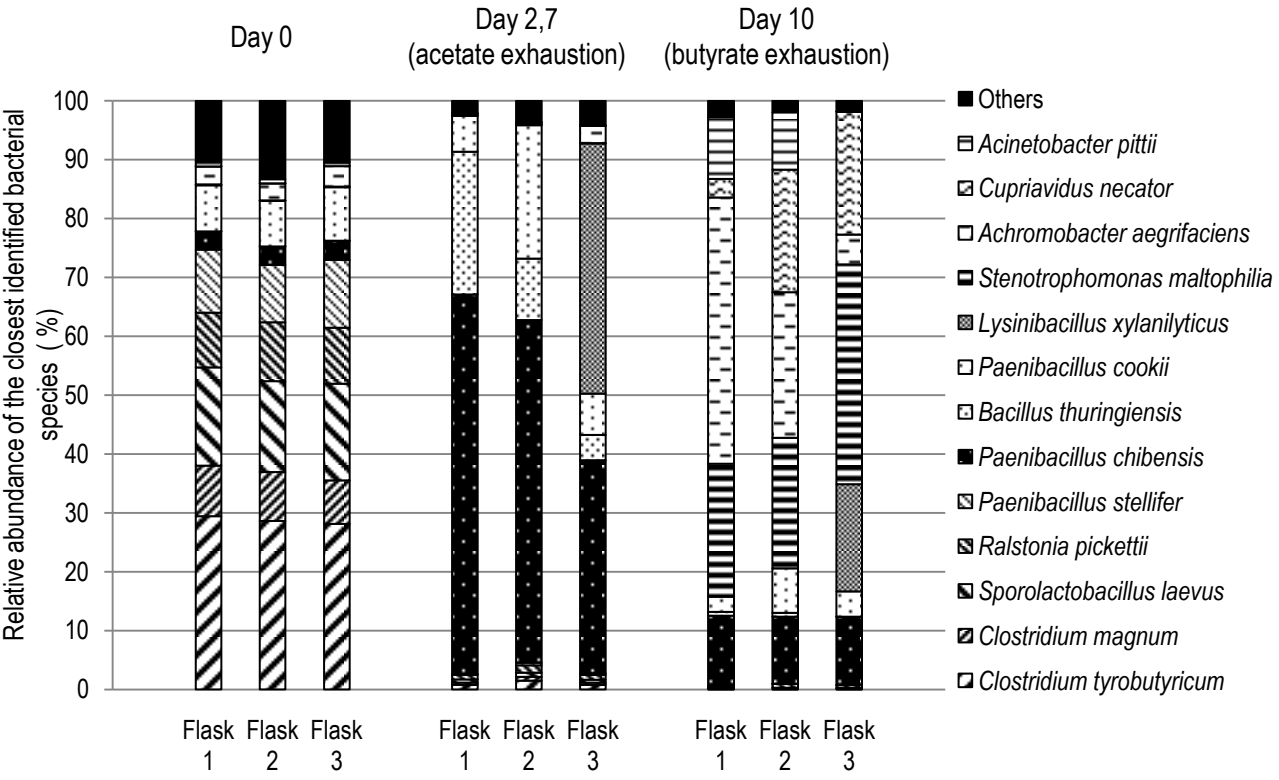
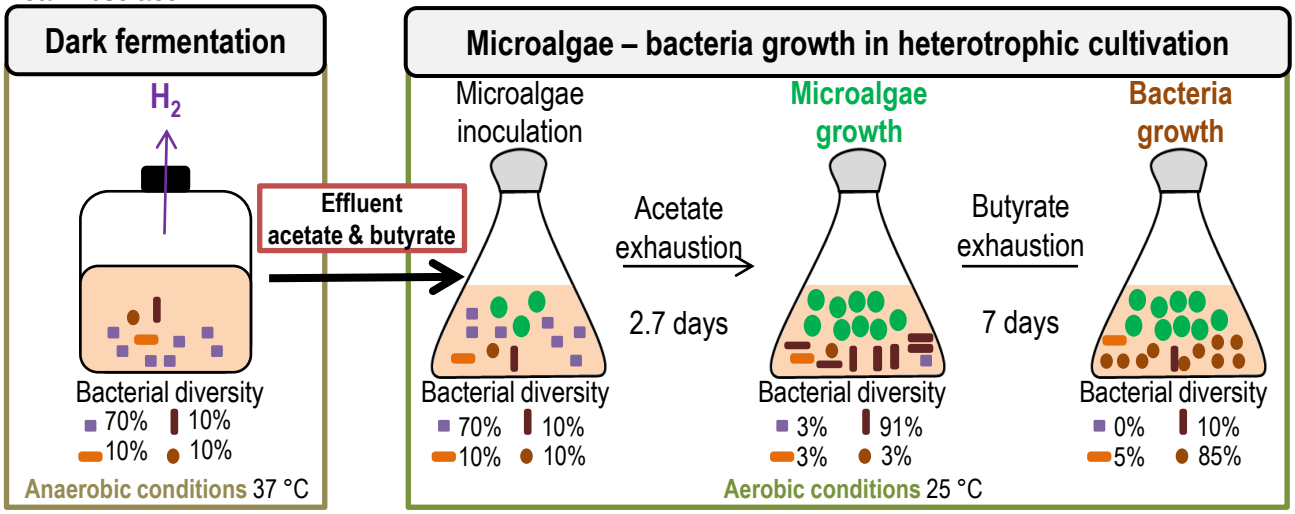


Graphical Abstract



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.