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

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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.

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

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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. *C. 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

C. 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