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Production and storage of biohydrogen during sequential batch fermentation of *Spirogyra* hydrolyzate by *Clostridium butyricum*

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ABSTRACT

The biological hydrogen production from *Spirogyra* sp. biomass was studied in a SBR (sequential batch reactor) equipped with a biogas collecting and storage system. Two acid hydrolysis pre-treatments (1N and 2N H_2SO_4) were applied to the *Spirogyra* biomass and the subsequent fermentation by *Clostridium butyricum* DSM 10702 was compared. The 1N and 2N hydrolyzates contained 37.2 and 40.8 g/L of total sugars, respectively, and small amounts of furfural and HMF (hydroxymethylfurfural). These compounds did not inhibit the hydrogen production from crude *Spirogyra* hydrolyzates. The fermentation was scaled up to a batch operated bioreactor coupled with a collecting system that enabled the subsequent characterization and storage of the biogas produced. The cumulative hydrogen production was similar for both 1N and 2N hydrolyzate, but the hydrogen production rates were 438 and 288 mL/L.h, respectively, suggesting that the 1N hydrolyzate was more suitable for sequential batch fermentation. The SBR with 1N hydrolyzate was operated continuously for 13.5 h in three consecutive batches and the overall hydrogen production rate and yield reached 324 mL/L.h and 2.59 mol/mol, respectively. This corresponds to a potential daily production of 10.4 L H_2/L *Spirogyra* hydrolyzate, demonstrating the excellent capability of *C. butyricum* to produce hydrogen from microalgal biomass.

1. Introduction

The demand for alternative energy sources has been growing in the last few decades. H_2 (hydrogen) is an interesting alternative to the increasingly scarce and polluting fossil fuels as it is easily and efficiently converted into energy. Furthermore, it has a high heating value (142–120 MJ/kg) and produces only water as a by-product [1]. Hydrogen can be produced biologically ($bioH_2$) through anaerobic fermentation of organic substrates, particularly those rich in carbohydrates [2], a process known as DF (dark fermentation). The production of $bioH_2$ is also accompanied by the production of organic acids with industrial use [3].

Microalgae have been recently regarded as a prime substrate for third generation bioconversion processes [4,5]. They are photosynthetic CO_2 (carbon dioxide) consuming organisms, easy to grow in fresh, salt or wastewater streams and capable of storing carbohydrates and lipid compounds in the intracellular space and as part of the cell walls [6,7]. Microalgae also display high photosynthetic efficiencies, can be cultivated all year round and harvested daily, and grow faster when compared to higher plants, without the need for arable land [5,7]. Furthermore, the use of microalgal biomass as a feedstock for DF is recommended, as the CO_2 produced in the fermentation can be reused for the autotrophic culture of microalgae [4,8]. Several studies have already shown that this type of feedstock is adequate and easily convertible in a $bioH_2$ production setting. *Scenedesmus obliquus*, a microalga with the capacity to store glucose-based carbohydrates, was efficiently converted into hydrogen by *Clostridium butyricum* and *Enterobacter aerogenes* [9,10]. The production process required no biomass pre-treatment, relied solely on wet or dry microalgal biomass as a carbon and energy source and the hydrogen production yield by *C. butyricum* and *E. aerogenes* was 69% and 34% of the maximum theoretical yield from glucose (4 mol/mol), respectively. In a similar way, the thermophilic *Thermotoga neapolitana* and *C. butyricum* CGS5 were able to produce hydrogen from pre-treated *Chlamydomonas reinhardtii* and *Chlorella vulgaris* biomass, respectively [11,12]. Comparatively, the high-potential for sugar accumulation that the microalga *Spirogyra* presents, makes it extremely adequate as a fermentative substrate. In a recently proposed model of a microalgae biorefinery for hydrogen and pigments production, 156 mL H_2/g of total sugars

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were produced from milled *Spirogyra* biomass without any acid or enzymatic pre-treatment [13].

The referred studies were performed, in their vast majority, in batch systems, which are relatively simple to set-up while allowing an easy monitoring of the biogas production. Nevertheless, the dark fermentation for industrial hydrogen production should be addressed at a larger scale and under a continuous production mode, such as in CSTR (continuous or intermittently stirred tank reactors) or SBR (sequential batch reactors). These systems are much more reliable for an in-depth characterization of the kinetic behavior of the hydrogen production from microalgal biomass [4]. Liu et al. [14] adapted a CSTR for the fermentation of *C. vulgaris* by *C. butyricum* CGS5, in which the microalgal biomass was converted into hydrogen, and the CO_2 and organic acids were recirculated as nutrients for the microalga culture. By using *C. vulgaris* acid hydrolyzate as carbon and energy source, the maximum hydrogen production rate achieved approximately 250 mL/L.h. Jung et al. [15] developed a two-stage fermentation system that included a stable intermittent-CSTR for hydrogen production from pre-treated *Laminaria japonica* biomass, a brown macroalga. Under optimal conditions, the hydrogen production rate attained was 3.1 L/L.d. While both CSTR and SBR are valid systems for large-scale hydrogen production from microalgal biomass, the SBR is less prone to cellular wash-out, requiring little equipment maintenance and operator control, and it is capable of supporting strong variations in hydraulic and organic loadings [16]. A semi-continuous fermentation was successfully conducted by Xia et al. [4] for the fermentation of acid pre-treated *Chlorella pyrenoidosa* biomass to produce hydrogen. The fermentation was stable for a total period of 22 days, and the hydrogen production reached 51.7–62.0 mL/g total volatile solids once the microbial community was stabilized.

In this work, a sequential batch fermentation system was developed to monitor in detail the hydrogen production throughout the fermentation of *Spirogyra* biomass by *C. butyricum*. The system enabled the continuous removal, collection, and further characterization and storage of the produced biogas. *Spirogyra* sp. was chosen as the fermentative substrate, due to its capability to accumulate starch at a high concentration (up to 64%) [17]. Two biomass pre-treatments consisting of acid hydrolysis with 1N and 2N H_2SO_4 were compared and both hydrogen production yields and rates were evaluated. The best hydrolysis condition was used for SBR operation, targeting the establishment of a reliable and scalable system of hydrogen production. This is the first report of bioH_2 production from *Spirogyra* biomass in bioreactor. The stability of the SBR operation was established, and the *C. butyricum* performance as cell factory adapted to this crude microalgal hydrolyzate was assessed for future process optimization.

2. Materials and methods

2.1. Spirogyra culture and biomass hydrolysis

The microalga used in this work was *Spirogyra* sp. (division *Chlorophyta*, family Zygnemaceae) acquired from SAG (Sammlung von Algenkulturen Göttingen) of the University of Göttingen (reference: SAG 170.80). The microalga was grown in an open raceway pond and the biomass was harvested and dried as previously described [13]. The biomass was characterized in terms of moisture, total sugars, crude protein, fat, and ash contents, according to the A.O.A.C standard methods [18]. The *Spirogyra* biomass used in this work had the following average composition (% (w/w) dry weight basis): 45.1 ± 4.2% total sugars, 22.0 ± 0.3% crude protein, 3.6 fat ± 0.1%, 25.9 ± 0.3% ash and 3.4% others (by difference).

The microalgal biomass was submitted to two conditions of acid hydrolysis with H_2SO_4 1N for 60 min and H_2SO_4 2N for 30 min. Both were performed at 121 °C with a solid to liquid ratio of 1:7.5. After neutralization with NaOH, the hydrolyzates were sterilized through filtration (0.2 μm, Pall Life Sciences, USA) and kept at 4 °C.

2.2. C. butyricum culture conditions

The strain *C. butyricum* DSM 10702 was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). *C. butyricum* was pre-cultured in RCM (reinforced Clostridial medium, Difco Laboratories, USA), prepared under anoxic conditions, with replacement of the gas phase by nitrogen gas by using a gassing manifold system. The MCM (modified Clostridial medium) was adapted from RCM and was composed of 10 g/L of TSB (trypticase soya broth) without dextrose (Difco Laboratories, USA), 5 g/L of sodium chloride (Panreac Quimica SA, Spain), 5 g/L of yeast nitrogen base (Fluka Analytical, USA), 3 g/L sodium acetate (Pronalab, Portugal) and 0.5 g/L cysteine hydrochloride (Merck, Germany) in 50 mM of phosphate buffer at a pH 6.8.

2.3. Fermentation experiments

2.3.1. Small scale assays

Small scale fermentations were performed in 120 mL serum flasks prepared under anoxic conditions and stoppered with butyl rubber stoppers. Each flask contained 20 mL of MCM medium supplemented with starch or *Spirogyra* hydrolyzate added at a known volume to obtain an initial sugar concentration of approximately 5 ± 1 g/L. Fourteen serum flasks were prepared for each condition and two independent flasks were analyzed at each sampling time (t = 0, 6, 12, 18, 24, 30 and 36 h) for a total incubation time of 36 h. At the referred time, gas samples were removed through the stoppers with a glass-tight syringe for GC (gaseous chromatography) analysis. Subsequently, the flasks were opened and the pH was measured with a pH meter (Micro pH 2002, Barcelona, Spain). The liquid fractions were filtered through 0.2 μm (Pall Life Sciences, USA) prior to HPLC (high-pressure liquid chromatography) analysis and total sugar quantification by the phenol-sulphuric acid method [19]. The filters were dried at 100 °C for 16 h and weighed for cell dry weight quantification.

2.3.2. Bioreactor set-up

A 1.65 L lab scale double jacketed bioreactor with a working volume of 500 mL was used for hydrogen production. The bioreactor was fitted with a pH electrode, one inlet for nitrogen gas, a second inlet for the liquid medium and/or microalgal hidrolyzate, one exit for the effluent biogas connected to the biogas collecting system, and one additional exit for the liquid fermentate when operated as SBR (Fig. 1). The biogas collecting and storage system was based on the principle of water displacement. It comprised an inverted serum flask filled with water, closed with a butyl rubber stopper and sealed with an aluminum cap through which two 21G needles were inserted (Fig. 1). One of the needles was connected to the biogas exit of the bioreactor through a neoprene tube, to allow the flow of the produced biogas to enter into the collecting flask. As this took place, water was displaced through the second needle. The volume of the biogas was determined by the volume of water displaced and collected in a measuring cylinder.

The MCM was sterilized by autoclave (30 min, 121 °C) inside the bioreactor whereas the microalgal hydrolyzates were filter-sterilized (0.2 μm, Pall Life Sciences, USA) into a communicating vessel that was connected to the liquid inlet of the bioreactor. The hydrolyzates were added to the bioreactor at a known volume that