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Technical Communication

Growth and hydrogen production characteristics of *Caldicellulosiruptor saccharolyticus* on chemically defined minimal media

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ABSTRACT

Caldicellulosiruptor saccharolyticus is an extreme thermophilic bacterium recognized for its saccharolytic ability and superior ability to produce high yields of hydrogen. However, most studies have been made using yeast extract (YE) as a rich but expensive nutrient source. For the first time, we show that *C. saccharolyticus* is able to grow on defined minimal media, including essential vitamins, provided that CO_2 was allowed to accumulate sufficiently in the culture broth to activate growth. Growth and hydrogen production performance on minimal media was analyzed in both batch and continuous mode. Absence of YE resulted in similar or higher hydrogen yields and specific hydrogen productivities but lower volumetric hydrogen productivities than with YE. The results also indicate that YE is used as a carbon- and energy source thus affecting metabolic flux calculations. This study clarified that YE is not essential making *C. saccharolyticus* more attractive for fundamental studies on its metabolism and future industrial exploitation.

1. Introduction

Biological hydrogen production from cellulosic feedstock is considered one of the promising avenues for future bioenergy production. Major economical issues for such a process, however, are the low substrate conversion efficiency [1,2] and high chemical cost [2]. The extreme thermophilic bacterium *Caldicellulosiruptor saccharolyticus* can produce hydrogen at yields close to the theoretical maximum of the dark fermentation process (i.e., 4 mol H_2/mol hexose) [3]. The organism is able to ferment an array of mono-, di- and polysaccharides [4–8]and is relatively tolerant to high partial hydrogen pressures, making it a promising candidate for exploitation in a biohydrogen process [9]. It has also been subjected to several fundamental studies due to (i) its extraordinary energy metabolism where pyrophosphate plays a central role [10], (ii) its carbon source-dependent redox metabolism [11]and (iii) its strong link between its redox- and energy metabolism [9,12]. So far metabolic and growth analysis has been performed on media supplemented with yeast extract (YE). The use of YE increases the overall cost of the process significantly [2]. In addition, herein we demonstrate that YE can be used as a carbon- and energy source. Therefore, to adequately estimate metabolic fluxes it is essential to

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grow cells on chemically defined media. Defined compounds replacing YE that can support both growth and high hydrogen yields are therefore required. In the current study the specific nutrient requirements of *C. saccharolyticus* are specified and the effect of YE on hydrogen and by-products formation is evaluated.

2. Material and methods

2.1. Microorganism

C. saccharolyticus DSM 8903 was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Routine subcultures and inoculum development were conducted in 250-mL serum bottles containing 50-mL of medium.

2.2. Medium composition

The effect of nutrient composition on growth and hydrogen production was evaluated in four different medium compositions, namely (i) the DSM 640 medium including YE (1 g/L) as previously described [13], (ii) the DSM 640 medium but replacing YE with peptone (1 g/L), (iii) the DSM 640 medium excluding YE and peptone and (iv) the DSM 640 medium excluding YE and peptone but with a vitamin solution defined elsewhere [14]. Anoxic solution of glucose was autoclaved separately and added to the sterile medium at the required concentration, i.e. 10 g/L in batch cultures and 4 g/L in serum flasks and continuous cultures.

2.3. Fermentation setup

For cultivation on minimal media, cultures were continuously re-cultivated (five subcultures) in serum flasks (250 mL flask, with 50 mL working volume), which were sparged with nitrogen gas and closed with a rubber stopper, prior to inoculation. Cultures from the fifth subculture were then inoculated in a jacketed, 3-L bioreactor equipped with an ADI 1025 Bio-Console and an ADI 1010 Bio-Controller (Applikon, Schiedam, The Netherlands) at a working volume of 1 L, for growth in either batch or continuous mode. The pH was maintained at 6.5 ± 0.1 at 70 °C by automatic titration with 4 M NaOH. The temperature was thermostatically kept at 70 ± 1 °C and the stirring rate was set to 250 rpm. A condenser with 5 °C cooling water was fitted to the bioreactor's headplate. Prior to inoculation, the medium was either sparged with N_2 (100 mL/min) or CO_2 (100 mL/min) and supplemented with an anoxic solution of cysteine-HCl at a final concentration of 1 g L^{-1} to render the medium completely anaerobic. For continuous cultivations, the bioreactor was fed at the end of the logarithmic growth phase with a fresh medium, having a similar composition to the batch start-up medium, except with 0.35 g/L cysteine instead of 1 g/L, at the dilution rate (D) of 0.05 h^{-1}. Steady states were assessed after at least 5 volume changes based on the criteria of constant H_2 and CO_2 production rates and constant biomass concentration. The investigation of whether or not YE is used as a carbon- and energy source was performed in serum flasks (triplicates) in parallel without sugar and with or without YE. Statistical analysis, i.e. *t*-test and confidence intervals 95%, were performed in Microsoft Excel and estimations of hydrogen productivity and product yields were performed as described previously [3,14]. The carbon and electron balances were calculated as described previously [3].

2.4. Analytical methods

Headspace gas samples were analyzed for CO_2 and H_2 by gas chromatography, using a dual channel Micro-GC (CP-4900; Varian, Middelburg, The Netherlands), as previously described [14]. The results were analyzed with a Galaxie Chromatography Workstation (v.1.9.3.2). The optical density of the culture was measured at 620 nm (OD_{620}) using a U-1100 spectrophotometer (Hitachi, Tokyo, Japan). Cell dry weight (CDW) was determined by filtration as previously described [12]. Glucose, acetate, lactate, succinate and ethanol were analyzed by HPLC (Waters, Milford, MA, USA) on an Aminex HPX-87H ion exchange column (Bio-Rad, Hercules, USA) at 45 °C, with 5 mM H_2SO_4 (0.6 ml·min^{-1}) as the mobile phase. The column was equipped with a refractive index detector (RID-6A; Shimadzu, Kyoto, Japan).

3. Results and discussion

3.1. Nutrient requirement for growth

Growth requirements of *C. saccharolyticus* were evaluated by testing different additives in batch cultures with glucose (10 g/L) as carbon source (Table 1). Growth and H_2 production in the presence of YE (1 g/L) progressed without any problem (Fig. 1A). Similar results were obtained by replacing YE with peptone (1 g/L; data not shown), suggesting that peptone could substitute YE without any addition of vitamins. However, in the medium without any protein source growth did not initiate. To establish growth on this medium, two different experimental setups were taken. Primarily, five subsequent cultivations in serum flasks were performed as a means of medium adaptation, as was previously applied to establish growth of *Caldicellulosiruptor owensensis* on minimal media [14]. Secondly, the N_2 gas flow rate was decreased to 10 mL/min until a partial CO_2 pressure (P_{CO_2}) of approx. 1 kPa was reached in a controlled bioreactor (Fig. 1B). After this point, the stripping rate was increased to 100 mL/min to avoid interference of increased partial hydrogen pressure (P_{H_2}) [9].

In these experimental setups, growth was initiated on lean medium, of which only the data of the controlled bioreactor are shown (Fig. 1B). However, the OD reached a maximum value of only 0.2, indicating that the culture underwent only one generation and that another nutrient was required. From the genome sequence, it can be deduced that *C. saccharolyticus* is unable to synthesize cobalamin (IMG pathway 00478; http://img.jgi.doe.gov). In addition, a similar study on *C. owensensis* has shown that this species required a vitamins cocktail for growth on minimal media [14]. When considering the same vitamins cocktail used for *C. owensensis* [14], growth of *C. saccharolyticus* continued for many generations (OD 2.9; Fig. 1C) and glucose was completely consumed