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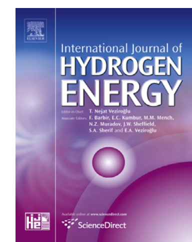

Hydrogen photoproduction by co-culture *Clostridium butyricum* and *Rhodobacter sphaeroides*

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ABSTRACT

The starch-utilizing co-culture *Clostridium butyricum* and *Rhodobacter sphaeroides* demonstrated the higher H_2 yield than *C. butyricum* (4.9 and 1.5 mol/mol hexose, respectively). Two species in co-culture were probably in balance since volatile fatty acids were not accumulated. The co-culture was as efficient in H_2 production from glucose as *R. sphaeroides* monoculture. A short-term test was suggested to determine the current activity of either species in co-culture. During the early phase, the H_2 production was mediated by both species, or *C. butyricum* accompanied with H_2 consumption by *R. sphaeroides*. Glucose addition did not influence the current rates of *Clostridium*-mediated and *Rhodobacter*-mediated H_2 production arguing against the limitation/competition for glucose between two species. During the late phase, the H_2 production was completely attributed to *R. sphaeroides*. The current rates of *Clostridium*-mediated H_2 production by co-culture were definitely reduced as compared to *C. butyricum* monoculture due probably to adverse effect of purple bacteria.

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Introduction

Hydrogen is considered as a promising fuel for the future. Various ways of biological H_2 production are under intensive investigation [1–4]. The schemes for biological H_2 production from complex substrates involve diverse microorganisms cooperating either in tandem (two-stages) or in co-culture (one stage). The advantages and disadvantages of different approaches are a subject of wide discussions [1–4]. Combining dark- and photo-fermentation in co-culture is simpler in manipulation and cost-effective because there is no need in pre-treatment of the dark fermentation effluent. Quite successful hydrogen production from glucose by co-cultures of *Lactobacillus* and *Rhodobacter sphaeroides* RV or *Clostridium butyricum* and *Rhodopseudomonas faecalis* RLD-53 has been reported before [5,6]. It should be emphasized that some purple bacteria (monoculture) produce H_2 from glucose with high yield as well [7]. The most efficient single-stage conversion of glucose to H_2 with a yield of 9.0 mol/mol glucose was recently demonstrated in continuous culture of *Rhodobacter capsulatus* JP91, hup$^-$ [8]. Data on photofermentation of complex substrates such as starch, distillery effluent and cellulose using co-culture of DF and purple bacteria have been presented earlier [9–17]. However, in a number of studies with defined co-cultures and mixed cultures (based on anaerobic sludge) the H_2 yield was quite low [2]. It was likely due to the non-trivial challenge of maintaining stable relationships between bacterial species in co-culture. Apparently, any environmental factor (pH, temperature, light intensity, substrate concentration, nitrogen

Abbreviations: Bchl, bacteriochlorophyll; DF, dark fermentation; VFAs, volatile fatty acids.

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source) can significantly affect co-culture by changing the ratio of species growth rates. To optimize H_2 production much attention was paid to the appropriate initial ratio of DF bacteria to purple bacteria in inoculum. Recommendations were to keep this ratio as high as 1:4 or even 1:10 because prevailing of DF bacteria inevitably resulted in fast pH decrease unfavorable for purple bacteria [2,6,12,18]. Low-efficient H_2 production by co-culture sometimes correlate with accumulation of VFAs produced during carbohydrates fermentation by DF bacteria [2,12,18,19]. It was attributed to inability of purple bacteria to utilize VFAs in presence of glucose, to the point of glucose depletion [2]. On the contrary, other data proved that purple bacteria preferred VFAs to glucose and no VFAs accumulation was observed in well-balanced co-cultures [20,21]. Previously it was suggested that in co-culture the purple bacteria *R. sphaeroides* could not compete with *C. butyricum* for glucose even at the ratio 1:6 [19]. It was attributed to the higher glucose-utilization rate of the latter in monoculture [19]. Nevertheless, the question still exists whether purple bacteria in co-culture are capable of glucose utilization along with DF bacteria or they only utilize VFAs produced.

The understanding of interaction between two species is crucial for improvement of H_2 production by co-culture. The common approach is to quantify either species during the process using molecular biological, biochemical, and microbiological methods and analyze the population dynamics [22,23]. Notice that the H_2-producing co-cultures including purple bacteria have not been adequately explored and results are dissimilar. It was demonstrated that *Clostridium cellulolyticum* grew twice as fast in co-culture with *Rhodopseudomonas palustris* as in monoculture, when using cellulose [13]. It seems that in this co-culture both species had mutual advantage: purple bacteria utilized fermentation products thus eliminating the inhibitory effect of these products on *Clostridium* [13]. In another example, the growth of *C. butyricum* in glucose-grown co-culture with *R. sphaeroides* was slowed down as compared to monoculture [19]. Apart from the number of either species, one can estimate their H_2-production activity in co-culture especially because the cell number and the current activity need not be in close agreement. This information can provide additional insight into the interaction between species in co-culture and limitations of the process.

The aim of this study was to compare the long-term H_2 production by co-culture *C. butyricum* and *R. sphaeroides* with that by monocultures using starch or glucose. The further challenge was to estimate the contribution of each species to the current hydrogen production by co-culture at different stages using the simple short-term test. The comparison of these results to H_2 production by monoculture would be useful for understanding of interspecies interaction and finding the new room to enhance hydrogen production.

Material and methods

Bacterial strains and media

The strain of *C. butyricum* was isolated from the dark starch-hydrolyzing consortium [24] by routine anaerobic technique and identified using MALDI mass spectrometry [21]. The basic cultivation medium was identical to that used for initial consortium but contained 5 g/L starch and 10 mM glutamate instead of peptone. The strain of purple bacterium *R. sphaeroides* N7 [25] was grown 3 d (30 W/m^2, 30 °C) on Ormerod medium [26] with ammonium sulfate and lactate.

Basic mineral medium for co-culture contained $FeSO_4$, $MgSO_4$, EDTA and microelements according to Ormerod medium. It was supplemented with 5 g/L starch. Following solutions were sterilized separately and added before inoculation: yeast extract 0.2 g/L, 100 mM phosphate buffer (pH 7.4) and 1–10 mM glutamate. Glucose (4 g/L, sterilized separately) was used instead of starch when indicated. Co-cultures were grown at 30 °C in illuminated by incandescent lamp (30 W/m^{-2}) Hungate tubes, under the Ar gas phase. Total tube volume was 16 mL, culture volume 8 mL. When required, tubes were incubated in the darkness. The control experiments with monocultures *R. sphaeroides* and *C. butyricum* were performed under the same conditions. The volumetric inoculum percentage was 0.25–1.25% (as specified in Tables and Figures). Data in Tables represent mean ± 95% confidence interval calculated for 5–8 experiments.

Hydrogen production by growing cultures

Gas production by growing cultures in Hungate tubes was recorded manometrically at 25°C. Measurements were made every 1–5 days (as indicated) till the end of gas production. Depending on the substrate, glutamate concentration and bacterial species (*Clostridium*, *Rhodobacter*, co-culture), the H_2 production was recorded 3–30 days till the end of H_2 production. The hydrogen percentage was determined by gas chromatography and the H_2 amount (mL/mL culture) and yield (mol/mol hexose or mol/mol glucose) were calculated.

Short-term H_2 production test

To estimate the current H_2 production rate of the growing cultures, the 3 mL culture aliquots were withdrawn anaerobically from the Hungate tubes (Section Hydrogen production by growing cultures) and injected into 12 mL vials filled with argon. Vials were repeatedly evacuated, filled with Ar and incubated with shaking at 30 °C in saturating light (40 W/m^2) or in the darkness (as indicated). Glucose (10 mM) or H_2 (100 μL) were added anaerobically as indicated in Figures. The duration of experiments was 2–7 h with every 30–60 min gas sampling. Hydrogen concentration was determined by gas chromatography and the H_2 amount (μl) and production rate (μl/h*mL of culture) were calculated based on linear part of the kinetics.

Other measurements

Bchl concentration was measured spectrophotometrically at 772 nm after extraction with 7:2 (v/v) acetone:methanol mixture [27]. The protein concentration was measured by Lowry method in washed cells after freezing/thawing and sonication (22 kHz, 30 W, 20 s/1 mL). If grown cultures were heterogeneous, all the measurements were carried out after the thorough mechanical homogenization of cell aggregates. The concentrations of acetate and butyrate were determined

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