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Different types of H_2 photoproduction by starch-utilizing co-cultures of *Clostridium butyricum* and *Rhodobacter sphaeroides*

*Tatyana Laurinavichene, Anatoly Tsygankov**

Institute of Basic Biological Problems, RAS, Pushchino, Moscow Region, 142290, Russia

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ABSTRACT

Co-culture of *Clostridium butyricum* and *Rhodobacter sphaeroides* displayed different types of H_2 photoproduction from starch: quick low-yield (2.6 mol/mol glucose), prolonged high-yield (6.1 mol/mol glucose) and delayed low-yield (4.2 mol/mol glucose). It depended primarily on the ratio of two species and, besides, some other factors. The first type H_2 photoproduction was similar to that in *Clostridium*, observed at high clostridia/purple bacteria ratio and promoted by low light intensity, temperature above and below optimum, reduced conditions, low initial pH or buffer concentration, high concentration of yeast extract (320 mg/L). The second type, high yield H_2 production, was favored by low clostridia/purple bacteria ratio, high light intensity, and high concentration of yeast extract (320 mg/L). The third type, delayed H_2 photoproduction, was observed at the lowest clostridia/purple bacteria ratio and was promoted by low concentration of yeast extract (25 mg/L). Thus, a well-known crucial effect of a species ratio on H_2 photoproduction was in turn controlled by other factors, especially yeast extract concentration.

Introduction

Hydrogen is considered as a promising future fuel and different ways of H_2 production including biological ones are under consideration. Integration of dark fermentative H_2 production using organic wastes in the first stage and H_2 photoproduction by purple bacteria using fermentation effluent in the second stage attracted much attention recently. Theoretically, it is possible to get as much as 12 molecules H_2 per mole of glucose. Technologically, it can be realized as a sequential two-stages process or a single-stage process using mixing culture or co-culture, each having its pros and cons [1–5]. The single-stage process (co-culture) seems to be simple in operation and cost-effective, because it occurs in one bioreactor and there is no need in pre-treatment of fermentation effluent for following photofermentation. Photofermentation of glucose, sucrose, starch, and cellulose have been studied earlier using co-culture of purple bacteria and dark-fermentative (DF) bacteria or consortia. Unfortunately, there are only few reports demonstrating H_2 yield above 4 mol/mol hexose [6–9]. In most instances the H_2 yield was below this value (for review: [2]). The explanation of such a low yield is still unclear.

To improve the H_2 yield, the influence of different factors on H_2 photoproduction by co-culture should be elucidated. Any factor is supposed to affect differently the growth rate or lag-phase of either species, which could imbalance co-culture

* *Corresponding author*. Tel.: +7 4967 732791; fax: +7 4967 330532.
E-mail address: ttt-00@mail.ru (A. Tsygankov).

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resulting in suppressed H_2 photoproduction. Besides, under some conditions the reverse process, H_2 consumption, may take place, especially when using consortia of unknown composition.

Actually, DF bacteria have advantage due to higher growth rate and substrate consumption rate [10]. The ratio of DF bacteria to purple bacteria in inoculum is the most explored factor. The influence of inocula ratio (anaerobic sludge/*Rhodobacter*) was studied in fermentation of ground wheat. While the ratio 1/4–1/10 was considered as optimal, it nevertheless did not provide the H_2 yield above than either component of inocula alone [2]. Apparently, the low proportion of *Clostridia*/purple bacteria prevented the metabolic domination of *Clostridia*, which, if occurred, resulted in fast pH decrease unfavorable for purple bacteria. Accumulation of VFAs is another result of such domination. It was assumed that purple bacteria in mixed culture were initially adapted to fermentation of carbohydrate and the presence of glucose repressed VFAs fermentation [2].

Statistically based experimental designs identified three factors for improvement of H_2 yield: substrate concentration, initial pH, and inoculum ratio [9]. Co-culture of *Clostridium acidisoli* and *Rhodobacter sphaeroides* produced H_2 at 5.08 mol/mol hexose under optimized conditions with sucrose [9]. In addition, some other factors were proved to be important based on the experimental results. Effect of glutamate concentration was studied in the mixed culture of *Clostridium butyricum* and *Rhodobacter* sp. using starch. The H_2 yield did not reliably depend on the glutamate concentration over the range 5–20 mM and went down at 2 mM glutamate [6]. Similarly, in co-culture of *C. butyricum* and *R. sphaeroides* we found the reduced H_2 yield at 1 mM compared to 10 mM [11]. The dependence of H_2 production on yeast extract (YE) concentration was shown in the above-mentioned mixed culture with optimum at 200 mg/L [6]. In co-culture of anaerobic sludge and *Rhodospirillum rubrum* the hydrogen yield was maximum at initial pH 7 as compared to pH 6 and 5 [12]. This is quite understandable because purple bacteria usually prefer neutral pH. It should be noted that most factors influence either species but to a different extent and even, probably, in opposite direction. Furthermore, the effect of each factor is dependent on other conditions, thus, the overall result in co-culture is mainly unpredictable.

Apparently, for high H_2 production metabolic activity of *Clostridia* and purple bacteria should be in balance: inhibition of purple bacteria activity by *Clostridia* leads to low-yield H_2 production and accumulation of volatile fatty acids. Complete inhibition of *Clostridia* activity by purple bacteria (if occurs) may result in the shortage of substrates for purple bacteria to produce H_2 since they do not hydrolyze complex substrates such as cellulose and starch (in most cases). Earlier we demonstrated a partial inhibitory effect of purple bacteria on *Clostridia* activity [11].

To summarize, although the effect of some factors on co-culture including DF bacteria and purple bacteria have been studied, the efficient H_2 production is still a challenging task. It is worthwhile to re-estimate the potential of co-culture for high H_2 photoproduction by taking into account impact of various factors on their interaction.

The aim of this study was to demonstrate different types of H_2 photoproduction by co-culture of *C. butyricum* and *R. sphaeroides* using starch and to reveal conditions leading to one or another type of H_2 photoproduction.

Materials and methods

Bacterial strains and media

The strain of *C butyricum* was isolated earlier and cultivated using 5 g/L starch and 5 mM glutamate [13]. The strain of purple bacterium *R. sphaeroides* N7 [14] was grown on Ormerod medium [15] with 10 mM ammonium sulfate and 20 mM lactate (30 W/m^2, 30 °C).

Basic mineral medium for co-culture of *C. butyricum* and *R. sphaeroides* contained $FeSO_4$, $MgSO_4$, EDTA and microelements according to Ormerod medium. It was supplemented with 5 g/L starch. Following solutions were added to the sterile medium before inoculation: yeast extract 80 mg/L, 75 mM phosphate buffer (pH 7.3 unless otherwise stated) and 5 mM glutamate. Glucose (4 g/L) was used instead of starch when indicated. Co-cultures (8 mL) were grown in Hungate tubes (16 mL) under Ar gas phase at 30 °C illuminated by incandescent lamp (30 W/m^2). When specified, growth conditions were changed in terms of temperature, light intensity, YE concentration, addition of redox components. Monocultures of *R. sphaeroides* and *C. butyricum* were grown under the same conditions when comparison was aimed. The *C. butyricum* inoculum was added at 1.25% v/v invariantly [16]. The *R. sphaeroides* inoculum was added at various percentage to provide different Bchl content but no more than 2.5% v/v. To obtain high Bchl content, *R. sphaeroides* culture (initial Bchl 16–20 mg/L) was preliminary concentrated by centrifugation. Data represent mean ± 95% confidence interval calculated for 5–8 experiments.

Hydrogen production by growing cultures

Gas production by co-cultures in Hungate tubes was recorded manometrically at 25°C. Measurements were made every 1–3 days (as indicated) during 9–11 days. The hydrogen percentage was determined by gas chromatography and the H_2 yield (mL of H_2/mL culture) or molar yield (mol/mol glucose utilized) was calculated.

Other measurements

Bacteriochlorophyll (Bchl) concentration was measured spectrophotometrically at 772 nm after extraction with 7:2 (v/v) acetone:methanol mixture [17]. Extraction was carried out after thorough mechanical homogenization of cell pellet.

Results

Different types of H_2 production by co-culture

It is well-known that kinetics of H_2 production by co-cultures vary substantially depending on the process conditions. Our