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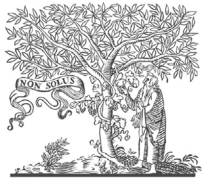

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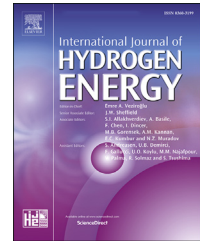

Inoculum density and buffer capacity are crucial for H_2 photoproduction from acetate by purple bacteria

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ABSTRACT

The increase of inoculum density of *Rhodobacter sphaeroides* B-3059 above 2.0 mg bacteriochlorophyll/l in 15 mM K-phosphate buffer inhibited hydrogen photoproduction but favored PHB synthesis. This inhibition was substrate-specific and was observed on media containing acetate. The inhibition resulted from sharp increase in pH above 10 due evidently to fast acetate consumption. The lower the buffer concentration, the lower the inhibitory inoculum density was. The dependence of H_2 photoproduction from acetate on buffer concentration at low and high inoculum density was different for different strains of purple bacteria. However, conditions were found to provide H_2 production by each of 9 tested strains. Acetate-dependent hydrogen photoproduction in 10 mM K-phosphate buffer was unreliable even with low inoculum density. Ammonium traces and high acetate concentration also facilitated medium alkalization and, consequently, inhibited H_2 production. The increase of buffer concentration above 20 mM helped to prevent pH rise independent of the triggering factor.

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Introduction

The strategies of microbial cultivation involve optimization of quite a few parameters. Inoculum density is not usually considered as an important factor of microbial growth. However, inoculum density might affect duration of lag phase and the whole process, growth rate, yield, productivity and set of target metabolites or products [1,2]. The use of high inoculum density is also a promising approach for nonsterile fermentation [3].

One of biotechnological processes aiming to biofuel production is the photofermentation of organic acids by purple bacteria resulting in hydrogen production. The inoculum density is one of the conditions to be met for the highest hydrogen photoproduction rate [4]. Oh et al. [5] reported that volumetric H_2 production rate increased but specific H_2 production rate decreased with increase of inoculum density. Beside that, high inoculum density was shown to affect the cumulative H_2 production positively [6,7]. Alternatively, there is information about certain inhibitory effect of high inoculum density [8,9]. Such inhibition was also observed when using co-culture of purple and heterotrophic bacteria with high total inoculum density [10]. General explanation is the light or substrate limitation in dense cultures, due in particular to flock formation and poor availability of substrates inside the flocks [6,8–10]. It is well known that the main recommendation for effective co-culture was to maintain the high ratio of purple bacteria to *Clostridia* [11]. However, in starch-utilizing co-culture of *Clostridium butyricum* and *Rhodobacter sphaeroides* it was observed that at high proportion of purple bacteria the H_2 production decreased under specific conditions [12]. In

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this case, the main products of starch fermentation by *C. butyricum* were acetate and butyrate. We supposed that the inhibitory effect could be due to some features of acetate metabolism in *R. sphaeroides* B-3059 when using high inoculum density.

Acetate, a product of anaerobic fermentation of organic wastes, is considered as a promising substrate for H_2 photoproduction by purple bacteria. Acetate utilization is advantageous to couple energy production with wastewater treatment. Therefore, the study of H_2 production from acetate and attempts to reach high yield and rate of H_2 production have been already undertaken. Along with it, the screening of new strains was aimed at selection of the most appropriate ones, with high H_2 production activity and wide range of organic acids used. Malate or lactate were definitely utilized by the majority of purple bacteria. As for acetate, in different strains (and obviously under non-similar conditions) the results were either positive [7,8,13–17] or negative [18–21]. Even under the same conditions, some strains demonstrated reliable H_2 production from acetate while others did not [22–24]. Earlier it was found that photofermentation in acetate-containing media resulted in drastic pH increase which could be fatal for the process [6,20,25,26].

We hypothesized that the amplitude of such a pH burst might depend on the inoculum density which was not studied earlier. This is why the aim of this study was to examine the effect of inoculum density of purple bacteria and buffer capacity on pH and photofermentation products when using acetate and other organic acids.

Materials and methods

Bacteria and media

The following purple bacteria were used in this study: *R. sphaeroides* strains VKM B-3059, 2R, GL, RV, *Rhodobacter capsulatus* strains B10, BK5, AD2, *Rubrivivax gelatinosus* RL2 and *Rhodospirillum rubrum* S1. Bacteria were grown on Ormerod medium [27] with ammonium sulfate and 30 mM lactate (30 W/m^2, 30 °C). Experiments were mainly done with *R. sphaeroides* VKM B-3059 unless otherwise stated. For inoculum, 2-3 day-grown bacteria (~10 ml) were centrifuged and resuspended in H_2-production medium (~0.7 ml). The latter contained $FeSO_4 \cdot 7H_2O$, 10 mg/l; $MgSO_4 \cdot 7H_2O$, 200 mg/l; EDTA, 20 mg/l, microelements (according to Ormerod medium). It was supplemented with 15 mM K-phosphate buffer, 5 mM glutamate, 300 mg YE/l (unless otherwise specified), and organic acid (acetate, lactate, succinate, butyrate or their mixtures) at concentration indicated. All supplements (except buffer) were filter-sterilized and added before inoculation. In some experiments (as indicated) bacteria were additionally washed and centrifuged.

Hydrogen production experiments

Hydrogen production experiments were made in Hungate tubes (16 ml) with 8 ml of medium. Inoculum was added at a final BChl (bacteriochlorophyll) concentration 0.3 ± 0.1 or 2.0 ± 0.3 mg/l, hereinafter referred to as low or high inoculum density. Gas phase was replaced by argon via repeated vacuuming and flushing. Hungate tubes were incubated at 30 °C under illumination (30 W/m^2). Incubation continued till the end of gas production (7–14 days depending on substrate and its concentration). The main object was *R. sphaeroides* strains VKM B-3059. First set of experiments was aimed at examination of H_2 production from mixtures of organic acids at their different ratio (acetate + butyrate or acetate + lactate, 40 mM in total). Then H_2 production from individual acids was compared, any acid concentration being 90 mM in relation to C atoms. Thereafter attention was focused on acetate photofermentation with analysis of its products. The impact of buffer concentration (10–20 mM) at low and high inoculum density was compared in different bacteria. The influence of inoculum density over the range from 0.2 to 3.0 mg BChl/l at 10 and 20 mM buffer was analyzed. Effect of acetate concentration (15, 30, 45 and 60 mM) at 10 and 20 mM buffer was also studied. A result of small additions of ammonium was revealed in order to simulate possible input of ammonium with high density inoculum.

Analytical methods

Gas production was recorded manometrically at 25 °C every 2–3 days. However, in short-term it was recorded during 2–24 h. Hydrogen percentage was assayed with gas chromatograph (LkhM80, Moscow, Russia), equipped with a thermal conductivity detector and a 1 m × 3 mm molecular sieve column [28]. Hydrogen production (accumulation) was then calculated taking into account H_2 percentage and expressed in ml H_2/ml of culture volume. Acetate concentration was determined by gas chromatography (TCVET800, Russia) as described earlier [28]. Total polysaccharide concentration (presented as glucose equivalents) was assayed by anthrone method [29]. PHB concentration was measured by a rapid gas chromatography method [30]. BChl concentration was measured spectrophotometrically at 772 nm after extraction with 7:2 (v/v) acetone:-methanol mixture [31]. If grown cultures were heterogeneous, all the measurements were carried out after thorough mechanical homogenization of cell aggregates. Ammonium content was analyzed by the micro-diffusion method [32]. Data in Tables and Figures represent mean ± 95% confidence interval calculated for 3–15 experiments.

Results

Hydrogen photoproduction from acetate-containing mixtures by R. sphaeroides B-3059 at different inoculum densities

Earlier, we found the effect of high inoculum density of purple bacteria in co-culture with *C. butyricum* utilizing starch [12]. Since the main substrates for purple bacteria in this case were probably acetate and butyrate (products of starch fermentation by *Clostridia*), the effect of mixed organic acids on H_2 production by purple bacteria at different inoculum densities was tested. Two types of mixtures were applied: acetate + butyrate and acetate + lactate with different proportions of acetate, total substrate concentration being 40 mM.

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