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Effect of growth conditions on advantages of *hup*⁻ strain for H_2 photoproduction by *Rubrivivax gelatinosus*

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ABSTRACT

H_2 photoproduction by growing cultures of *hup*⁻ mutant and parental strain RL2 of *Rubrivivax gelatinosus* was compared. We checked the influence of different substrates, presence of air and N_2, culture shaking, inoculum concentrations. At low inoculum concentration, *hup*⁻ strain demonstrated significant advantage over the parental strain in microaerobic conditions, while under N_2–Ar atmosphere it was lower and vanished in anaerobic conditions (Ar only). This advantage was evident when using substrates with low degree of reduction (malate and succinate). Culture shaking under microaerobic conditions and in presence of N_2 completely prevented H_2 production by both strains. The high inoculum concentration inhibited H_2 production under microaerobic conditions and in presence of N_2, unlike to anaerobic conditions. With inoculum concentration increase, H_2 production decreased not gradually but stepwise which means some metabolic shift. H_2 production by *hup*⁻ strain seems to be more tolerant to air traces than by parental strain.

Introduction

Molecular hydrogen is a valuable alternative source of energy since it can be generated from water, and water is the end product of it practical utilization. Several technologies for H_2 production including photobiological ones are under consideration. Purple nonsulfur bacteria (PNSB) can photoproduce H_2 at high rates via nitrogenase reaction. However, these rates are still insufficient for practical application [1,2]. There are several ways to address this problem based mainly on genetic modification of bacteria: using strains with reduced pigment content, strains incapable of Calvin cycle flux, insensitive to ammonium, incapable of storage compound synthesis [3–8]. One of the ways explored is using mutants with deletion of uptake hydrogenases (Hup) or even double mutants that lack both uptake hydrogenase and poly-hydroxybutyrate (PHB) synthase. Hup hydrogenase re-directs electrons from H_2 to bacterial metabolism while PHB synthesis is a process competing with H_2 production for electrons. In *hup*⁻ strains of *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, *Rhodopseudomonas palustris* an increase in total H_2 production varied

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from 0 to above 100% [5,9–15]. The reasons of variability are not clear. The *hup*$^-$ mutant of *R. capsulatus* was successfully applied in large-scale photobioreactors in outdoor conditions [16].

On the basis of general consideration one can suggest that the difference in H_2 production rate, yield and total accumulation between parental and *hup*$^-$ strains might depend on a number of factors. For example, the degree of reduction of substrate can define the need to recycle H_2. Actually, the demonstration of *hup*$^-$ strain advantages was mainly done using more oxidized substrate, malate [5,10,11]. The comparison of different substrates for H_2 production by both strains was reported by Wu [15], but no correlation could be found between reduction degree and H_2 production rate. On the contrary, Rey [10] showed that the difference between H_2 production by *hup*$^-$ and *hup*$^+$ strains was huge when using substrates with higher O/H ratio (malate and succinate vs acetate).

Another factor to take into account is the substrate concentration at given N-source concentration. The lower the substrate concentration, the higher the relative substrate portion appears in biomass and lower relative portion goes to H_2 production. As far as H_2 recycling resulted in somewhat increase in biomass production [5,11,17,18], it can be reasonably expected that H_2 production in *hup*$^-$ strain would increase substantially as compared to minute H_2 production in parental strain. To illustrate this, the impressive improvement of H_2 production in *hup*$^-$ strain of *R. palustris* was achieved by Rey [10]. In this work, the increase amounted to even 5–100-fold when using succinate or malate. However, it should be noted that substrate concentrations were as low as 10 mM. Unfortunately, this concentration is too low if we take into consideration the large-scale process of intensive H_2 production. H_2 production by two strains at different substrate concentrations was not compared yet.

We hypothesize that H_2 recycling and consequently the negative role of Hup hydrogenase in H_2 production should be higher under microaerobic conditions, at which point an advantage of *hup*$^-$ strain in H_2 production should increase. In addition, higher H_2 uptake can be expected under conditions of culture shaking (mixing) and at high inoculum concentration.

The aim of this study was to verify some factors determining the improvement of H_2 production in *hup*$^-$ strain compared to parental strain of *Rubrivivax gelatinosus*. For this purpose, we explored the influence of substrate degree of reduction, microaerobic growth conditions, presence of N_2, inoculum concentration and culture shaking on H_2 accumulation by both strains.

Materials and methods

Bacterial strains and media

The strains of purple phototrophic bacteria *R. gelatinosus* RL2 [19] and RL2*hup*$^-$, were provided by K.V.P. Nagashima. The latter *hup*$^-$ mutant lacking *hupSL* without any insertions of antibiotics-resistant cartridges was created using the pJPCm plasmid and the plasmid pJPΔ*hupSL*-Skm followed by kanamycin screening and by sucrose resistance screening, which will be detailed elsewhere [20]. *R. gelatinosus* RL2 was shown to synthesize reaction centers and antenna pigment complexes even under semiaerobic conditions [19]. Both strains of *R. gelatinosus*, RL2 and RL2*hup*$^-$, were pre-grown photosynthetically on YPS medium supplemented with Na succinate (5 g/l) and K-phosphate buffer (10 mM, pH 7.0).

H_2 production by growing cultures

For H_2 photoproduction experiments, bacteria were inoculated into Ormerod mineral medium [21] with glutamate (5 mM), yeast extract (0.4 g/l), K-phosphate buffer (20 mM, pH 7.0) and NaCl (0.23 g/l). The medium was supplied with succinate or other organic acid (as indicated in the text and Tables) with equal concentration of carbon atoms, 90 mM. Medium aliquots (8 ml) were placed in Hungate culture tubes (16 ml) supplied with butyl rubber stoppers and screw caps. For inoculum, one-day-old cultures were centrifuged aseptically in Eppendorf tubes (10,000 rpm, 3 min), the cell pellet was resuspended in Ormerod medium and added to Hungate tubes at 0.4–5% v/v, bacteriochlorophyll (BChl) concentration was specified in Tables and Figures. Depending on the aim of experiment, tubes were incubated under air or Ar gas phases (microaerobic or anaerobic conditions, correspondingly). In the latter case, tubes were repeatedly evacuated and filled with Ar or Ar + 50% N_2 using sterile filters. Cultivation was carried at 30 °C for 7–14 days until H_2 production ceased, light intensity of 30 W m^{-2} (incandescent lamps). Tubes were placed at the angle ~30° to horizon and shaken manually once a day. In some experiments tubes were placed horizontally on the platform and permanently shaken at 70 rpm (as indicated in Tables). Gas production was measured manometrically, and H_2 production was calculated on the basis of H_2 percentages measured by gas chromatography. H_2 amounted to approximately 85% of the produced gas. Each independent experiment was made in duplicates or triplicates (two or three tubes with identical composition in identical conditions). Number of experiments is specified in figure legends and table footnotes.

Short-term H_2 production/consumption

The observation of short-term H_2 production/consumption was performed in 9-ml vials. Culture aliquots (2 ml) were transferred anaerobically to the vials filled with Ar, repeatedly evacuated, refilled with Ar and supplied with ~25% H_2. Additions of N_2 or air were made as indicated in Figure legends. Vials were incubated at 30 °C under light (30 W m^{-2}) on a shaker platform (70 rpm). Experiments continued up to 10 h. The initial and final H_2 percentage was measured in each vial.

Other measurements

The total content of saccharides (as glucose residues) was analysed in the medium at the end of experiments and in cell pellet by anthrone assay [22]. BChl concentrations were measured spectrophotometrically at 772 nm following extraction in a 7:2 (v/v) acetone:methanol mixture [23].

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