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Hydrogen production during stationary phase in purple photosynthetic bacteria

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ABSTRACT

The merit of a hydrogen production system was investigated, where cells in the stationary phase of growth were treated as live enzymes, continually catalyzing hydrogen production in the absence of growth. Batch cultures of the purple photosynthetic bacteria *Rhodospirillum rubrum* UR2 were grown photoheterotrophically with succinate as the electron donor. Hydrogen evolved during growth, via the enzyme nitrogenase, at a rate of 21 mL gas L^{-1} culture h^{-1}, and continued to evolve at high rates for about 70 h after cells had ceased growth. Hydrogen production stopped precisely when succinate was depleted from the medium. Upon replenishment of succinate to the cultures, hydrogen production resumed but cells did not grow further; however, the rate and yield of hydrogen production was lower with successive succinate additions than that measured during growth. These results suggest that hydrogen production is not strictly coupled to growth. Nevertheless, the results also establish the necessity for cell growth in order to maintain maximal hydrogen production rates. Supplementation of cultures with limited amounts of fresh growth medium, given in addition to the succinate replenishment, partially restored the hydrogen production rate and yield, along with a proportional increase in cell biomass. Results were confirmed in parallel experiments with *Rhodopseudomonas palustris* CGA009. A strategy is suggested for enhancing the biofuels to biomass production ratio under conditions of continuous cultivation with minimal cell growth (about 10% of the control), allowing a greater proportion of the cellular metabolic activity to be directed toward H_2-production.

1. Introduction

Photosynthesis is the only efficient biological process evolved for the harvesting of solar irradiance and its conversion into chemical energy. Therefore, exploitation of photosynthesis is a promising approach in the production of renewable biofuels. Hydrogen, particularly biohydrogen, is purported to be a suitable energy carrier for the needs of the global economy, generated directly from the photosynthesis of microorganisms with energy from the sun [1–3]. Photosynthetic microorganisms, in turn, are strong candidates for biofuel production due to their tractable genetics and ease of metabolic manipulation.

Amongst photosynthetic microorganisms, purple photosynthetic bacteria are well known for the photoproduction of

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Abbreviations: BChl, bacteriochlorophyll; LH, light-harvesting complex; PCV, packed cell volume.
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large amounts of hydrogen [4,5]. Under anaerobic conditions, purple photosynthetic bacteria express their photosynthetic apparatus and, via bacteriochlorophyll [6] and the associated bacteriopheophytin-quinone type reaction center [7–11], absorb and utilize sunlight [12]. This photochemical event drives a cyclic electron transport process for the generation of a proton and electrochemical gradient across the photosynthetic membrane [13–15]. The potential energy stored in this gradient is utilized for the generation of ATP, which, together with the reductant from the photosynthetic metabolism, is important for driving cellular biochemistry, thereby enabling cell growth, as well as repair and maintenance reactions.

Under nitrogen fixation conditions [16], ATP and reductant are also pivotal for driving the function of the enzyme nitrogenase [17], which simultaneously converts molecular nitrogen (N_2) to ammonia (NH_3), and protons (H^+) to hydrogen (H_2), respectively [18–20]. The overall reaction catalyzed by this enzyme is shown in Eq. (1):

$$N_2 + 8e^- + 8H^+ + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i \quad (1)$$

Of interest is the observation that, under conditions where nitrogenase genes are expressed [16] but N_2 is absent, all of the high potential-energy electrons and ATP may be directed toward the proton-reducing function of the enzyme, leading to greater than normal hydrogen production [17,21]. According to this scenario, the nitrogenase enzyme may function strictly as a hydrogenase, with the overall biochemical Eq. (2):

$$2e^- + 2H^+ + 4ATP \rightarrow H_2 + 4ADP + 4P_i \quad (2)$$

In purple non-sulfur photosynthetic bacteria, the source of electrons for reactions (1) and (2) can ultimately be derived from small organic acids, such as succinate, provided exogenously. However, carbon substrate also serves to provide reductant for the processes of photosynthetic electron transport and anaerobic cellular metabolism [15,22]. In the absence of carbon substrate, cell growth stops and cells slow down their metabolism to enter a phase known as stationary phase [23]. Thus, purple photosynthetic bacteria intimately rely on the supply of exogenous electron-donating substrate in order to sustain metabolism and growth.

The stationary phase of bacteria may be reached under a variety of conditions, but often is the result of a nutrient limitation. During the later stages of batch cultivation in the laboratory [24], but also in nature, nutrients such as electron donors, essential minerals, carbon, or nitrogen sources may be depleted from the surrounding environment, thus preventing growth and inducing stationary phase. Stationary phase tends to include several physiological events: diminished bioenergetic activity, storage polymer accumulation, secretion of scavenging enzymes, up-regulation of high-affinity transporters and antioxidants, and gradual loss of cell vitality and viability [23]. Slow growth may also involve many of the same processes [25]. Long-term maintenance of a culture in the stationary phase eventually leads to mutations, sterility, and cell death [26–28].

However, in scaled-up commercial application of any microbial production process, economic considerations would favor an approach where high-density cells in the stationary phase of growth are used as live enzymes, continually performing a specific catalytic reaction (e.g. hydrogen production) in the absence of growth [29]. Such a production system, envisaged in liquid culture or with immobilized cells, would require input of a single feedstock, leading to the generation of a specific product. A requirement for the successful application of this stationary-phase production model is that catalysis performed by the cells would be sustained in the absence of cell growth.

In this work, experiments were conducted in order to address the question of whether hydrogen production could proceed in the absence of photosynthetic bacterial cell growth. Particularly, it was posited that the onset of stationary phase might affect the ability of cells to sustain catalytic production of hydrogen. Substrate-replenishment experiments were performed with purple photosynthetic bacterial batch cultures in the early stationary phase, with or without other nutrient supplementation, to assess the effect on hydrogen production.

2. Materials and methods

2.1. Organisms and growth media

Rhodospirillum rubrum UR2 (gift from Dr. Paul Ludden) and *Rhodopseudomonas palustris* CGA009 (gift from Dr. Caroline Harwood, University of Washington) were used in this study. Ormerod Medium [30] was used for cell growth and hydrogen production, with 16 mM Na-succinate serving as the organic carbon substrate and photosynthetic electron donor. The growth medium contained 3 mM glutamate as a nitrogen source, serving to enhance growth and hydrogen production without repressing the nitrogenase enzyme [31]. The growth medium was supplemented with 0.06 μM biotin for the growth of *R. rubrum* or 3 μM para-aminobenzoic acid for the growth of *R. palustris* [32]. Starter cultures were grown in supplemented minimal medium (SMN), containing 0.3% bacto-tryptose (Difco) and 0.3% bacto-yeast extract (Difco) supplemented to Ormerod medium [33].

2.2. Culture conditions

Starter cultures were inoculated with 500 μl frozen aliquots of purple bacterial strains into 20 mL screw-cap glass tubes containing SMN medium. Cells were grown photoheterotrophically with mixing by inversion every 12 h. 24 h after cells turned purple in the glass tubes, 3 mL of these starter cultures were inoculated into flat Roux-type bottles containing 1 L Ormerod medium. Inoculated cultures were sealed with a three-port silicon stopper and placed in a temperature-controlled room at 24 °C on magnetic stir-plates. Irradiance from a combination of fluorescent (15 W m^{-2} intensity) and incandescent (45 W m^{-2} intensity) light bulbs was set up, such that total irradiance was 60 W m^{-2}, as measured with a LI-COR 185B Radiometer equipped with a pyranometer-type sensor. Cultures were initially bubbled with Argon aseptically for 5 min through the long-needle port in the stopper while stirring.

2.3. Measurement of hydrogen

Sealed culture bottles were tested to be air-tight for hydrogen prior to inoculation. One of the ports in the silicon stopper was connected to an inverted 1 L graduated cylinder filled with water, serving as a gas collection trap. Gas levels were recorded volumetrically by water displacement at several points throughout each day. Purity of hydrogen in the gas phase was measured to be greater than 85% by gas chromatography of samples taken from both the trap and from the culture headspace. Gas chromatography was performed using an SRI 8610C Multiple Gas Analyzer equipped with a Molecular Sieve column and a TCD filament detector, using Ar as the carrier gas.

2.4. Measurement of cell growth

Cell growth in the culture was determined by dry cell weight, bacteriochlorophyll (BChl) accumulation, and packed cell volume (PCV) measurements. Packed cell volume was measured by centrifugation of a 10 mL sample aliquot at 1000 g for 15 min, concentration of pellets (30–50x) by resuspension in a known volume, loading into a 2 mm inner diameter glass tube plugged at one end with modeling clay and sealed with duct tape, and upon centrifugation at 1000 g for 15 min in a swing-bucket centrifuge (Beckman TJ-6). Packed cell volume was then expressed as a percent of the original volume by comparing the height with a metric ruler and correcting for the concentration factor. Only purple biomass was measured, excluding occasional precipitates from the medium. This method was also used to examine whether cultures were free of contaminants (axenic). Bacteriochlorophyll concentrations were measured by pigment extraction in a 7:2 acetone:methanol solvent, followed by spectrophotometry at 775 nm in a Shimadzu UV160U spectrophotometer [34]. Alternatively, BChl concentrations *in vivo* were estimated from the absorbance spectra of culture aliquots, after correction for light scattering (Fig. 1).

2.5. Determination of organic acids

The concentration of succinate was analyzed by HPLC (Modular HPLC, Varian, Inc., Mulgrave, Victoria 3170, AU), with an Aminex HPX-87 H column, 300 mm × 7.8 mm (I.D.) and a photodiode array detector (Prostar 335, Varian, Inc.). The column temperature was kept at 65 °C by an HPLC column chiller/heater (ECHO therm, model C030) and an aqueous solution of 0.013 N H_2SO_4 was used to obtain an isocratic elution with a flow rate of 0.6 mL min^{-1}.

2.6. Succinate replenishment experiments

Upon cessation of hydrogen production or after 200 h of cultivation (whichever occurred first), cultures were injected with 16 mmol succinate, pH 6.8, contained in a 10 mL solution. A series of two such 10 mL succinate replenishments were performed in each culture. Some of the culture bottles received supplementation of fresh Ormerod medium along with the succinate addition, i.e., 10 mL Ormerod medium (1% supplement), 50 mL Ormerod medium (5% supplement), or 20 mL 10x concentrated Ormerod medium (20% supplement). Ormerod medium without succinate was used in these "Ormerod supplementation" experiments. In these cases, an equivalent volume of culture was removed from the bottle. Additionally, a complete replacement of the Ormerod medium (100%) was implemented upon centrifugation of the cells from a 1 L culture at 5000 g for 10 min (Beckman J2-21M centrifuge with a JA-10 rotor) and resuspension in 1 L fresh Ormerod medium.

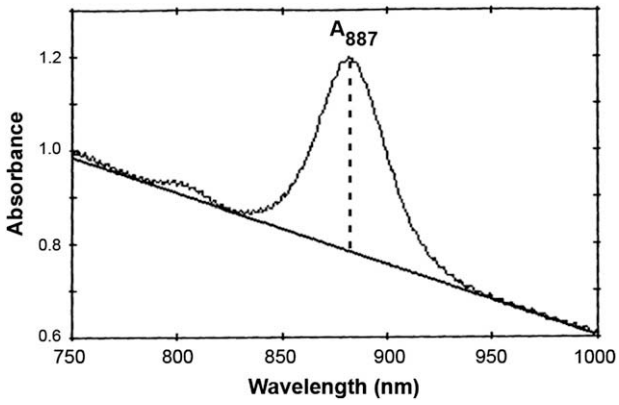

Fig. 1 – *In vivo* absorbance spectrum (750–1000 nm) of *R. rubrum* cells in culture medium. Baseline correction for scattering was achieved from the slope of the non-absorbing linear 1000–950 nm region of the recording. The BChl peak maximum at 887 nm (A_{887}) was used as a measure of cell growth. *R. rubrum* possesses a single light-harvesting bacteriochlorophyll antenna (LH1), and thus only absorbs at the LH1 peak of 887 nm [54].

3. Results

The efficacy of different organic carbon substrates for hydrogen production in *R. rubrum* was first investigated. A variety of small organic molecules, including succinate, malate, pyruvate, and lactate were examined for their ability to serve as electron donors in bacterial anoxygenic photosynthesis and hydrogen production. On the basis of equal organic C supplied to 1 L cultures, succinate was found to yield the highest amount of gas (1600 mL, Fig. 2), and thus was selected as the small organic molecule of choice for the further experimentation reported in this work. Experiments were also conducted with acetate, ethanol, glycolate, propionate, and butyrate; however, no gas was produced on these substrates, and some substrates (acetate, ethanol, glycolate) were unable to support substantial growth.

Cell growth and culture productivity was measured in a variety of different but independent ways. Fig. 3 shows results relating the packed cell volume, measured as a percent of the total culture volume [20], and extracted BChl content (in μg per mL) to the *in vivo* absorbance of the culture at 887 nm, as measured in Fig. 1. A linear relationship between these variables was found, suggesting that any of these measurements can provide estimates of cellular growth. Moreover, these

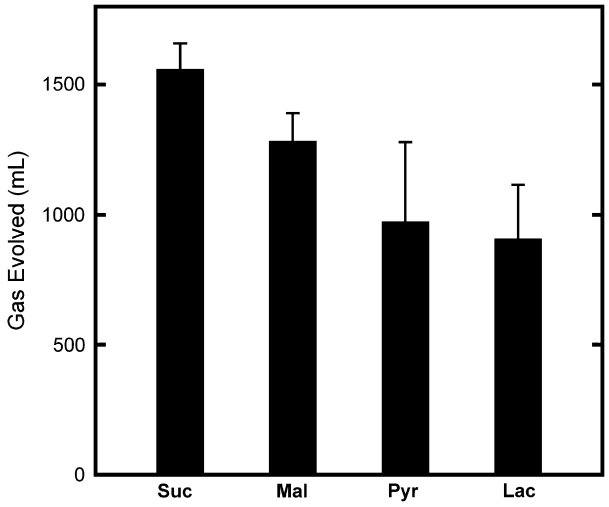

Fig. 2 – Comparison of H_2 yield in batch cultures of *R. rubrum* supplied with different carbon sources. Batch cultures were grown with different carbon sources in order to ascertain their relative H_2-production efficacies. Organic carbon substrate was each supplied on the basis of equal C (64 mM). Values shown are the average of three to four replica measurements and results show the total yield of H_2 produced. (Suc = succinate; Mal = malate; Pyr = pyruvate; Lac = lactate).

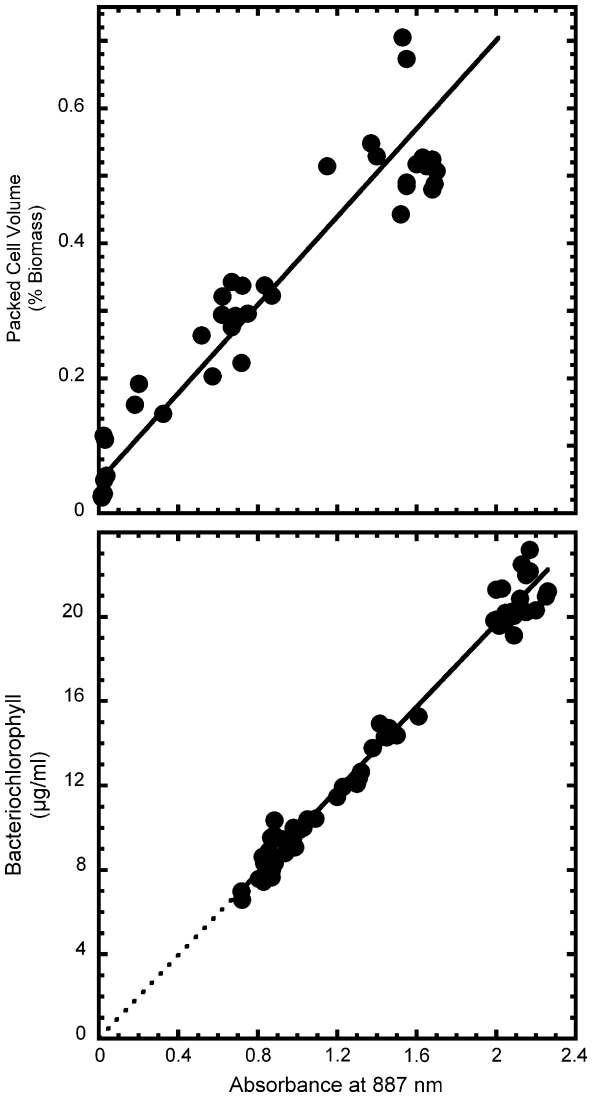

Fig. 3 – Packed cell volume and extracted bacteriochlorophyll as a function of the *in situ* absorbance at 887 nm. Packed cell volume (upper panel; expressed as a percent of culture volume) and extracted bacteriochlorophyll (μg per mL, lower panel) were plotted as a function of the *in situ* 887 nm absorbance of cultures of *R. rubrum*. The linear relationships suggested that readings of culture absorbance at 887 nm can be used as measurements of cellular growth. The trend lines shown have the formulae: upper: Y (PCV) = 0.07623 + 0.29282 X (A887), R^2 = 0.89604 and lower: Y (BChl) = −0.012097 + 9.8497 X (A887), R^2 = 0.9812.

results showed that the BChl content of a culture can be estimated by either method and that the packed cell volume is a good indication of biomass accumulation. Additionally, protein increase in cultures was found to parallel that of bacteriochlorophyll increase. The relationship between these two cell variables was described by Eq. (3):

$$[\text{Protein}] = 82 + 18.5 \times [\text{BChl}] \quad (3)$$

where [Protein] and [BChl] concentrations were measured in $\mu g\,ml^{-1}$. The linear relationship between these two variables suggested a fairly constant [Protein]/[BChl] ratio of 18.5:1 throughout the photosynthetic growth of *R. rubrum*.

In order to address whether photosynthetic hydrogen production by purple bacteria could be sustained in the absence of growth and biomass accumulation, batch cultures were first grown photoheterotrophically on Ormerod medium. Experiments were performed with *R. rubrum* UR2 and with *R. palustris* CGA009. Fig. 4 shows biomass and BChl accumulation, and H_2 productivity of *R. rubrum* cultures as a function of incubation time under defined growth conditions. In the initial stage, growth commenced with a lag time of about 30 h after inoculation. Concomitant with cell growth, biomass accumulated to 0.32% (packed cell volume per culture volume (Fig. 4, top panel)), or to an $A_{887} = \sim 0.7$ of the cells in the culture (Fig. 4, lower panel). Stationary phase was reached within about 100 h after inoculation. Gas evolution in the cultures commenced with a lag time of about 60 h after inoculation, i.e., 30 h later with respect to the accumulation of biomass (Fig. 4). Maximal rates of hydrogen gas production were about 21 mL L^{-1} culture h^{-1}, sustained for 100–150 h, and progressing well into the stationary phase, i.e., well after cell growth and biomass accumulation had stopped. Yields typically reached 1200–1600 mL H_2 L^{-1} culture. From these results, it appears that cells were capable of producing hydrogen well into the stationary phase.

To assess the relationship between hydrogen production and substrate availability, parallel measurements of succinate concentration and hydrogen production were undertaken. Fig. 5 shows that succinate consumption and hydrogen production displayed an inverse relationship, with hydrogen

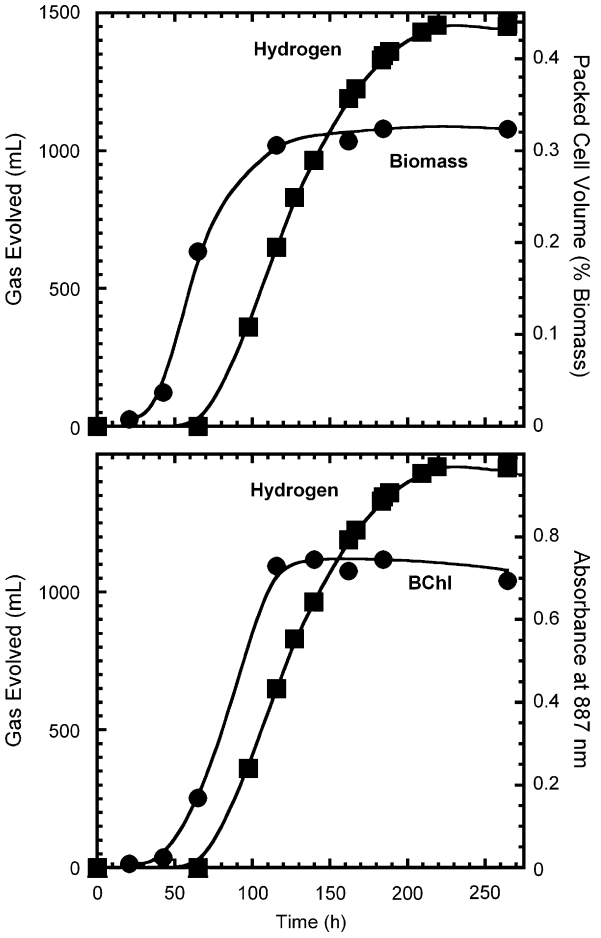

Fig. 4 – Cell growth and hydrogen production following culture inoculation. Growth of *R. rubrum*, as evidenced by accumulation of biomass (packed cell volume, upper panel) and of bacteriochlorophyll (absorbance at 887 nm, lower panel), began after a lag of about 30 h and reached stationary phase at about 100 h following culture inoculation. Hydrogen production commenced after a 50–60 h lag and stopped after about 220 h following inoculation. Hydrogen production thus continued for 100 h beyond culture entrance to stationary phase.

production depending strictly on the presence of succinate, and lasting for as long as this substrate was present in the culture. These results suggested that, in a batch culture (e.g. Fig. 4), a nutrient other than succinate becomes growth limiting, thereby inducing stationary phase, and that succinate consumption continues to drive hydrogen production during the early stationary phase.

In order to further explore the capacity of purple photosynthetic bacterial hydrogen production during stationary phase, cultures were replenished to 16 mM succinate upon succinate depletion and cessation of hydrogen production, typically every 200 h. Fig. 6 shows that hydrogen production resumed upon succinate replenishment at 200 h and also at 400 h after inoculation (Fig. 6, upward arrows). However, rates and yields of hydrogen accumulation were lower with each successive succinate addition than those of the control in each strain tested. In *R. rubrum*, the resumed rate was lowered

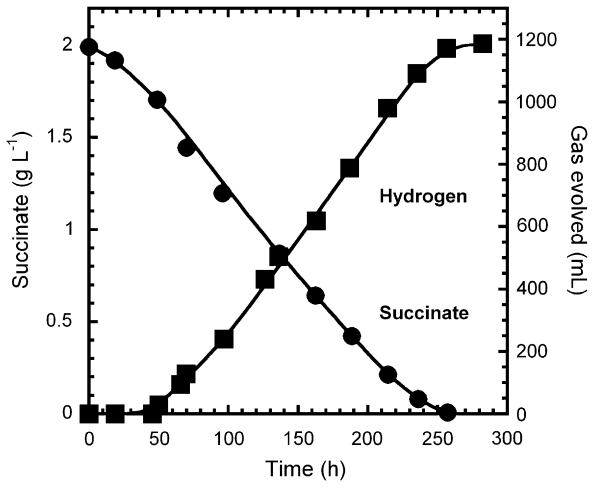

Fig. 5 – Succinate consumption versus hydrogen production. Succinate concentration declined with time and was inversely correlated with the accumulation of hydrogen. Hydrogen evolution ceased at about 270 h after culture inoculation, concomitant with the depletion of succinate from the medium. Experiments were performed with *R. palustris*.

from 20.7 to 7.9 ml L^{-1} h^{-1} (38% of the control) upon first succinate addition, and further lowered to 3 ml L^{-1} h^{-1} (14% of the control) upon the second succinate addition (Table 1A, 0% Ormerod). In *R. palustris*, the resumed rate was lowered from 4.3 to 3.3 ml L^{-1} h^{-1} (79% of the control) upon first succinate addition and further lowered to 2.3 ml L^{-1} h^{-1} (67% of the

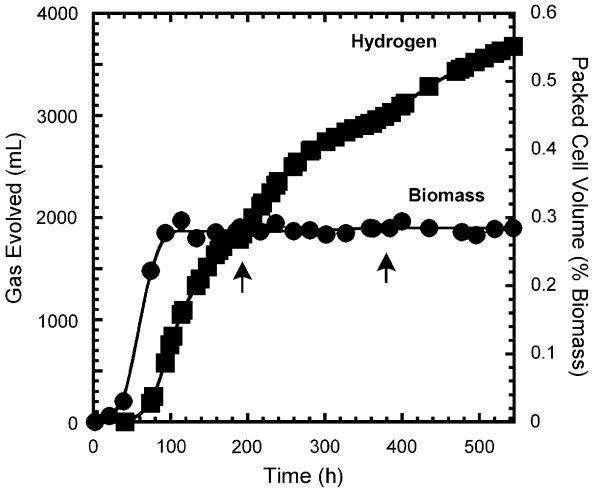

Fig. 6 – Effect of succinate replenishment on hydrogen evolution and cell growth. Cultures of *R. rubrum* were succinate-replenished upon addition of 10 mL solution containing 16 mmol succinate, typically every 200 h (indicated by arrows). There was no stimulation of growth, as indicated by the packed cell volume measurements. However, hydrogen was produced following each of the succinate replenishments. The rate and yield of hydrogen production diminished with each successive replenishment.

Table 1 – Rates of H_2-production in the purple photosynthetic bacteria *R. rubrum* (A) and *R. palustris* (B)

	Initial succinate batch	First succinate replenishment	Second succinate replenishment
(A) Rate of H_2-production in *R. rubrum* (mL gas evolved L^{-1} culture h^{-1})			
0% Ormerod	20.67 ± 6.03	7.88 ± 3.93	2.96 ± 2.40
1% Ormerod	20.67 ± 6.03	7.50 ± 2.33	5.16 ± 1.61
5% Ormerod	20.67 ± 6.03	10.60 ± 2.24	7.10 ± 1.96
Complete resuspension	20.67 ± 6.03	13.64 ± 4.32	–
(B) Rate of H_2-production in *R. palustris* (mL gas evolved L^{-1}culture h^{-1})			
0% Ormerod	4.31 ± 1.04	3.30 ± 1.12	2.28 ± 0.53
5% Ormerod	4.31 ± 1.04	5.20 ± 1.41	5.3 ± 0.31
20% Ormerod	4.31 ± 1.04	8.82 ± 3.39	8.38 ± 1.45

Succinate (16 mmol in 10 mL volume) was supplied during "replenishment" and the main experimental variable was the quantity of supplemental Ormerod medium. Rates were measured from the slope of the steep portion of the H_2-production curve. Number of replicates varied from 2 to 19. Standard deviation is given as ±.

control) upon the second succinate addition (Table 1B, 0% Ormerod). The average yield was lowered for *R. rubrum* from 1600 to 780 ml L^{-1} culture (49% of the control) upon first succinate addition, and further lowered to 580 ml L^{-1} culture (37% of the control) upon the second succinate addition (Table 2A, 0% Ormerod). In *R. palustris*, the average yield was lowered from 1285 to 1199 ml L^{-1} culture (93%) upon first succinate addition, and further lowered to 535 ml L^{-1} culture (45%) upon the second succinate addition (Table 2B, 0% Ormerod). Interestingly, growth was not stimulated at all by such additions of succinate and the cell cultures remained in stationary phase (e.g. Fig. 6, Biomass). It is therefore concluded that hydrogen production may be induced in the absence of growth upon the supply of a suitable electron-donor substrate, albeit with a gradually diminishing capacity throughout stationary phase.

A quantitative discrepancy in the rate of H_2-production between *R. rubrum* and *R. palustris* was noted, seen during the initial succinate batch. At 4.31 mL gas evolved L^{-1} culture h^{-1} (Table 1B), this *R. palustris* rate is substantially slower than the 20.67 mL gas evolved L^{-1} culture h^{-1} rate of *R. rubrum* (Table 1A). Such yield differences could not be attributed to discrepancies in biomass concentration between *R. rubrum* and *R. palustris* during these measurements, as the cultures had similar packed cell volumes. Analysis as to why *R. palustris* shows such a slow "initial batch" rate relative to the *R. rubrum* is given in Section 4. Irrespective of the reason, however, it is clear from the results (Table 1B) that succinate replenishment by itself failed to fully restore the rate of H_2-production measured during the initial succinate batch.

In order to investigate the effect of growth-limitation on stationary-phase hydrogen production, succinate replenishment experiments were also conducted with the simultaneous addition of small amounts of Ormerod medium (Fig. 7). Table 1 summarizes the findings with *R. rubrum* and *R. palustris*, showing that rates of H_2-production upon succinate replenishment were improved with the concomitant addition of increasing amounts of Ormerod medium (0, 1, 5 and 20%). More specifically, the rate of H_2-production upon first succinate replenishment in *R. rubrum* improved from 7.9 ml H_2 L^{-1} culture h^{-1} (0% Ormerod) to 10.6 ml H_2 L^{-1} culture h^{-1} (5% Ormerod). A similar pattern was observed for the rate of H_2-production upon the second succinate replenishment, as the rate improved from 3.0 ml H_2 L^{-1} culture h^{-1} (0% Ormerod) to 7.1 ml H_2 L^{-1} culture h^{-1} (5% Ormerod) (Table 1A).

The rate of H_2-production in *R. palustris* improved from 3.3 ml H_2 L^{-1} culture h^{-1} (0% Ormerod) to 5.2 ml H_2 L^{-1} culture h^{-1} (5% Ormerod), to 8.8 ml H_2 L^{-1} culture h^{-1} (20% Ormerod). The same pattern was observed for the rate of H_2-production upon the second succinate replenishment, as the rate improved from 2.3 ml H_2 L^{-1} culture h^{-1} (0% Ormerod) to 5.3 ml H_2 L^{-1} culture h^{-1} (5% Ormerod), to 8.8 ml H_2 L^{-1} culture h^{-1} (20% Ormerod) (Table 1B).

Fig. 8 plots the resumption rates of hydrogen production after first and second succinate replenishment in *R. rubrum* and *R. palustris* cultures, as a function of the Ormerod

Table 2 – Yield of of H_2-production in the purple photosynthetic bacteria *R. rubrum* (A) and *R. palustris* (B)

	Initial succinate batch	First succinate replenishment	Second succinate replenishment
(A) Yield of hydrogen production in *R. rubrum* (mL gas evolved L^{-1} culture)			
0% Ormerod	1600	782	584
1% Ormerod	1600	870	838
5% Ormerod	1600	800	650
Complete resuspension	1600	1460	–
(B) Yield of hydrogen production in *R. palustris* (mL gas evolved L^{-1} culture)			
0% Ormerod	1285	1200	535

Succinate (16 mmol in a 10 mL volume) was supplied during "replenishment" and the main experimental variable was the quantity of supplemental Ormerod medium. Yields were measured upon cessation of hydrogen production or upon succinate replenishment, whichever occurred first. Number of replicates varied from 2 to 19.

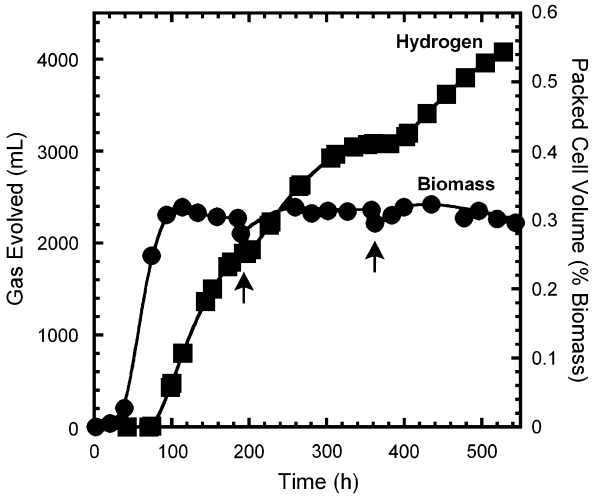

Fig. 7 – Effect of combined Ormerod medium supplementation and succinate replenishment on H_2 evolution and cell growth. At 200 and 400 h after inoculation (indicated by arrows), cultures of *R. rubrum* were subjected to removal of 60 mL culture and replacement with 50 mL fresh Ormerod medium (5% volume of the culture) supplemented with 10 ml succinate (16 mmol). Stimulation of growth, as measured by the packed cell volume method, was equivalent to 14% and 9% upon first and second succinate addition, respectively. Hydrogen production rates and yields were partially restored by this supplementation.

supplement given to the medium. It is seen that small amounts of Ormerod medium, when supplied to the cultures during the first and second succinate replenishment, substantially enhance the resumed rates of H_2-production. On the basis of such plots, it was evident that minimal bacterial growth (about 10% of the control) would suffice to permit high rates of H_2-production in the presence of replenishing amounts of endogenous substrate (succinate).

Although rates of H_2-production were improved upon partial (1–5%) Ormerod medium supplementation (Fig. 8 and Table 1), H_2 yields in *R. rubrum* were not substantially affected upon such supplementation (Table 2). Cultures grew by an amount roughly proportional to the percent Ormerod supplementation, as evidenced by packed cell volume (Fig. 7) and BChl concentration measurements (data not shown).

As an additional experiment, *R. rubrum* stationary phase cultures were also resuspended in 1 L complete Ormerod medium (100% Ormerod). Fig. 9 shows that such complete medium replacement was sufficient to restore both biomass accumulation and hydrogen production, which were high and sustained over 100 h. More specifically, the resumed rate of H_2-production was 13.6 ml H_2 L^{-1} culture h^{-1} (66% of the control, Table 1A), whereas yield of H_2-production was 1460 ml H_2 (90% of the control, Table 2A). The slower rate of H_2-production upon 100% Ormerod medium replacement may be due to the physiology of cultures grown to high cell density [35–37]. It is therefore concluded that H_2-production capability in stationary phase can be improved by the supplementation of succinate with nutrients essential for growth.

4. Discussion

The question of whether cell growth is essential for continuous hydrogen production in purple photosynthetic bacterial cultures was investigated. Cells were brought to the stationary phase and, subsequently, replenished with the electron donor, succinate. Results showed that purple photosynthetic bacteria retain their capacity to photoproduce hydrogen in stationary phase. However, rates and yields were lower with repeated succinate replenishment than those of cells in the exponential growth phase (Fig. 6). Upon stimulation of growth by supplementation with a small volume of growth medium, along with succinate replenishment (Fig. 7), cultures exhibited restoration of hydrogen production rate and yield. Thus, although growth does not appear to be a requirement for hydrogen production, some growth is essential for prolonged

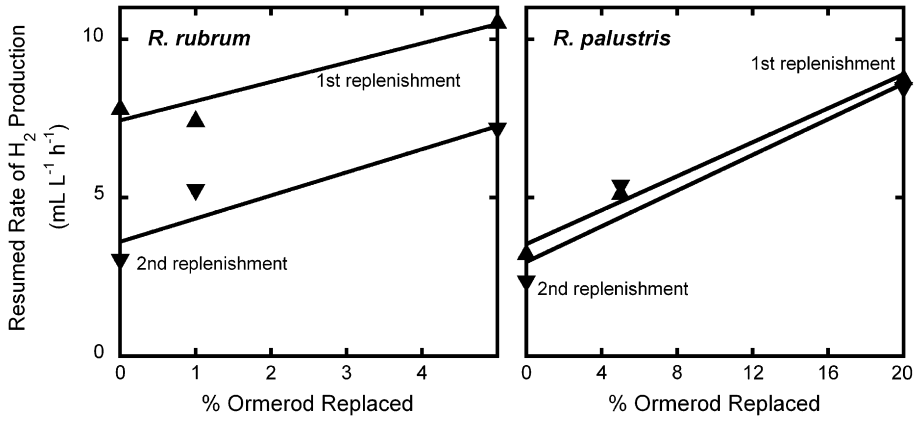

Fig. 8 – Rates of hydrogen production after 1st and 2nd succinate replenishment in *R. rubrum* and *R. palustris* cultures, as a function of the Ormerod medium supplement given. Varying amounts of Ormerod medium were supplied to the cultures during the first succinate replenishment (upward triangles) and second succinate replenishment (downward triangles). The corresponding initial succinate batch rates of H_2-production for *R. rubrum* were 21 mL L^{-1} h^{-1}, and for *R. palustris* 4.3 mL L^{-1} h^{-1}.

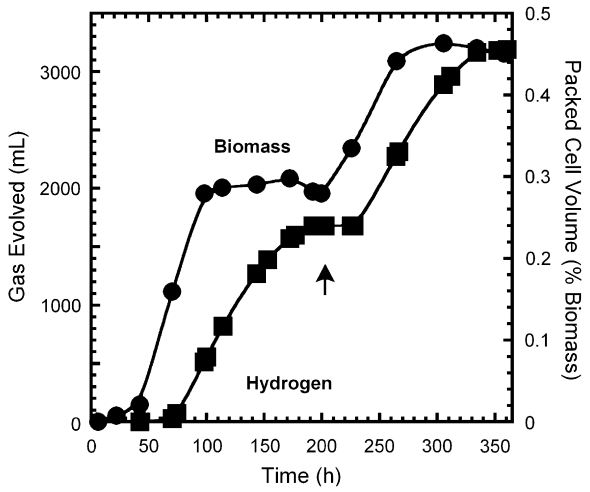

Fig. 9 – Effect of full Ormerod medium replacement on hydrogen evolution and cell growth. At 200 and 400 h after inoculation (indicated by arrow), cells in an entire culture of *R. rubrum* were pelleted by centrifugation and resuspended in the same volume of fresh Ormerod medium. Stimulation of growth, as measured by the packed cell volume, was equivalent to 66% upon Ormerod medium replacement. Hydrogen production rate and yield were also restored to a substantial degree upon this treatment.

hydrogen production at maximal rates. These conclusions hold true for two divergent species of purple photosynthetic bacteria: *R. rubrum* UR2 and *R. palustris* CGA009, conferring breadth to the findings.

A plausible hypothesis for the declining rates and yields of hydrogen production in stationary phase is that the primary physiological role of nitrogenase, i.e., converting atmospheric nitrogen to ammonia for amino acid biosynthesis, is eclipsed in the absence of growth. Accumulation and storage of ammonia is not a viable option for the cells, as ammonia is toxic to living organisms. However, as shown in Fig. 6, H_2-production capability remains in stationary phase, albeit with diminished activity. An explanation for the residual presence and activity of nitrogenase is that now it functions only as a hydrogenase via which cells dissipate excess reductant brought about by the high C:N ratio during succinate replenishment. It could be argued that, under our experimental conditions, where cultures are deprived of nitrogen, cells maintain an active nitrogenase enzyme, which utilizes reductant from an excess of endogenous substrate to produce hydrogen. It has been shown that an excess of reducing power can circumvent the repression of nitrogenase, using the hydrogen-evolving capability of the enzyme to maintain redox poise [38]. Moreover, it has also been shown that *R. palustris*, cultivated under conditions of high C:N ratio, preferentially dissipates reducing power through release of gaseous H_2 instead of the biosynthesis and accumulation of storage polymers, poly-hydroxybutyrate (PHB) and glycogen [39]. It is of interest that cells would employ the nitrogenase as a ''redox-valve'' over a process that stores energy in a polymer [40].

It is also of interest that a quantitative discrepancy in the rate of H_2-production in *R. palustris*, relative to that in *R. rubrum*, was evidenced in the initial succinate batch sample (Table 1). This may be attributed to different nutrient requirements and differences in the induction of H_2-production by the two strains [4], such that the Ormerod medium is more suitable in supporting H_2-production in *R. rubrum* relative to *R. palustris*. Other investigators have also reported variability in hydrogen production rates within purple non-sulfur photosynthetic bacteria and, further, differences among strains belonging to the same genus or species. Indeed, Barbosa et al. [41] reported that three photosynthetic bacteria, *Rhodopseudomonas* sp., *R. palustris* and one unidentified strain, showed differences in their hydrogen production capability. A similar result was also reported by Asada et al. [42] for five strains of purple non-sulfur bacteria, four *Rhodobacter sphaeroides* and one strain of *R. palustris*. Irrespective of the reason for the underlying hydrogen production rate among strains, however, it is also clear from the results (Table 1B) that succinate replenishment along with Ormerod supplementation was more effective at resuming the rate of H_2-production than succinate replenishment alone.

Results in this work, showing sustained-but-diminishing hydrogen production beyond the cessation of growth are concordant with earlier studies. For instance, Koku et al. [43] have shown that hydrogen production rates and yields are lower in *R. sphaeroides* when inocula were taken from stationary phase cultures, in contrast to inocula harvested from mid-exponential phase. Additionally, Sasikala et al. [44] examined H_2-production as a function of culture age and found that mid-log phase cultures possessed the highest production capability. Results in this work are also consistent with the findings of Zürrer and Bachofen [45], showing that continuous H_2-production can be sustained upon frequent supplementation of a *R. rubrum* culture with small amounts of growth medium, although with rates that eventually decline over time. In contrast, one experiment in the work by Zürrer and Bachofen ([45], see Fig. 1 therein), performed as a single-replicate, showed a nearly-constant hydrogen evolution rate in 500 mL non-growing fed-batch cultures of *R. rubrum* periodically replenished with lactate every few days for a month.

Considerations presented above are especially relevant to the design of systems for the commercial exploitation of microbial hydrogen production [44,45]. A variety of strategies have been proposed [18,46–48], but many suggest fed-batch systems reminiscent of the succinate replenishments described in this publication, often involving non-growing and/or immobilized cells. For instance, Gosse et al. [49] have concentrated *R. palustris* 100-fold and embedded it in nanoporous latex, producing hydrogen from acetate in the light. Their study observed deterioration in hydrogen production after 2700 h of dark storage, despite high initial rates with an initial substrate batch. However, repeated substrate replenishments were not investigated. On the other hand, Vincenzini et al. [50] showed that *R. palustris* cells, immobilized in agar beads, maintained a constant H_2 evolution rate for at least 100 days under light–dark cycles, sustained by the daily addition of a nitrogen source and malate during the course of the experiment. However, Yetis et al. [51], working with continuous cultures of *R. sphaeroides*, demonstrated that cells

need to recover their physiological status in order to sustain a continuous H_2-production. Our results lead to similar conclusions for fed-batch cultures.

Relevant in this respect are experimental designs with other H_2-producing organisms, both heterotrophic and autotrophic. Examples include work with *Escherichia coli* overexpressing the enzyme formate hydrogen lyase [52], where concentrated but non-growing cells were treated as industrial catalysts by providing them with a continuous supply of formate and seeking to obtain continuous and high yields of hydrogen production. This was successful over the short term; however, long-term sustainability of this system remains to be investigated. In addition, results from the present work may find application in the design of green microalgal systems for long-term hydrogen production [53].

A conclusion from the present work is that diminishing rates and yields of hydrogen production should be expected with each repetitive substrate addition in the absence of cellular growth. This experimental observation ought to be taken into consideration by engineers when designing fed-batch, growth-independent hydrogen production systems. It is further concluded that vigorous growth may not be an absolute requirement for microbial hydrogen production. However, some growth is necessary for cellular enzymatic maintenance and repair processes, which are needed to sustain a high yield of H_2-production.

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