Novel properties of photofermentative biohydrogen production by purple bacteria *Rhodobacter sphaeroides*: effects of protonophores and inhibitors of responsible enzymes

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Abstract

**Background:** Biohydrogen (H\textsubscript{2}) production by purple bacteria during photofermentation is a very promising way among biological H\textsubscript{2} production methods. The effects of protonophores, carbonyl cyanide m-chlorophenylhydrazone (CCCP), 2,4-dinitrophenol (DNP), and inhibitors of enzymes, involved in H\textsubscript{2} metabolism, metronidazole (Met), diphenyleneiodonium (DPI), and dimethylsulphoxide (DMSO) on H\textsubscript{2} production by *Rhodobacter sphaeroides* MDC6522 isolated from Jermuk mineral springs in Armenia have been investigated in both nitrogen-limited and nitrogen-excess conditions.

**Results:** With the increase of inhibitors concentrations H\textsubscript{2} yield gradually decreased. The complete inhibition of H\textsubscript{2} production was observed in the presence of DPI and CCCP. DPI's solvent—DMSO in low concentration did not significantly affect H\textsubscript{2} yield. *N,N*\textsuperscript{′}-dicyclohexylcarbodiimide (DCCD)-inhibited the F\textsubscript{0}F\textsubscript{1}-ATPase activity of bacterial membrane vesicles was analyzed in the presence of inhibitors. Low concentrations of DPI and DMSO did not affect ATPase activity, whereas Met and CCCP stimulated enzyme activity. The effect of DNP was similar to CCCP.

**Conclusions and significance:** The results have shown the low concentration or concentration dependent effects of protonophores and nitrogenase and hydrogenase inhibitors on photofermentative H\textsubscript{2} production by *Rh. sphaeroides* in nitrogen-limited and nitrogen-excess conditions. They would be significant to understand novel properties in relationship between nitrogenase, hydrogenase and the F\textsubscript{0}F\textsubscript{1}-ATPase in *Rh. sphaeroides*, and regulatory pathways of photofermentation. The inhibitors of nitrogenase and hydrogenase can be used in biotechnology for regulation of H\textsubscript{2} production in different technology conditions and development of scale-up applications, for biomass and energy production using purple bacterial cells.

**Keywords:** *Rhodobacter sphaeroides*, Biohydrogen photoproduction, Protonophores, Nitrogenase and hydrogenase inhibitors, ATPase activity

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world production of \( \text{H}_2 \) is more than 50 million tons, and it is increased quickly over the world, it will decrease \( \text{H}_2 \) production costs, which can be competitive with other fuels, such as oil and natural gas [3]. Thus, shortly \( \text{H}_2 \) can become one of the main fuels in global energy economy.

In comparison with the traditional ways of \( \text{H}_2 \) production such as thermochemical and photochemical processes, biological \( \text{H}_2 \) production is known to be less energy intensive, because it carried out at ambient temperature and atmospheric pressure [2, 3]. \( \text{H}_2 \) can be produced (1) by chemotroph bacteria during “dark” or “mixed-acid” fermentation of various carbon sources; (2) by microalgae and cyanobacteria during “direct” and “indirect” biophotolysis, resulting in water splitting, and (3) by purple bacteria during “photofermentation” of organic carbon substrates using sunlight as energy source (Fig. 1) [1–3, 6–8]. Among these, purple bacteria are highly favorable microorganisms for \( \text{H}_2 \) production. Photosynthetic purple non-sulfur bacteria are able to produce \( \text{H}_2 \) during photofermentation of organic carbon sources under anaerobic conditions using light as energy source [7–9]. As known, two types of enzymes—nitrogenase and hydrogenase are involved in photofermentative \( \text{H}_2 \) metabolism in purple bacteria [2, 7, 8]. Purple nonsulfur bacterium \textit{Rhodobacter sphaeroides} contains only one form of nitrogenase—[Mo–Fe]-nitrogenase [7, 8]; this is a binary enzyme, consisting of two metalloproteins: [Fe]-protein and [Mo–Fe]-protein [2, 10, 11]. These bacteria also contain [Ni–Fe]-hydrogenases, which are classified according to their involving in \( \text{H}_2 \) metabolism: “\( \text{H}_2 \)-evolving”, “\( \text{H}_2 \)-uptake” and “bidirectional” hydrogenases [1, 11–13]. The latter can catalyse \( \text{H}_2 \) uptake or production depending on the growth conditions.

During photofermentation purple bacteria can oxidize some organic carbon substrates to \( \text{CO}_2 \), protons and electrons in tricarboxylic acid cycle (TCA) by generating NADH [7, 8]. The protons are pumped through the bacterial membrane during the photosynthetic electron transport with generation of proton motive force (\( \Delta p \)). Under nitrogen-limited conditions or in the absence of \( \text{N}_2 \), upon light \( \text{H}_2 \) production by purple bacteria is mainly mediated by nitrogenase, which catalyzes conversion of protons to \( \text{H}_2 \) by using energy from ATP, which is generated via the proton-translocating \( \text{F}_6\text{F}_1\text{-ATPase} \); while hydrogenases in \textit{Rh. sphaeroides} are usually involved in \( \text{H}_2 \) uptake. But these hydrogenases can be reversible depending on the conditions: the reversibility of hydrogenases might be similar to the situation with hydrogenases in \textit{Escherichia coli} or cyanobacteria [1, 3, 8, 13].

Carbonyl cyanide \( m \)-chlorophenylhydrazone (CCCP) and 2,4-dinitrophenol (DNP), protonophores functioning as uncouplers and dissipating \( \Delta p \), and metronidazole (Met), a low-range electron acceptor (redox potential \( E_p \) is equal to \(-325 \text{ mV}\)), have been shown to inhibit nitrogenase activity in cyanobacteria [14–20]. Diphenyleneiodonium (DPI) was established as an inhibitor of hydrogenase activity in \textit{Rh. capsulatus} and \textit{Chlamydomonas reinhardtii} [21, 22]. Dimethylsulphoxide (DMSO) (solvent of DPI) affected the bacterial growth properties and membrane stability [23, 24]. In our previous works, we demonstrated the inhibitory effects of high concentrations of DPI, DMSO and Met on \( \text{H}_2 \) production by \textit{Rh. sphaeroides} strain MDC6521 isolated from Arzni mineral springs in Armenia [25, 26]. The hydrogenase activity in \textit{Rh. sphaeroides} and its relationship with nitrogenase and the \( \text{F}_6\text{F}_1\text{-ATPase} \) were suggested. Moreover, light and dark alternations affected \( \text{H}_2 \) production by \textit{Rh. sphaeroides} [9]. However, there are no data on effects of protonophores and those inhibitors at low concentrations on \( \text{H}_2 \) production ability of \textit{Rh. sphaeroides}. It is known, that many chemicals show biological effects at low and ultra-low concentrations [27, 28]. This is very interesting phenomenon; however, the mechanisms of low and ultralow concentrations effects are not clear. It is interesting, how the effects of compounds used in low concentrations differ from those of relatively high concentrations and for other bacterial strains. Appropriate mechanisms of photofermentation and \( \text{H}_2 \) production are still not clear; further studies will be needed.

In the present study we have investigated the effects of protonophores such as CCCP, DNP, inhibitors of nitrogenase and hydrogenase such as Met, DPI, and DMSO at different concentrations on \( \text{H}_2 \) production ability depending on the nitrogen-limited and nitrogen-excess conditions in \textit{Rh. sphaeroides} strain MDC6522, isolated from the other mineral springs in Armenian mountains—Jermuk. Novel and significant experimental data about the concentration-dependent effects of protonophores and inhibitors of nitrogenase and hydrogenase in
Rh. sphaeroides have been obtained. The results would improve our understanding of mechanisms, regulatory pathways of bacterial H₂ metabolism and bioenergetics of photofermentation. They can be helpful for determining the role of various enzymes and the interaction between them in H₂ production depending on the growth conditions. Thus they might lead to optimization of the technology conditions for efficient H₂ production. Importantly, the effects might be applied in H₂ biotechnology, energy production using purple bacteria.

Results
Effect of various inhibitors on Rh. sphaeroides growth properties

Photofermentative H₂ production by purple non-sulfur bacteria is known to be catalyzed by nitrogenase and hydrogenase. During the photosynthetic electron transport protons are pumped through the membrane with generation of Δp, which is used to generate ATP via the F_0F_1-ATPase and to transfer electrons to ferredoxin (Fd). It is known that then Fd and ATP are used to generate H₂ via nitrogenase [2, 7, 8]. As it was shown in our previous papers [25, 26], H₂ production was strongly inhibited by high concentrations of DPI and Met. The effect of various compounds such as hydrogenase inhibitor DPI and its solvent DMSO, nitrogenase inhibitor Met, protonophore CCCP and their concentrations on growth peculiarities and photofermentative H₂ production by Rh. sphaeroides strain MDC6522, in comparison with the other strain MDC6521, isolated from Arzni mineral springs, was studied.

The growth properties were determined during anaerobic growth of Rh. sphaeroides MDC6522 upon illumination. The compounds used affected the specific growth rate of bacterial culture. Figure 2 shows a comparison of the growth specific rates of Rh. sphaeroides, grown in the presence of different inhibitors. With the increase of reagents concentrations, the specific growth rate gradually decreased. 1–2 µM DPI decreased the specific growth rate 6- to 12-folds (p < 0.001), whereas 1 mM Met suppressed growth rate ~5-fold (p < 0.001) (see Fig. 2). Rh. sphaeroides was unable to grow in the medium with CCCP and DNP, and both uncouplers were added after 24 h growth of culture, after then bacterial growth was strongly inhibited (not shown). The effect of DPI’s solvent DMSO on the culture specific growth rate was also studied for revealing the inhibitory effect of DPI. In culture with 1 mM DMSO this rate was 1.3-fold (p < 0.01) lower than that of the control, whereas 5–10 mM DMSO suppressed the specific growth rate ~1.5- to 2-folds (p < 0.01) in comparison with the control (Fig. 2). These data were similar to the results on DPI and DMSO effects obtained for the other strain MDC6521 [26], but Rh. sphaeroides MDC6521 was more sensitive to the inhibitors used.

Effect of various inhibitors for enzymes on medium pH and E_h

The growth medium pH is an important parameter for bacterial growth under different conditions [9, 29–31]. During the anaerobic growth of Rh. sphaeroides MDC6522 control cells up to 72 h in nitrogen-limited anaerobic conditions, the pH of medium has risen from 7.0 ± 0.2 (initial pH) up to 8.8 (Fig. 3a). This increase can be caused by the carbon source utilization and OH⁻ ions efflux or by the polyhydroxybutyrate formation [29]. After then, during growth up to 96 h, pH decreased, which can be caused by the generation of photofermentation end-products, particularly acids, which could decay with H₂ evaluation. Also during H₂ generation the co-evolved CO₂ can moderate pH change.

All inhibitors affected the pH of culture growth medium (Fig. 3). The pH value increased to ~9.0 by addition of 5–10 µM DNP (after 24 h growth) (Fig. 3a). The other kinetics of pH was observed by the addition of CCCP (after 24 h growth): pH increased to ~7.8–8.0 in the presence of 0.5–1 µM CCCP, whereas in the presence of 2 µM CCCP and 50 µM DNP pH of medium was not changed (see Fig. 3a). In the presence of 0.5–1 µM DPI the pH of medium was not changed much, and it decreased to ~6.7 in medium with 2 µM DPI (Fig. 3b). By addition of 1–5 mM DMSO pH changes during bacterial growth were similar to the control, and pH was not changed much in the presence of 10 mM DMSO (see Fig. 3b). In the presence of Met pH change during Rh.
sphaeroides anaerobic growth was similar to the control (not shown).

Redox potential ($E_h$) is another significant parameter of the bacterial growth medium, which can be determined as the ability of a biological system to oxidize or reduce different substrates [9, 31–33]. According to the Nernst equation, $E_h$ depends on the reduced and oxidized products of fermentation, as well as on pH [29–31]. $E_h$ of Rh. sphaeroides MDC6522 control cells decreased to $-650 \pm 20$ mV during growth up to 72 h in nitrogen-limited anaerobic conditions (Fig. 4a). In the medium with 5 µM DNP $E_h$ decreased to $-580 \pm 20$ mV; whereas with 50 µM DNP $E_h$ dropped to $-170 \pm 10$ mV only (see Fig. 4a). In contrast to DNP, the other protonophore—CCCP delayed drop in $E_h$. The inhibition of bacterial growth may arise from the effect of CCCP (1–2 µM) on $E_{hp}$ which was decreased to $-315 \pm 20$ and $-200 \pm 15$ mV, respectively, during 72 h culture growth (see Fig. 4a). In the medium with 0.5 mM Met $E_h$ decreased to $-410 \pm 5$ mV; whereas with 1 mM Met $E_h$ dropped to $-210 \pm 10$ mV only (not shown). Therefore, the used compounds affect the $E_h$ in a concentration-dependent manner. Change of $E_h$ can be caused by indirect effect of these reagents on $E_{hp}$ or by redox processes on a surface of bacterial membrane. Thus, the negative values of $E_h$ and reduced medium are required for bacterial growth.

$E_h$ of Rh. sphaeroides str. MDC6522 control cells, grown in nitrogen-excess anaerobic conditions up to 72 h, decreased to $-600 \pm 15$ mV (Fig. 4b). The addition of DPI into the growth medium also affected $E_h$: in the medium with 0.5 µM DPI $E_h$ decreased to $-520 \pm 10$ mV; whereas with 2 µM DPI $E_h$ did not change much (Fig. 4b). 1 mM DMSO increased $E_h$ up to $-640 \pm 20$ mV. At the same time $E_h$ gradually decreased during the growth from 5 to 10 mM DMSO: $E_h$ drop was more intensive in
the presence of 10 mM DMSO (up to $-470 \pm 25$ mV) (see Fig. 4b).

**Effect of various inhibitors for enzymes on $H_2$ photoproduction during *Rh. sphaeroides* anaerobic growth**

The analysis of $E_h$ changes gives information not only on main redox processes but also on $H_2$ yield during bacterial anaerobic growth. There is a direct relationship between changes of $E_h$ and $H_2$ production by these bacteria; the reduction of protons to $H_2$ is observed under strong reducing conditions [9, 31, 32, 34].

$H_2$ yield of *Rh. sphaeroides* MDC6522 control cells during growth up to 72 h in nitrogen-limited anaerobic conditions was 6.91 mmol $H_2$ (g dry weight (DW))$^{-1}$, whereas in nitrogen-excess conditions $H_2$ yield was $\sim$1.2-fold lower (Fig. 5). As it can be seen from Fig. 5, four reagents used, except 1–5 mM DMSO, inhibited $H_2$ production by *Rh. sphaeroides*. With the increase of inhibitors concentrations, the $H_2$ yield gradually decreased. By addition of 0.1 and 0.5 mM Met $H_2$ yield has decreased $\sim$1.6-fold ($p < 0.01$) and $\sim$7.6-fold ($p < 0.001$), respectively (see Fig. 5). The effect of Met on $H_2$ yield might be coupled with change of photosynthetic electron transfer with Met as a preferred acceptor of electrons, instead of nitrogenase.

CCCP and DNP are two well-known protonophores, which are used to dissipate proton gradient responsible for ATP generation via the $F_0F_1$-ATPase and to uncouple photophosphorylation from photosynthetic electron transfer [35]. The Fig. 5 shows the $H_2$ yield level in the presence of various concentrations of CCCP and DNP. In medium with 1–2 µM CCCP $H_2$ production by bacterium has not been observed during 72 h growth, and has decreased $\sim$5.5-fold ($p < 0.001$) in the presence of 0.5 µM CCCP (see Fig. 5). $H_2$ yield lowered $\sim$1.6-fold ($p < 0.01$) and $\sim$8.0-fold ($p < 0.001$) in the medium with 5 and 10 µM DNP, and was not observed in the presence of 50 µM DNP. It is suggested that protonophores can decrease $H_2$ generation by inhibiting of ATP synthesis by photophosphorylation, which is significant for nitrogenase-dependent photofermentative $H_2$ production. These data were similar to the results obtained by Skizim and co-workers for cyanobacteria *Cyanothece* [35].

In medium with 1–2 µM DPI, the production of $H_2$ by *Rh. sphaeroides* has not been observed during 72 h growth, and has decreased $\sim$3.1-fold ($p < 0.001$) by addition of 0.5 µM DPI in comparison with the control (see Fig. 5). As shown in Fig. 5, DPI’s solvent—DMSO in concentrations of 1–5 mM did not affect $H_2$ production by *Rh. sphaeroides*, whereas the high concentrations (10 mM) suppressed the $H_2$ yield ($\sim$3.3-fold). These results were similar to those obtained for MDC6521, and they indicated that DMSO could inhibit $H_2$ production depending on its concentration [26]. $H_2$ production by the other strain—*Rh. sphaeroides* MDC6521 is more sensitive to the inhibitors action. DPI, DNP and CCCP irreversibly repress the $H_2$ production; however DMSO and Met reversibly inhibit this process. When Met and DMSO was added into the growth medium, $H_2$ started to be produced after 144–168 h (not shown) growth, probably, according to the recovery of enzyme activity.

**Effects of various inhibitors for enzymes on ATPase activity of *Rh. sphaeroides* membrane vesicles**

The $F_0F_1$-ATPase activity of *Rh. sphaeroides* MDC6522 membrane vesicles was analyzed in the presence of inhibitors to reveal the role of ATPase in $H_2$ production. The $F_0F_1$-ATPase of purple bacteria belongs to F-type ATPase [36]. The membrane vesicles of bacteria, grown in the absence of inhibitors, demonstrated definite ATPase activity. By addition of 0.2 mM $N,N'$-dicyclohexylcarbodiimide (DCCD) ATPase activity was suppressed $\sim$2-fold ($p < 0.01$) (not shown). Low concentrations of DPI and DMSO did not affect the enzyme activity, whereas CCCP (2 µM) stimulated ATPase activity on 10 % (Fig. 6). Similar data were obtained by the addition of DNP (not shown) and Met (0.5 and 1 mM), enhancing this enzyme activity on $\sim$40–45 % (see Fig. 6). This effect can be attributed to the $F_0F_1$-ATPase, because DCCD specifically inhibits the $F_0F_1$-ATPase in various bacteria [31, 32]. It is possible, that these effects might be a result of inhibitors effect on ATPase via binding with enzyme and changing its activity, or on $E_{sp}$ which can regulate the $F_0F_1$-ATPase activity.

![Fig. 5](image)

**Fig. 5** The effects of inhibitors various concentrations on $H_2$ yield of *Rh. sphaeroides* MDC6522 during anaerobic growth in batch culture up to 72 h. The $H_2$ yield was calculated by decrease in $E_h$ (see "Methods")
nitrogen-excess conditions are presented. Two possi-
ble pathways of H$_2$ generation in nitrogen-limited and
nitrogen-excess conditions can be suggested (Fig. 7). In
nitrogen-limited conditions nitro-
genase catalyzes reduction of protons to H$_2$ according
to the reaction: 8H$^+$ + 8e$^-$ + 16ATP $\rightarrow$ 4H$_2$ + 16ADP + 16Pi. Thereby, in these conditions 4 times more H$_2$
can be produced.

In the presence of protonophores and Met H$_2$
yield has decreased ~5–8-folds. It is known, that CCCP and DNP
are protonophores, which dissipate the $\Delta$P and inhibit
the synthesis of ATP via the F$_{0}$F$_{1}$-ATPase [14–17, 35].
Indeed, these protonophores can inhibit nitrogenase-
dependent photofermentative H$_2$ production by inhibiting
the synthesis of ATP (Fig. 7a). The mechanisms of the
Met inhibition are not clear yet, but it is known, that Met
as a low-range electron acceptor can interact with the
low potential electron carriers (Fd, flavodoxin) in photo-
synthetic electron transfer chain. The inhibitory effects
of Met on the H$_2$ yield in *Rh. sphaeroides* may be asso-
ciated with dysfunction of the photosynthetic electron
transport chain (Fig. 7a). Met penetrates into the bac-
terial cell through passive diffusion, where its nitro-group
is reduced to reactive cytotoxic nitro-radicals by reduced
Fd [37, 38]. Fd works as electron acceptors of nitrogen-
ase, hydrogenase and other enzymes in anaerobic bac-
teria. It is known, that the selective toxicity of Met for
anaerobic microorganisms is due to the redox potential
of their electron transport components, which are suffi-
ciently negative to reduce the nitro-group of Met [38].

Reversibility of hydrogenases is suggested for vari-
ous chemotrophic and phototrophic bacteria [1, 3, 12, 13,
39], so hydrogenase in *Rh. sphaeroides* might be bidi-
rectional involved in H$_2$ production (Fig. 7b). The com-
plete inhibition of H$_2$ production by *Rh. sphaeroides*
was observed in the presence of 1–2 $\mu$M DPI, whereas DPI’s
solvent—DMSO did not significantly affect H$_2$ yield. The
results with the inhibitory effects of DPI on H$_2$ yield in
*Rh. sphaeroides* provide a new evidence of involvement
of hydrogenase in H$_2$ production by these bacteria.

Then, to understand the role of ATPase in H$_2$
production by *Rh. sphaeroides* the F$_{0}$F$_{1}$-ATPase activity
of bacterial membrane vesicles was investigated. Low con-
centrations of DPI and DMSO did not affect the enzyme
activity, whereas CCCP (2 $\mu$M) and Met (0.5–1 $\mu$M)
enhanced ATPase activity on 10 % and ~40–45 %,
respectively. These effects might be a result of inhibi-
tors effect on ATPase via binding with the enzyme and
changing its activity. Indeed, CCCP suppressed transfer
of H$^+$ by whole cells of *Rh. sphaeroides*, as shown before

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**Discussion**

Purple non-sulfur bacteria are the most studied pho-
tosynthetic bacteria due to their demonstrated high H$_2$
production yield. Two enzymes—nitrogenase and hydro-
genase are involved in H$_2$ metabolism in these bacteria.
In the anaerobic nitrogen-limited conditions, during
bacterial non-oxygenic photosynthesis, organic carbon
sources are oxidized to CO$_2$, protons and electrons. H$^+$
can be recombined by a nitrogenase to produce H$_2$
using energy of ATP, which is generated by the F$_{0}$F$_{1}$-ATPase
during the work of photosynthetic apparatus [2, 7, 8].
The participation of hydrogenase in H$_2$ production is also
suggested under the other—nitrogen-excess conditions
[1, 25, 26]. But appropriate mechanisms of photofer-
mentation and H$_2$ production depending on conditions
are not clear yet. Thereby, two aspects of photofer-
mentative H$_2$ production by *Rh. sphaeroides* are interesting:
1st—type of enzyme (nitrogenase or hydrogenase), which
is responsible for H$_2$ production depending on the nitrogen-
limited or nitrogen-excess conditions, and the relation-
ship between these enzymes; and 2nd—a role of the
F$_{0}$F$_{1}$-ATPase in photofermentation and H$_2$
production by *Rh. sphaeroides*.

In this study the comparative analysis of protonophores
and various inhibitors low concentrations effects on photo-
ermentative H$_2$ production during *Rh. sphaeroides*
anaerobic growth in nitrogen-limited and
nitrogen-excess conditions are presented. Two possible
pathways of H$_2$ generation in nitrogen-limited and
nitrogen-excess conditions can be suggested (Fig. 7).
H$_2$ production from various carbon sources by purple
bacteria is observed in anaerobic conditions under illu-
mination (see Fig. 1). H$_2$ yield of *Rh. sphaeroides* con-
trol cells during growth up to 72 h in nitrogen-excess
anaerobic conditions was ~1.2-fold lower in comparison
with nitrogen-limited conditions (see Fig. 5). In nitrogen-
excess conditions nitrogenase catalyzes the reduction of
N$_2$ to ammonia according to the reaction: N$_2$ + 8H$^+$ +
8e$^-$ + 16ATP $\rightarrow$ 2NH$_3$ +H$_2$ + 16ADP + 16P$_i$, which
leads to the generation of 1 mol H$_2$ per mole of N$_2$ fixed
[4, 7, 8]. However, in nitrogen-limited conditions nitro-
genase catalyzes reduction of protons to H$_2$ according
to the reaction: 8H$^+$ + 8e$^-$ + 16ATP $\rightarrow$ 4H$_2$ + 16ADP + 16
Pi. Thereby, in these conditions 4 times more H$_2$
can be produced.

The ATPase activity of *Rh. sphaeroides* membrane vesi-
cles, which was calculated by colorimetric determination of liberation
of inorganic phosphate (P$_i$) per time and protein upon ATP adding
(see “Methods”).
[40], which confirms the role of the Δp in the activity of enzymes responsible for H⁺ transfer and H₂ production. Thus, two possible routes of H₂ production by *Rh. sphaeroides* can be suggested (Fig. 7). Oxidation of organic acids generates electrons, which are passed through various photosynthetic electron transfer carriers to Fd, and protons, which are pumped through the membrane generating a Δp. The latter derives the synthesis of ATP from ADP and inorganic phosphate (Pᵢ) via the FₒF₁-ATPase. Then Fd and ATP are used to generate H₂.
via nitrogenase. Protonophores used can inhibit nitrogenase-dependent H₂ production by suppressing synthesis of ATP, whereas the Met can interact with Fd in photosynthetic electron transfer chain and can work as an alternative electron acceptor, instead of nitrogenase.

Conclusions and significance
The data have shown low concentration or concentration dependent effects of protonophores and nitrogenase and hydrogenase inhibitors on photofermentative H₂ production by *Rh. sphaeroides* in nitrogen-limited and nitrogen-excess conditions. The results obtained are significant to understand the relationship between nitrogenase, hydrogenase and the F₀F₁-ATPase and their roles during photofermentation and H₂ production in *Rh. sphaeroides*. The relationship if any can be considered as a novel property of these enzymes. Importantly, the relationship depends on the nitrogen-excess and nitrogen-limited conditions. Thus, protonophores and nitrogenase and hydrogenase various inhibitors at different concentrations can be applied in the development of scale-up H₂ production biotechnology, for biomass and energy production using purple bacterial cells.

Methods
Bacterial strain and growth conditions
In the present work we used *Rh. sphaeroides* strain MDC6522 (Microbial Depository Center, Armenia, WDCM803), which was isolated from Jermuk mineral waters (pH 6.5–8.5, 57–64 °C) in Armenian mountains [9, 30]. Bacteria were grown in batch culture anaerobically upon illumination (~36 W m⁻²) in Ormerod medium with succinate as a carbon source and yeast extract as a nitrogen source as described previously [9, 30, 31]. The growth of bacterial culture was recorded by changes in optical density (OD 660) using a Spec- tro UV–Vis Auto spectrophotometer (Labomed, USA), and by determining DW of bacterial biomass, which was correlated with OD₆₆₀ according the equation: 

\[
\text{DW (g L}^{-1}\text{)} = \text{OD}_{660} \times 0.48. 
\]

The specific growth rate was calculated, as described previously [9, 26, 31].

In order to create conditions of nitrogen source limitation, the media was supplied with yeast extract (2 g L⁻¹); whereas to create nitrogen-excess conditions the concentration of yeast extract was increased 2.5-fold (the media was supplied with 5 g L⁻¹ yeast extract). Yeast extracts contain various amino acids, vitamins and other growth stimulating compounds and therefore it can be used as a component of growth media for the cultivation of various microorganisms [30, 41].

The concentrations of DPI and CCCP added into the growth medium ranged from 0.5 to 2 µM; DNP—from 5 to 50 µM; Met—from 0.1 to 2 mM, DMSO—from 1 to 10 mM.

Determinations of pH, *Eₕ*, and H₂ yield
The initial pH of the culture medium was maintained to 7.0 ± 0.1 by 0.1 M NaOH or 0.1 M HCl and determined at certain time intervals (0–96 h) by a pH-meter (HANNA Instruments, Portugal) with selective pH electrode, as described [9, 25, 31].

The medium *Eₕ* was determined during *Rh. sphaeroides* growth using a pair of redox (platinum (Pt) and titanium–silicate (Ti–Si)) and reference (Ag/AgCl) electrodes, as described before [9, 26, 31]. Note Ti–Si electrode measures the overall *Eₕ*, whereas Pt electrode (sensitive to O₂ and H₂) under anaerobic conditions detects only H₂ [30, 32]. *Eₕ* kinetics determined using redox electrodes during culture growth gives information about main redox processes and also H₂ generation [31, 32]. The H₂ yield was evaluated by the drop of *Eₕ* to low negative values using correlation between *Eₕ* change and H₂ evolution and was expressed in mmol H₂ (g DW)⁻¹ [9, 31]:

\[
\text{H}_2 \text{ yield} = \frac{\text{Amount of produced H}_2 \text{ (mmol L}^{-1}\text{)}}{\text{Dry weight (g L}^{-1}\text{)}}. 
\]

This determination of H₂ is close to the method with Clark-type electrode employed by other authors [42, 43]. H₂ generation was confirmed by the chemical method, as described [26, 44].

ATPase activity assay
ATPase activity of *Rh. sphaeroides* bacterial membrane vesicles was determined by the liberation of inorganic phosphate (*Pₐ*) in the reaction with ATP by the spectrophotometric method, as described [31, 32], and it was expressed in nmol *Pₐ* per L per μg protein in 1 min. Membrane vesicles were prepared by the method, as described previously [26, 31]. For inhibitors effects studies, the membrane vesicles were incubated with inhibitors for 10 min.

Reagents and data processing
CCCP, Met, DPI, DNP, DMSO, DCCD, ATP were obtained from Sigma, Aldrich (USA); yeast extract was purchased from Carl Roth GmbH (Germany) and succinic acid was obtained from Unichem (China). The other reagents of analytical grade were used in this study.

Each experiment was repeated three times to determine deviations, which are displayed as error bars on figures. The standard errors were calculated and Student criteria (p) were employed to validate the difference in average data between different series of experiments, as described previously [26, 31].
Abbreviations
CCCP: carbonyl cyanide m-chlorophenylhydrazone; DCCD: N,N'-dicyclohexylcarbodiimide; DMSO: dimethylsulphoxide; DNP: 2,4-dinitrophenol; DPI: diph-nylideniodonium; DW: dry weight; $E_{\text{r}}$: redox potential; Met: methionine; OD: optical density; TCA: tricarboxylic acid cycle; $\Delta p$: proton motive force.

Authors’ contributions
LG performed the cultivation and biohydrogen production study in Rh. sphaeroides: HS carried out preparation of membrane vesicles and ATPase activity assay. LG and HS interpreted the obtained data and drafted the manuscript. AT supervised and coordinated the research, reviewed and edited the manuscript. All authors have read and approved the manuscript.

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The authors declare that they have no competing interests.

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