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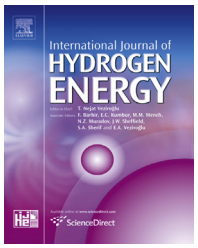

Light–dark duration alternation effects on *Rhodobacter sphaeroides* growth, membrane properties and bio-hydrogen production in batch culture

Harutyun Sargsyan [a], *Lilit Gabrielyan* [a,b], *Lilit Hakobyan* [b], *Armen Trchounian* [a,*]

[a] *Department of Microbiology & Microbe and Plant Biotechnology, Biology Faculty, Yerevan State University, 1 A. Manoukian Str., 0025 Yerevan, Armenia*

[b] *Department of Biophysics, Biology Faculty, Yerevan State University, 1 A. Manoukian Str., 0025 Yerevan, Armenia*

ARTICLE INFO

Article history:
Received 13 November 2014
Received in revised form
13 January 2015
Accepted 28 January 2015
Available online 21 February 2015

Keywords:
Hydrogen photoproduction
Light–dark alternation
Redox potential
Membrane potential and proton conductance
Rhodobacter sphaeroides

ABSTRACT

The effects of light–dark duration alternation on bacterial growth and hydrogen (H_2) production performance of *Rhodobacter sphaeroides* MDC 6522 from Armenian mineral springs were firstly investigated in batch culture. Five types of light–dark duration were applied: 1st culture was illuminated continuously (control); 2nd and 3rd cultures were illuminated after inoculation during 24–48 h, then dark conditions were applied; 4th and 5th cultures were illuminated after 24–48 h dark period. *R. sphaeroides* did not grow well in the dark conditions, but growth and H_2 production were restored upon illumination. When culture was illuminated after 24 h dark period, H_2 yield was maintained at a constant high level during 96–144 h, and duration of photo-fermentation was delayed up to 144 h, compared to the control. When culture was illuminated till H_2 production started (48 h), and then dark conditions were applied, H_2 production ability of *R. sphaeroides* persisted during the growth up to 120 h, whereas in the control cells H_2 production was not observed. Moreover, the results indicated that light–dark alternation affected the formation of photosynthetic pigments and membrane H^+ conductance. This study is of significant for understanding of bacterial metabolism regulation mechanisms and for application of purple bacteria in H_2 production biotechnology.

Introduction

At the present the production of bio-hydrogen (H_2) is considered as one of the perspective biotechnologies, suggesting the generation of renewable and ecologically clean energy from a variety of substrates and in different environmental conditions [1–5]. It is well known, that produced H_2 amount depends on the type and age of bacterial

* *Corresponding author*. Tel.: +374 60 710520.
E-mail address: Trchounian@ysu.am (A. Trchounian).
http://dx.doi.org/10.1016/j.ijhydene.2015.01.163

culture, carbon and nitrogen sources and their ratio, and various physicochemical factors, such as anaerobic conditions, temperature, pH, light intensity and others [6–12]. The light intensity is a significant physical factor of environment affecting the growth, the yield and rate of H_2 production by photosynthetic bacteria [9–12]. ATP required for bacterial growth and H_2 photoproduction is obtained during photophosphorylation, and sufficient intensity of light supplies more reductive power and ATP via membrane bound photosynthetic electron transfer, which is involved in photo-fermentative H_2 production [1–3,12].

It has been shown that the best growth and the maximal production of H_2 by purple bacteria were observed at the illumination about 6500–8500 lux, but the generation of H_2 occurred also at light intensity about 540 lux [6,13,14]. Increasing illumination stimulated H_2 photoproduction, but at lower intensities of light H_2 production was usually low [1,2,6,9] whereas the high intensities of light might inhibit the H_2 production by photosynthetic bacteria [10,11]. Koku with co-workers [15] reported that during dark periods of light–dark cycle H_2 production was not observed; but bacteria could survive and the H_2 production recovered once illumination was restored. Kondo et al. [16,17] found out that the mutant of *Rhodobacter sphaeroides* MTP4 with reduced pigments produced more H_2 under high illumination than wild type. Varying light intensities also affected the H_2 yield. Wakayama with co-workers [18] reported that the H_2 yield in *R. sphaeroides* showed dependence on the intensity of illumination. And Uyar with co-workers [10] and Li with co-workers [12] obtained that the maximum H_2 production rate and the total H_2 yield in *R. sphaeroides* during various light–dark cycles were lower than continuously illuminated cultures. It can be connected to deficiency of light energy to produce ATP needed for H_2 photoproduction during the dark period of the growth [2–4]. The bacterial cells metabolism might be changed to adapt to dark conditions as reported by Eroglu et al. [19], who showed that under limited illumination H_2 production was not observed, whereas *R. sphaeroides* performed fermentation of malate with the producing of formate as the end product. Light intensity also affected the rates of organic acids utilization. Shi and Yu [20] found that butyrate utilization needed higher intensities of light than acetate and propionate utilization. However, dark and light conditions duration and their alternations have been not clear in regulation of bacterial growth and H_2 production by purple bacteria.

In the present work novel data about light–dark duration effects on photo-fermentative H_2 production by purple bacterium *R. sphaeroides* str. MDC 6522 grown in batch culture under anaerobic conditions are presented. This strain is newly isolated from Jermuk mineral springs in Armenia non-sulfur bacterium, which can produce H_2 upon illumination and in anaerobic conditions [21,22]. However, the effects of light–dark duration on the growth and H_2 production ability of *R. sphaeroides* MDC 6522 have been never reported. The effects of light–dark alteration on membrane potential ($\Delta\varphi$) and membrane proton (H^+) conductance are also revealed. This study will be significant for understanding of mechanisms of bacterial metabolism regulation and for application of purple bacteria in biotechnology.

Materials and methods

Bacterial strain and growth conditions

In the present work *R. sphaeroides* strain MDC 6522 (Microbial Depository Center, Armenia, WDCM803) isolated from Jermuk mineral waters in Armenian mountains [21,22] was used. The bacterium was grown in batch culture anaerobically in glass vessels of 150 ml capacities with plastic press caps at 30 ± 2 °C under illumination in Ormerod medium with carbon source – succinate (30 mM) and nitrogen source – yeast extract (0.2%) as described previously [21,22]. Atmospheric and dissolved O_2 and N_2 were bubbled out from media by autoclaving, and then vessels were closed by press caps. The illumination of ~36 W/m^2 was provided by a halogen lamp (60 W). Light intensity was measured by a luxmeter (U-116 type, Russia). The initial pH of the bacterial growth medium was maintained to 7.0 ± 0.02 by 0.1 M NaOH or 0.1 M HCl and determined at time intervals 0 h–144 h by a pH-meter (HI 122-02, HANNA Instruments, Portugal) with pH electrode as described [21,22].

The growth of bacterial culture was monitored spectrophotometrically using a Spectro UV–Vis Auto spectrophotometer (Labomed, USA). It was found that optical density (OD_{660}) of 1.0 at 660 nm corresponded to a cell density of 0.52 g dry weight (DW) per liter of culture.

Adsorption spectra of bacteria

For study the light–dark alternation effects on the photosynthetic apparatus formation, the absorption spectra of *R. sphaeroides* were recorded in the 400 nm–1000 nm wavelength region on Spectro UV–Vis Auto spectrophotometer (Labomed, USA) in cuvette with path length of 1 cm. For obtaining comparable data, the original spectra were subtracted of the scattering and normalized to the same cell concentration.

Determinations of redox potential and H_2 yield assay

The medium redox potential (E_h) was determined during *R. sphaeroides* anaerobic growth using a pair of redox (platinum (Pt) and titanium-silicate (Ti–Si)), and reference (Ag/AgCl) electrodes as described [21–23]. The use of a pair of Pt and Ti–Si electrodes has certain advantages: Ti–Si electrode measures the overall E_h, whereas Pt electrode (sensitive to O_2 and H_2) under anaerobic conditions detects also H_2 [23,24]. Therefore, the difference between Pt and Ti–Si electrodes readings indicated H_2 evolution in the bacterial suspension. The H_2 yield was evaluated by the drop of E_h to low negative values (up to −720 mV) during bacterial anaerobic growth and expressed in mmol/g DW as before [21,22,24].

E_h of both electrodes were tested in the control solution as described [23]: E_h at 25 °C was of 245 ± 10 mV. E_h decrease did not depend on organic carbon substrate nature in medium. In addition, E_h value measured by Pt and Ti–Si electrodes is not affected by changes of medium pH (Table 1). Moreover, the changes of bacterial count in the growth medium had no marked effect on E_h value (see Table 1). This electrochemical determination of H_2 is close to the method with Clark-type

Table 1 – The effects of Ormerod medium pH changes on E_h variation measured by Pt and Ti–Si electrodes without and with *R. sphaeroides* MDC 6522 grown under anaerobic conditions.

E_h Pt (mV)				E_h Ti–Si (mV)		
pH	Ormerod medium (without bacteria)	With bacteria		Ormerod medium (without bacteria)	With bacteria	
		0.026 DW (g/L)	0.052 DW (g/L)		0.026 DW (g/L)	0.052 DW (g/L)
6.5	+227	+217	+191	+112	+120	+120
7.0	+223	+205	+190	+89	+96	+95
7.5	+213	+196	+180	+71	+80	+78
8.0	+186	+175	+173	+51	+57	+55

electrode employed by the other authors during the last decades [25–27].

Besides, E_h kinetics determined using pair of redox electrodes during culture growth gives information about main redox processes and also H_2 generation in time [23].

H_2 production during the growth of *R. sphaeroides* was also confirmed by the chemical method as described [21,28].

For study of the effects of light–dark duration on H_2 production ability and anaerobic growth properties of *R. sphaeroides* five types of light–dark alternation in the batch cultures were applied in parallel: the 1st culture was illuminated continuously (control); the 2nd culture was illuminated after inoculation of bacteria during 24 h, and then dark conditions were applied; the 3rd culture was illuminated after inoculation of bacteria till H_2 production started (48 h), and then was moved under dark conditions; the 4th and 5th cultures were illuminated after 24 h and 48 h dark period, respectively.

Determination of membrane potential

The membrane potential ($\Delta\varphi$) was determined from the tetraphenylphosphonium cation (TPP^+) distribution between the cytoplasm and the external medium as described [29,30].

1 μM TPP^+ was added in the assay medium, containing 150 mM Tris-phosphate buffer, then the bacteria was added and changes of the concentration of this probe was determined by using TPP^+-selective electrode as described [29,30].

Determination of membrane H^+ conductance

The membrane H^+ conductance was evaluated by registration of H^+ flux through the membrane up to achievement of electrochemical balance in the H^+ distribution on both sides of membrane by addition of small amounts of HCl (so-called "acid pulse" technique) as described [31–33].

The equilibration in the H^+ distribution on both sides of the membrane was determined by the absence of pH changes by addition of 2 μM protonophore, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), over 30 s after "acid pulse". Buffering capacity was negligible (did not affect the H^+ flux). Membrane permeability for other ions was increased using valinomycin and solutions with K^+ high content as described [31–33]. That led to the fast drop of their concentration gradient across the membrane, which was important for free passage of H^+ through membrane. The H^+ flux was measured using a pH-meter (HI 122-02, HANNA Instruments, Portugal) with selective electrode. The membrane H^+ conductance was expressed in nmol transferred H^+ per time (s) per unit of pH and the DW of bacteria as before [31–33].

Reagents and data processing

N,N'-dicyclohexylcarbodiimide (DCCD), CCCP, potassium thiocyanate, valinomycin (Sigma, Aldrich, USA), yeast extract (Carl Roths GmbH, Germany) and the other reagents used were of analytical grade.

Each experiment was repeated three times to determine deviation, which is presented as error bars on the figures. The standard errors and Student criteria (p) were employed to validate the difference in average data between various series of experiments as described previously [21,22].

Results and discussion

Effects of light–dark duration on E_h kinetics during R. sphaeroides anaerobic growth

E_h is very important factor of the environment, which can be defined as the biological system ability to reduce or oxidize various compounds [34,35]. Positive and negative values of E_h indicate the oxidized and reduced states of systems. Negative values of E_h are required for bacterial anaerobic growth [33–35]. In our previous work it was shown that during photofermentation of succinate the E_h drop of *R. sphaeroides* growth medium was of ~ −680 mV [22]. This negative value of E_h is coupled with H_2 production, because for the $2H^+ \rightarrow H_2$ reaction E_h equals to −420 mV [23,34–36].

The light–dark alteration affected the E_h of *R. sphaeroides* in a different manner. E_h of control culture, grown under continuous illumination, measured by a Pt electrode, was gradually decreased during the growth (0–72 h) up to −720 ± 20 mV (Fig. 1a). This decrease indicates the enhancement of reduction processes, which characterize bacterial metabolism under anaerobic conditions, and generation of H_2 [21,22]. Then E_h of *R. sphaeroides* control cells was gradually increased during the growth (96–144 h) up to −110 ± 5 mV (Fig. 1a). This increase might be coupled with activity of other enzyme – hydrogenase, which is usually involved in H_2 uptake in *R. sphaeroides* under illumination conditions. E_h of the 2nd culture was not changed much during the growth up to 144 h (see Fig. 1a). E_h was gradually decreased during the growth (0–120 h) from 150 ± 10 mV up to −298 ± 10 mV, and then increased up to −200 ± 5 mV. This testifies that under dark conditions bacterium is also able to carry out

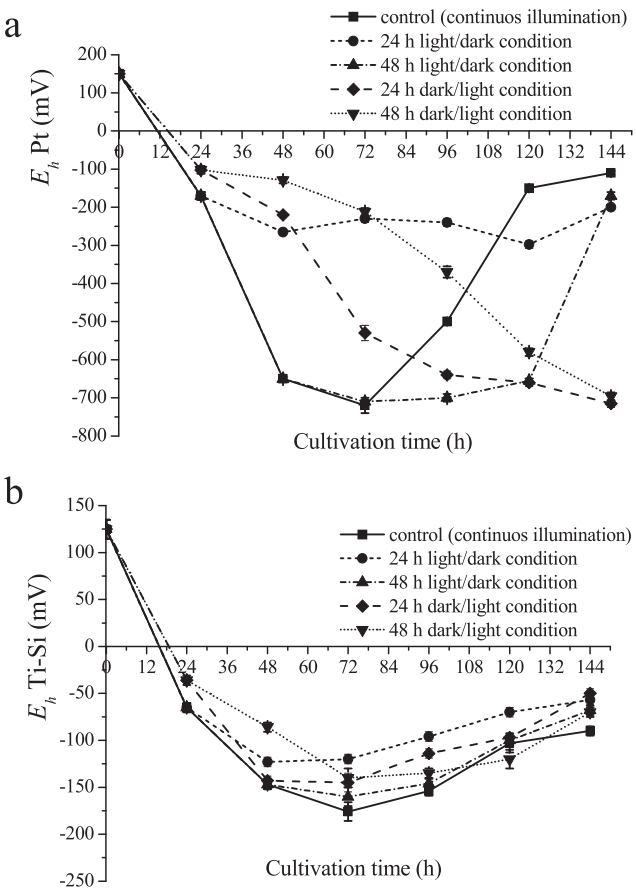

Fig. 1 – Effect of light–dark duration on E_h kinetics, measured by Pt (a) and Ti–Si (b) electrodes, during *R. sphaeroides* MDC 6522 anaerobic growth in batch culture.

fermentation. E_h of the 3rd culture was gradually decreased during the growth (0–120 h) up to −655 ± 6 mV, and then increased up to −170 ± 10 mV (see Fig. 1a). The change of E_h kinetics was coupled with the enhancement and inhibition of H_2 production by *R. sphaeroides*. Whereas E_h of the 4th and 5th cultures were gradually decreased during growth up to 144 h in anaerobic conditions to −715 ± 10 and −696 ± 10 mV, respectively (see Fig. 1a).

On the other hand, E_h of *R. sphaeroides* cells, grown under various light–dark continuous conditions, measured by Ti–Si electrode, was gradually decreased (up to −120 to −176 mV) during the growth up to 72 h, and then increased (up to −50 to −90 mV) (Fig. 1b).

Such changes of E_h during bacterial anaerobic growth can be connected with the production of reducing equivalents such as NADH or $FADH_2$, which can have a pronounced effect on the bacterial metabolism, because greater availability of NADH considerably alters the nature of end-products.

Effects of light–dark duration on H_2 yield in R. sphaeroides

The analysis of E_h changes during anaerobic growth of bacteria gives information not only on basic redox processes but also about H_2 generation by bacteria. It was found that the direct relationship between change of E_h and H_2 production for anaerobic bacteria; the reduction of protons to H_2 is observed under strong reducing conditions [6,7,36].

The data in Table 2 illustrate the variation of H_2 yield by *R. sphaeroides* under various light–dark alternations. In control cells, grown under continuous illumination, during 72 h anaerobic growth H_2 yield was 8.28 mmol/g DW, which was decreased ~5 fold during the growth up to 96 h (Table 2). Then, during the growth up to 144 h, H_2 production by *R. sphaeroides* control cells was not observed. The increase in E_h level and, therefore, the decrease of H_2 production might be coupled with activity of H_2 uptake hydrogenase, which is also involved in H_2 metabolism in purple bacteria [2–5]. The 2nd culture, which was illuminated during 24 h, then dark conditions were applied, was unable to produce H_2 during 144 h anaerobic growth (Table 2), which can be connected to absence of light energy to generate ATP and to synthesis of nitrogenase, the key H_2 producing enzyme of purple bacteria, involving in H_2 production process, which was strongly stimulated by light [3,37]. H_2 production ability of the 3rd culture (which was illuminated during 48 h, and then moved in dark conditions) was continued during the 120 h growth, and then H_2 production was not observed. That is possible due to the utilization of available substrates and can be connected with formation of reductive power and ATP synthesis [12]. H_2 production lag times of the 4th and 5th cultures (which were illuminated after 24 and 48 h dark periods) were 72 h and 120 h compared to 48 h of the control. H_2 production by the 4th culture was not observed during 48 h growth and increased ~2.2–2.6-folds after 96–144 h growth, compared to 72 h culture (see Table 2). Duration of photo-fermentation of 4th culture was

Table 2 – The effects of light–dark duration on H_2 yield and membrane H^+ conductance of *R. sphaeroides* MDC 6522 during anaerobic growth.

	H_2 yield[a], mmol/g DW					Maximum H^+ conductance (nmol of H^+/s/pH unit/mg protein)
	48 h	72 h	96 h	120 h	144 h	
Control (continuous illumination)	6.15	8.28	1.70	–	–	15.20 ± 1.0
24 h light/dark condition	–	–	–	–	–	ND
48 h light/dark condition	6.15	7.00	7.10	6.44	–	3.70 ± 0.1
24 h dark/light condition	–	3.45	7.74	8.15	8.80	9.80 ± 0.5
48 h dark/light condition	–	–	–	5.11	8.00	4.06 ± 0.1

Minus (–) sign represented the H_2 yield not observed.
ND – not determined.
[a] The mean values calculated by decrease in E_h (see Materials and methods) are presented.

delayed up to 144 h, compared to continuous illuminated culture, and H_2 production was maintained at a constant high level during 96–144 h. H_2 yield of the 5th culture determined during 144 h growth (8.00 mmol/g DW) was ~1.6 fold higher than that during 120 h (5.11 mmol/g DW) (Table 2). This increase of H_2 yield is possible due to enhanced biosynthesis of nitrogenase. The results showed that during dark conditions H_2 production was not observed, but H_2 yield was restored, when illumination was turned on.

Effect of light–dark duration on R. sphaeroides *growth properties*

The growth and pH changes were monitored during *R. sphaeroides* cultivation under various light–dark conditions. As for the H_2 production, various light–dark conditions resulted in growth properties changes. During the anaerobic growth of *R. sphaeroides* dry weight of the bacterial suspension was increased, that testified growth of the culture (Fig. 2). *R. sphaeroides* MDC 6522 (the 2nd culture) was unable to grow well in the dark conditions, but 4th and 5th cultures survived and restored their growth, when illumination was started (see Fig. 2).

Under various light–dark ratios, the color of the bacterial culture was turned from brown to red brown and light brown, which was related to the decrease of amount of synthesized pigments. Illumination conditions have been shown to affect the synthesis of the photosynthetic apparatus of purple bacteria [9,21]. The photosynthetic apparatus of *R. sphaeroides* is known to lies in the cell membrane and contains antenna pigments, which absorbed light energy; reaction centers, which converted absorbed light energy to redox energy; two pigment-protein light-harvesting (LH) complexes (LH I or B875 and LH II or B800-850, which are identified by the bacteriochlorophyll *a* (Bchl *a*) absorbance maxima), and associated electron transport chain [1–4,38]. The membrane bound photosynthetic electron transfer process is involved in photofermentative H_2 production [1–3,38].

To observe the effect of light–dark duration on formation of *R. sphaeroides* MDC 6522 photosynthetic apparatus, room temperature light absorbance spectra were determined first for bacterium grown in various light–dark conditions during 72 h (Fig. 3). The multiple peaks in the 400–500 nm wavelength region such as 420, 450, 478 and 510 nm, observed in the absorbance spectrum of control cells, correspond to carotenoids, and the absorbance maxima at 590, 800 and 850 nm in the near infrared region are due to Bchl *a* (see Fig. 3). The LH II Bchl *a* absorption can be seen as two peaks at 800 nm and at 850 nm, while the LH I Bchl *a* as an absorption peak shoulder at 875 nm [9,38,39]. But the peaks at 850 and 875 nm often merge in one large peak, which has the maxima at intermediate wavelength in between [9]. As shown in Fig. 3, absorbance spectrum of bacterial culture, which was illuminated during 48 h, and then moved in dark conditions (24 h), was the same as the control, but peaks, typical for carotenoids (except peak at 510 nm) disappeared in the dark conditions. In the culture of *R. sphaeroides*, which was illuminated during 24 h, then moved in the dark conditions, the formation of the photosynthetic apparatus structural components, such as LH pigments, was not observed (not shown). In spectra of the cultures, which were illuminated after 24 and 48 h dark period, all typical peaks appeared. In these spectra the decreases in the levels of carotenoids and Bchl *a* were observed. Whereas in bacterial culture, moved under light after 48 h dark period, the shoulder at 875 nm appeared (see Fig. 3). These results indicate that light–dark ratios affect the formation of photosynthetic pigments.

pH is very essential parameter of environment determining the anaerobic growth of purple bacteria [3,4,11,21]. In our previous studies we have shown the correlation between increase of pH and decrease of E_h [21,22,30]. Drop of E_h and change of pH are known phenomena which occur during growth of the majority of bacteria and their ability to live [33–35]. As per Nernst equation, E_h depends on pH by: $E_h = E_0 + (RT/nF)ln([ox/[red]) + (RT/nF)\ ln[H^+]$, following E_h decrease with increasing pH [34]. This was considered for two-basic ionizing system, and was observed for the assay medium without bacteria. For growth of purple bacteria during photo-fermentation the character of changes of E_h and pH

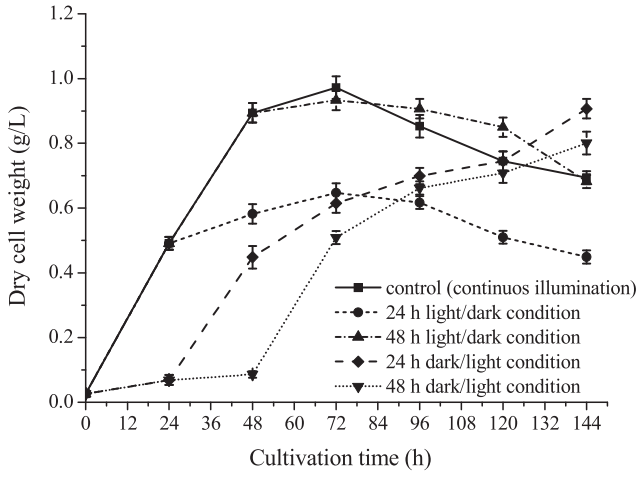

Fig. 2 – Effect of light–dark duration on *R. sphaeroides* MDC 6522 growth in batch culture.

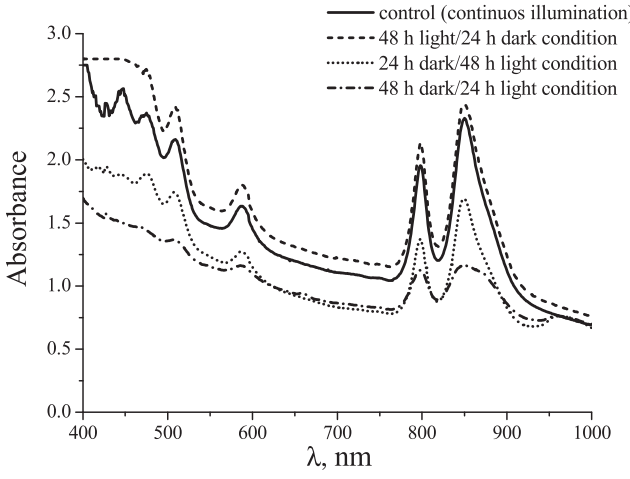

Fig. 3 – Effect of light–dark duration on the absorbance spectra of *R. sphaeroides* MDC 6522. The absorbance spectra were recorded as described in Materials and methods.

could not be described by the equation above specifying set and complexity of processes and making interplay between these parameters ambiguous. The kinetics of these interrelated parameters during *R. sphaeroides* growth can be caused by oxidation-reduction processes within the membrane such as electrons transfer and H^+ flux, which are coupled with generation of proton motive force, formation and maintenance of various ion gradients on the bacterial membrane surface [21,30,35].

During the anaerobic growth of *R. sphaeroides* MDC 6522 control cells up to 144 h pH of medium has risen from 7.0 ± 0.2 (initial pH) to ~9.15 (Fig. 4a). pH of growth medium was changed during growth under various light–dark ratios (see Fig. 4a). pH of the 2nd culture was not changed during the growth up to 144 h ΔpH (the difference between initial pH of growth medium and value of pH after 48–144 h bacterial growth) of the 3rd culture was lower than that of the control (Fig. 4b). ΔpH of the 4th and 5th cultures were gradually increased during anaerobic growth up to 144 h (see Fig. 4b). pH variation can be connected with the uptake of organic substrates and the formation of various products of fermentation such as H_2 [21].

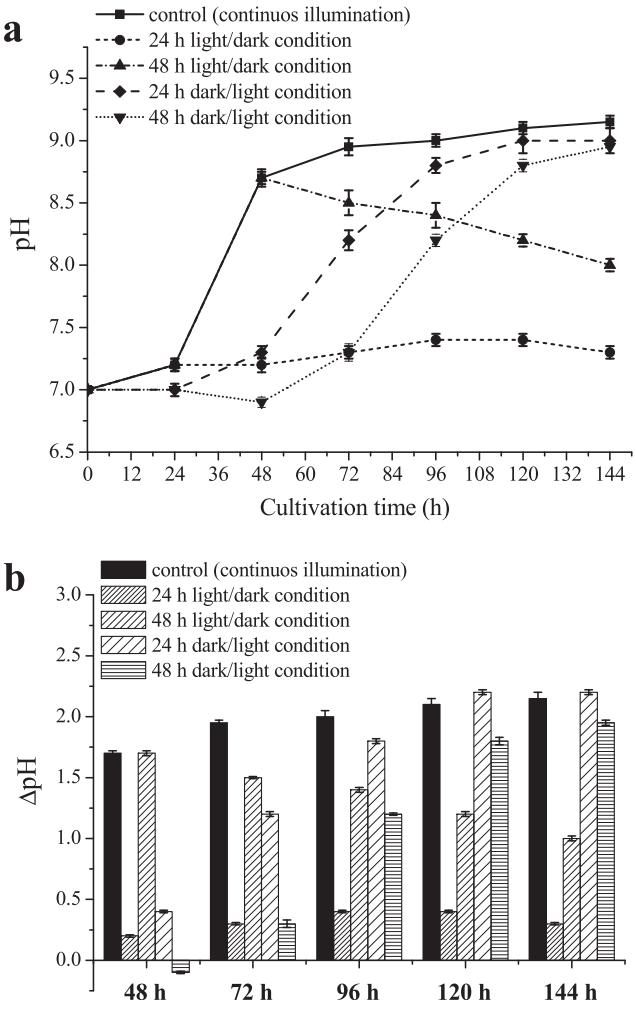

Fig. 4 – Effect of light–dark duration on medium pH (a) during *R. sphaeroides* strain MDC 6522 anaerobic growth in batch culture. ΔpH (b) is the difference between initial pH and pH value after 48–144 h bacterial growth.

Effects of light–dark conditions on membrane potential (Δφ) in R. sphaeroides

The effects of light–dark conditions on growth properties and H_2 production observed under anaerobic conditions can be explained by the variation of the bioenergetic characteristics and ATP yields for these conditions. In the present study $\Delta\varphi$ was measured in *R. sphaeroides* MDC 6522, grown in anaerobic conditions under light and dark conditions. The $\Delta\varphi$ in *R. sphaeroides* was −105 ± 5 mV under anaerobic dark conditions (not shown). Upon illumination with light intensity of ~36 W/m^2 a depolarization of the $\Delta\varphi$ was observed: $\Delta\varphi$ was decreased from −105 ± 5 mV to −68 ± 5 mV. The F_0F_1-ATPase inhibitor DCCD did not affect $\Delta\varphi$ in dark conditions, but under light in the presence of DCCD a depolarization of $\Delta\varphi$ up to −92 ± 5 mV was observed. Similar data were obtained by Abee and co-workers [40].

Changes of membrane H^+ conductance during R. sphaeroides *growth in various light–dark ratios*

It is known that membrane H^+ conductance can serve as an indicator of bacterial membrane state [30]. In the processes of energy transformation coupled with transmembrane H^+ transfer membrane H^+ conductance carries out the significant role [31–33].

The membrane H^+ conductance was considerably changed during the bacterial growth in various conditions [31–33]. The data in Table 2 show the variation of membrane H^+ conductance to different light–dark alterations. In *R. sphaeroides* cells, which were illuminated during 48 h, and then moved in dark conditions, membrane H^+ conductance was ~4-fold lower than that in continuous illuminated culture (Table 2). In the bacterial culture, which was illuminated during 24 h, then dark conditions were applied, membrane H^+ conductance was not detected. The membrane H^+ conductance of cultures, which were illuminated after 24 and 48 h dark periods, were ~1.6- and ~3.7-folds lower, respectively, compared to the control (see Table 2).

Thus, various light–dark ratios caused different values of membrane H^+ conductance. Change of H^+ conductance during the anaerobic growth of *R. sphaeroides* under various light–dark ratios might be related to modification of membrane lipid and protein composition, which can be reflected by changes in lipid bilayer and led to appearance of membrane defects, causing an increase in ion conductance [31,32]. The light stimulated photosynthetic electron transfer, which is coupled with H^+ flux through membrane [1–4,38]. In the continuous illuminated *R. sphaeroides* culture enhancement of membrane H^+ conductance was observed. Whereas the membrane H^+ conductance of cultures, which grown under various light–dark conditions (3rd, 4th and 5th cultures), was lower compared to the continuous illuminated control. Moreover, in the 2nd culture, which was illuminated during 24 h, then moved in dark conditions, membrane H^+ conductance was not detected.

The H^+ conductance variation during the bacterial growth under various light–dark ratios can be also coupled with pH changes. The relationship between membrane H^+ conductance and pH value was shown for other bacteria such as

Escherichia coli, *Enterococcus hirae* and lactic acid bacteria [30–32]. Moreover, Akopyan and Trchounian [32] detected some correlation between H^+ conductance, $\Delta\varphi$ and E_h: it was shown that change of membrane H^+ conductance is related to decrease of $\Delta\varphi$ and E_h.

In our current work we have shown, that under continuous illumination $\Delta\varphi$ and E_h values of *R. sphaeroides* were decreased and H^+ conductance was increased. As known E_h determines the redox state of lipids lateral groups and protein thiol groups and can affect the protein-lipid interaction in the membrane by changing the ion conductance [31]. Thus, low values of E_h, observed during anaerobic growth of *R. sphaeroides*, led to the modification of thiol groups of membrane proteins, and therefore to the enhancement of H^+ conductance through membrane.

Concluding remarks

It was shown, that purple bacterium *R. sphaeroides* MDC 6522 from Armenian mineral springs performs a photofermentation of succinate with H_2 production. Under various light–dark alternations the variations of H_2 yield by *R. sphaeroides* was observed. It is possible, that alternation of dark and light conditions resulted in the more stable activity of nitrogenase. Then, the results indicate, that various light–dark conditions have different effects on the photosynthetic pigments level in comparison to control. Energy required for H_2 production by purple bacteria was obtained from succinate during anaerobic light-dependent trycarboxylic acid (TCA) cycle. During assimilation of succinate *R. sphaeroides* produces reduced equivalents to promote H_2 production. When the nitrogenase activity was inhibited, the activity of TCA cycle is limited, and various alternative pathways, such as the polyhydroxybutyrate synthesis, can be stimulated [3,11].

Moreover, the membrane H^+ conductance considerably decreased during the bacterial growth in various light–dark alterations. This decrease can be related with change of E_h, because E_h determined the redox state of lipids lateral groups and protein thiol groups [34,35], and can affect the protein-lipid interaction in the membrane by changing the H^+ conductance through membrane.

Thus, alternation of light and dark conditions can change the photosynthetic pigments level, modify the bacterial anaerobic metabolism and affect H_2 production. The light–dark duration alternation especially illumination after 24 h dark period in comparison with continuous illumination at inoculation of bacteria can be used for optimization of the conditions for enhancing H_2 yield, and for application of purple bacteria in H_2 production biotechnology.

Acknowledgments

This study was supported by Research grant from the State Committee on Science, Ministry of Education and Science of Armenia, to AT (#13-1F002).

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