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Characterization of light-dependent hydrogen production by new green microalga *Parachlorella kessleri* in various conditions

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ABSTRACT

Nowadays, hydrogen (H_2) production by green microalgae seems to be a very perspective, as stocks of water and solar energy are practically inexhaustible and renewable. The aim of this study was the optimization of conditions (organic carbon sources and lighting regime), which can provide light-dependent H_2 production by green microalga *Parachlorella kessleri* RA-002 newly isolated in Armenia. The results indicated that carbon sources and lighting regimes affected H_2 production. In the presence of used carbon sources H_2 production was observed, but the highest yield of H_2 was obtained in the presence of acetate. It was 2-fold higher than the H_2 yield determined in the presence of glucose. The increase of H_2 production might be connected with the stimulation of H_2-producing enzyme – [Fe]-hydrogenase synthesis. The data obtained show that acetate can be used as an effective carbon source in H_2 production. H_2 production by microalga (in the presence of acetate and glucose) was enhanced by 1.5–2.5-fold in comparison with continuously illuminated algal cells, when *P. kessleri* was illuminated during 24 h, and then was moved in the darkness. H_2 yield increase is possible due to hydrogenase activation and the creation of anaerobic conditions. This study was significant to find out available effective substrates and optimal lighting regime to provide with light-dependent H_2 production by microalgae.

1. Introduction

Biological hydrogen (H_2) production appears to be a good candidate for energy source from different substrates and wastes, because H_2 is an environment-friendly fuel, its combustion does not contribute the air contamination as there is no production of carbon dioxide, the only product of the reaction is water [1,2].

The light-dependent H_2 production is carried out by three groups of phototrophic microorganisms: in microalgae and cyanobacteria resulting in water splitting and in purple bacteria during photo-fermentation of organic substrates [3–6]. Two types of enzymes, hydrogenase and nitrogenase, are involved in H_2 metabolism in photosynthetic organisms, and H_2 production rate and yield depend on the various factors, such as type of microbial culture, carbon and nitrogen sources, anaerobic conditions, temperature, pH, light intensity, metal ions and others [7–10]. Manipulation of these factors will make it possible to significantly enhance the H_2 production.

The basis of light-depended H_2 production by green microalgae is the process of photosynthesis, during which under certain conditions H_2 production is observed [11–13]. Green microalgae use photosystems (PS) such as PSII and PSI to carry out plant-like oxygenic photosynthesis [12,13]. The processes of oxygenic photosynthesis, mitochondrial respiration, metabolism of carbon sources, and electron transport to [Fe]-hydrogenase lead to H_2 production [13,14]. This process is carried out by various green microalgae such as *Chlamydomonas reinhardtii*, *Scenedesmus obliquis*, *Chlorella vulgaris*, etc. [7,8,13–18]. Nowadays *C. reinhardtii* is the most studied photosynthetic H_2-producing green alga. Various approaches have been used to increase the H_2 production by *C. reinhardtii* such as optimization the environmental conditions (temperature, light and pH), optimization of the medium content, the nutrient deprivation, expression of PSII, etc. [7,14–17].

Nowadays, so-called algal H_2 production has a great interest due to various advantages, such as natural origin, efficiency and renewability of solar energy and the substrate – water, as well as having non-toxic by-product – oxygen [6,11–13]. It is known, that H_2 production by photosynthetic organisms is achieved by utilization of internally stored compounds and can be enhanced by addition of external carbon substrates. The selection of the carbon source for microalgae cultivation becomes a serious problem, because it strongly affects the growth, obtaining algal biomass and H_2 yield. The nature of carbon substrates is very important, because they can be effectively utilized by microalgae and would be providing the enhanced yield of H_2.

Light is a basic factor of the environment to regulate photosynthetic organisms' growth, obtaining of their biomass and producing H_2

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[8,9,19–21]. Sufficient intensity of light is suggested to supply more reductive power and ATP via photosynthetic electron transfer, which is involved in H_2 production [13,19]. However, the role of lighting regime on H_2 production by green microalgae has not been studied in detail. Lighting regime can change the photosynthetic pigments content, modify the metabolism of microalgae and affect H_2 yield.

Therefore, the optimization of factors, which can stimulate algal biomass and H_2 yield, such as various carbon sources and lighting regime, is very important. In the present study the effects of organic carbon sources and lighting regimes on pigments content, redox potential (E_h), and H_2 yield in new green microalga *P. kessleri* RA-002 were firstly investigated.

2. Materials and Methods

2.1. Algal Culture and Cultivation

Green microalga *P. kessleri* RA-002 was obtained from the Algae collection of the Microbial Depository Center, National Academy of Sciences of Armenia, Yerevan, Armenia. The alga was cultivated photoautotrophically in the Tamia medium with pH 7.5 ± 0.2 and 27.0 ± 0.2 °C upon continuous illumination (2000 lx or ~47 W m^{-2}) with shaking on an orbital shaker (100 rpm). The medium was autoclaved before use to reduce the risk of contamination. Halogen lamp (60 W) was used for illumination. Light intensity was measured by a lux-meter LM37 (Carl Roth, Germany). The growth of algae was monitored by changes in the optical density (OD_{680}) using a Spectro UV–Vis Auto spectrophotometer (Labomed, USA). Freshly prepared sterile solutions of acetate, glucose, and fructose in concentration 1 g L^{-1} were added into the medium before bacterial inoculation.

2.2. Absorption Spectra and Photosynthetic Pigments Concentration Measurements

The absorption spectra of *P. kessleri* cell extracts in ethanol were recorded in the 400 nm to 800 nm wavelength regions with a spectral resolution of 1 nm and the absorbance values were sampled at every 1 nm in standard cuvette with path length of 1 cm on Spectro UV–Vis Auto spectrophotometer (Labomed, USA) interfaced to a personal computer [22].

Photosynthetic pigments were extracted from the algal cells with 96% ethanol [22,23]. Then, the ethanol extract was subjected to the spectrophotometric determination of chlorophylls (Chl) *a* and *b*, and carotenoids concentration. Chl *a* and *b*, and carotenoids concentrations were determined using a Spectro UV–Vis Auto spectrophotometer (Labomed, USA) and expressed in mg L^{-1} [23].

2.3. The Redox Potential and Medium pH Determinations

The redox potential (E_h) of *P. kessleri* was measured using a pair of redox (platinum (Pt) and titanium-silicate (Ti–Si)) electrodes at anaerobic growth, as described before [10,19]. Pt electrode (sensitive to O_2 and H_2) under anaerobic conditions detected only H_2, whereas Ti–Si electrode measured the overall E_h. E_h of both electrodes were tested in the control solution, as described [10,19]: E_h at 25 °C was of 245 ± 10 mV.

The extracellular pH of *P. kessleri* was measured by a pH-meter (HANNA Instruments, Portugal) with selective pH electrode (HJ1131B), as described [10,19]. The initial pH was maintained at 7.5 ± 0.02 by 0.1 M NaOH or 0.1 M HCl.

2.4. The H_2 Production Assay

For study of H_2 production algal cells in the late logarithmic growth phase (10^6 cells mL^{-1}) were harvested by centrifugation at 2000 rpm for 7 min, washed twice, and then suspended in TAP medium (pH 7.5) in the presence organic carbon source. Four types of lighting regime were provided: 72 h of continuous illumination or continuous darkness; 24 h of dark conditions followed by 48 h of illumination, and 24 h of illumination followed by 48 h of dark conditions.

All assays were performed in anaerobic conditions. The experiments were carried out in glass bottles with a working volume of 100 mL. Atmospheric and dissolved O_2 was bubbled out from media by autoclaving (at 120 °C for 20 min), after which bottles were closed by press caps. To reach anaerobic conditions the bottles were kept sealed to maintain anoxic conditions, and all experiments were performed under the strict absence of O_2.

The H_2 yield in *P. kessleri* was calculated by the decrease of E_h to low negative values during algal growth, as described [10,19] and expressed in mmol H_2 L^{-1}. In addition, H_2 evaluation in algal suspension was visualized by the appearance of gas bubbles using Durham tubes and was confirmed by the chemical assay based on the bleaching of solution of potassium permanganate in H_2SO_4 in the presence of H_2 [24].

2.5. Reagents and Data Processing

Sodium acetate, glucose, fructose from Sigma Aldrich (USA); yeast extract, Tris (amino-methane) from Carl Roth GmbH (Germany), and other reagents of analytical grade were used. The average data are presented from three independent measurements; error bars were presented on figures. Standard errors were calculated using appropriate function of Microsoft Excel 2013 [4,10].

3. Results and Discussion

3.1. Carbon Sources and Lighting Regime Effects on P. kessleri Pigments Concentration and Medium pH

The effects of carbon sources and lighting regime on *P. kessleri* growth properties were studied. Acetate, glucose, and fructose were used as organic carbon source. Four types of lighting regime were applied in *P. kessleri*: 1st culture was kept upon constant illumination during 72 h (control); 2nd culture was kept in the darkness during 72 h; 3rd culture was illuminated during 24 h, and then was moved in the darkness; and 4th culture was illuminated after 24 h dark incubation.

Green microalgae are suggested to produce H_2 in photosynthetic reactions using water as electron source and the sunlight as energy source. The photosynthetic apparatus of green algae contains antenna pigments, which absorbed light energy; reaction centers, which converted absorbed light energy to redox energy; two pigment-protein light-harvesting complexes (LHCII and LHCI), and associated electron transport chain [6,11,25]. The electrons are transferred via electron transport chain from LHCII to LHCI, which transfers electrons to ferredoxin; the latter can donate electrons to [Fe]-hydrogenase, leading to H_2 production (so-called “direct biophotolysis”) [2,6,11].

To observe the effect of carbon source on the content of total photosynthetic pigments in *P. kessleri* the absorption spectra of algal cell extracts in ethanol were measured (see Materials and methods). The absorption peaks in the 400 to 500 nm wavelength region correspond to carotenoids, and the other peaks are due to Chl *a* and Chl *b* (Fig. 1). The change in the photosynthetic pigments such as Chl *a*, Chl *b*, and carotenoids content in *P. kessleri* cell extracts in ethanol using various carbon sources are shown in Table 1. Ethanol has been shown to be an effective extraction solvent in pigment analysis [22]. Indeed, Chl *a* content was found to be the highest one when *P. kessleri* was grown in the presence of acetate, whereas Chl *b* content was slightly higher in the presence of acetate than in fructose and glucose containing media (see Table 1). The carotenoids content was more (~1.2–1.4-folds) in alga grown in fructose containing medium in comparison with acetate and glucose containing media (see Table 1).

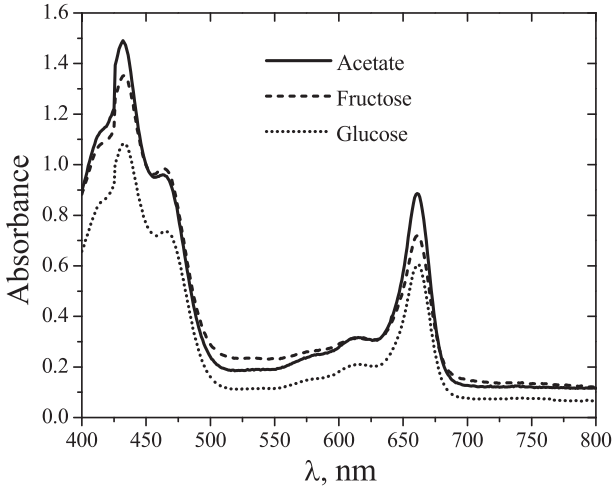

Fig. 1. Effects of various carbon sources on the absorption spectra of *P. kessleri* RA-002 cell extracts in ethanol.

Table 1
Changes of photosynthetic pigments concentration in green microalga *P. kessleri* RA-002 in the presence of organic carbon sources.

Carbon source	Chl *a*, mg L^{-1}	Chl *b*, mg L^{-1}	Carotenoids, mg L^{-1}
Acetate	15.10 ± 0.5	11.02 ± 0.5	3.89 ± 0.3
Fructose	14.00 ± 0.6	10.83 ± 0.2	4.58 ± 0.5
Glucose	12.54 ± 0.6	9.54 ± 0.3	3.28 ± 0.2

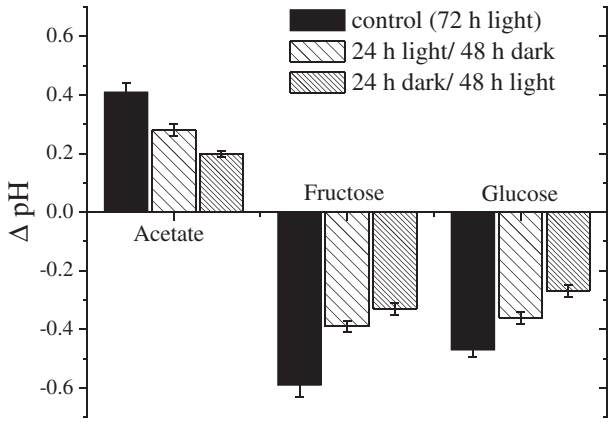

Fig. 2. Effects of various carbon sources and lighting regime on medium pH changes in *P. kessleri* RA-002 suspension (ΔpH is the difference between initial pH and pH value after 72 h algal growth).

pH is very essential parameter of environment, which can affect the activity of H_2-producing enzymes, as well as the H_2 metabolic pathways [8,9,20]. In acetate containing medium pH has risen from 7.5 ± 0.2 (initial pH) to ~8.0, whereas the decrease of pH up to ~7.0 was observed by the addition of two other carbon sources – fructose and glucose (Fig. 2). pH of *P. kessleri* medium was changed in different manner under various lighting regimes (see Fig. 2). Change of pH can be connected with the uptake of organic substrates and the formation of end-products of algal anaerobic metabolism such as CO_2 and H_2.

3.2. Carbon Sources and Lighting Regime Effects on Redox Potential and H_2 Yield in P. kessleri

E_h is another important parameter of the environment, positive and negative values of which were indicating the oxidized and reduced

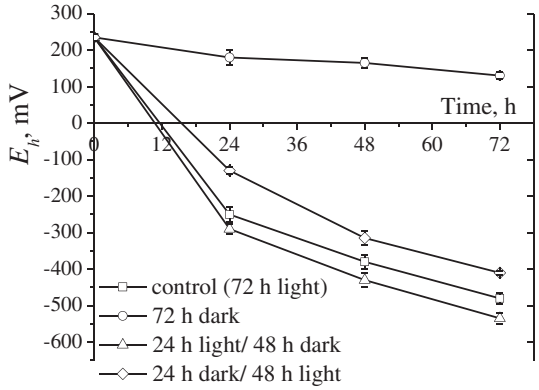

Fig. 3. Effects of lighting regime on medium E_h in *P. kessleri* RA-002 suspension in the presence of acetate.

states of biosystems [4,10]. Variation of the algal medium E_h was also examined. The organic carbon sources and lighting regimes affected the E_h in a different manner. E_h of algal culture, grown under constant illumination in the presence of acetate has gradually decreased during 72 h up to − 480 ± 15 mV (Fig. 3). This decrease indicates the enhancement of reduction processes characterizing algal metabolism under anaerobic conditions and generation of H_2. E_h of *P. kessleri* cells in the presence of fructose and glucose have decreased during up to − 455 ± 10 mV and − 430 ± 15 mV, respectively (not shown).

In acetate containing medium E_h of *P. kessleri* culture after exposure to 72 h of dark has not changed much and decreased up to 130 ± 10 mV. 24 h light/48 h dark period resulted in the E_h decrease up to − 535 ± 15 mV (see Fig. 3). At the same conditions in the presence of fructose and glucose E_h dropped up to − 510 ± 15 mV and − 525 ± 20 mV, respectively (not shown). The change of E_h kinetics was coupled with the decrease of oxygen production and enhancement of H_2 production by *P. kessleri*. Whereas E_h of *P. kessleri* culture after exposure to 24 h dark/48 h light has decreased in anaerobic conditions up to − 410 ± 5 mV (Fig. 3). In fructose and glucose containing media E_h decreased up to − 405 ± 10 mV and − 445 ± 10 mV, respectively (not shown). E_h kinetics is probably connected with the formation of different products of algal metabolism.

During growth in acetate, fructose, and glucose containing media *P. kessleri* culture was able to produce H_2. It is known, that the concentration of carbon source is an important factor, which can affect the H_2 production yield. 1 g L^{-1} of substrate is the optimal concentration for carbon source used to obtain H_2 by *P. kessleri*. Indeed, the high concentration of carbon sources inhibited the H_2 yield via modification the metabolic pathways and various by-products production [8]. The highest H_2 yield of ~1.40 mmol L^{-1} was obtained in *P. kessleri* in the presence of acetate upon continuous illumination (Fig. 4). For comparison, the H_2 yield (in TAP medium) of wild-type of *C. reinhardtii* at a light intensity of 50 W m^{-2} was ~0.30 mmol H_2 L^{-1} [16]. Thus, the data obtained indicate that *P. kessleri* can be as a promising object for the further research of mechanisms of H_2 production by green algae. Kosourov with co-workers [15] have also shown the stimulatory effect of acetate on H_2 production by *C. reinhardtii*. As known, H_2 production by green algae depends on two metabolic routes, which provide reductants for the [Fe]-hydrogenase-catalyzed reaction [15,26]. The 1st pathway includes the water-splitting process in PSII and subsequent electron transfer to the [Fe]-hydrogenase; the 2nd depends on the organic sources oxidation. According to Gibbs and co-workers [26], acetate and starch are the main sources supplying electrons for H_2 production; moreover, utilization of acetate can provide the algae with extra carbon for starch accumulation.

The H_2 yield in the presence of fructose and glucose were ~1.06 and 2.0-folds lower in comparison with algal cells, grown in acetate containing medium (see Fig. 4). The different behavior of carbon

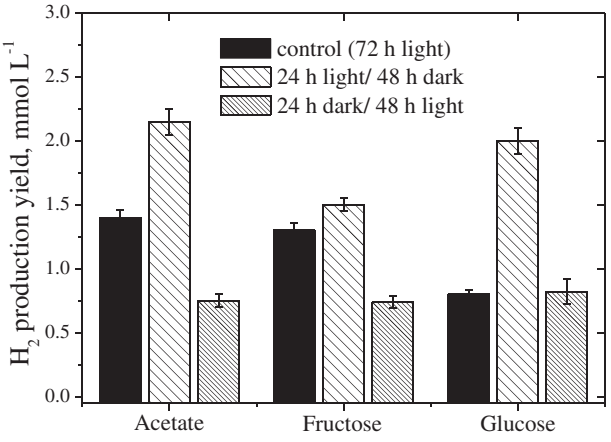

Fig. 4. Effects of various carbon sources and lighting regime on H_2 production yield by *P. kessleri*.

sources can be coupled with the efficiency of using these substrates as electron donors in H_2 production.

When the cultures (acetate and glucose containing media) were illuminated during 24 h, and then moved in the darkness, H_2 yields were ~1.5- and 2.5-folds higher in comparison with continuously illuminated algal cells (see Fig. 4). In the presence of fructose H_2 yield was ~1.2-fold higher in comparison with control (see Fig. 4). The increase of H_2 yield is possible due to expression of [Fe]-hydrogenase synthesis, which occurs in anaerobic dark conditions, as shown [12]. When cultures (in the presence of acetate and fructose) were illuminated after 24 h dark period, H_2 yields were ~2.0-fold lower in comparison with continuously illuminated culture, whereas in the presence of fructose H_2 yield was closed to the control (Fig. 4). The data obtained have shown that *P. kessleri* was not able to produce H_2 in the darkness.

Thus, used carbon sources and different lighting regimes affect the light-dependent H_2 production yield in different manner. The difference is coupled with the metabolism of carbon sources, which can be used as electron donors in H_2 production.

4. Conclusions and Significance

The presented data indicate that the various carbon sources (acetate, fructose and glucose) and lighting regimes demonstrate different behaviors in growth characteristics and H_2 production by green microalga *P. kessleri*. Carbon sources used affect the content of photosynthetic pigments. The highest concentration of Chl *a* and Chl *b* was found, when *P. kessleri* was grown in acetate containing medium. It was 1.2 fold higher than the content of chlorophylls in microalga grown in the presence of glucose. In the presence of various carbon sources H_2 production was observed. The highest H_2 yield was obtained in the presence of acetate: it was 2.0-fold higher than the H_2 yield in glucose containing medium. The nature of carbon sources plays a significant role in determination of metabolic pathways in green microalgae, which leads to H_2 production. *P. kessleri* didn't produce H_2 in the darkness, but H_2 production was enhanced by 1.5-fold during the dark incubation of microalga (acetate containing medium) after pre-illumination during 24 h. Such increase is possible due to activation of the responsible enzymes and the creation of anaerobic conditions.

The results obtained are significant for understanding H_2 production pathways in green microalgae for choosing optimal lighting regime and external carbon sources, which can be effectively consumed by algae and provide high yield of H_2. In this respect application of green microalga *P. kessleri* RA-002, isolated in Armenia, is important as a new approach for developing H_2 biotechnology.

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