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Relationship between the Photosystem II Regulation Mechanisms and Hydrogen Production in *Chlamydomonas reinhardtii* under Nitrogen or Sulfur Deprivation

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Abstract—Some microalgae are capable of light-dependent hydrogen production after a period of anaerobic adaptation, thus performing biophotolysis of water. The rate of hydrogen production the start of illumination has the rate equal to the maximum rate of photosynthesis. However, this process is short-lived: oxygen produced during photosynthesis quickly inactivates the key enzyme of biophotolysis, hydrogenase, and inhibits its expression. To date, approaches have been developed to achieve sustained hydrogen production by microalgae. The most studied are those based on transferring microalgae to nutrient-deficient conditions. However, it is known that hydrogen production under nutrient deficiency is always accompanied by the decrease in activity of photosystem II (PSII). Several mechanisms for suppression of PSII activity have been described in the literature, and there is no consensus on which mechanism is the determining one. The aim of this work was to test the hypothesis that realization of a particular mechanism of PSII suppression depends not only on the type of stress but also on the growth conditions. For this purpose, the photoautotrophic culture of the microalga *Chlamydomonas reinhardtii* was grown under nitrogen or sulfur deficiency under different light regimes, and realization of the following mechanisms of PSII activity suppression was analyzed: over-reduction of the plastoquinone pool (coupled with over-reduction of the entire photosynthetic electron transport chain), decoupling of PSII (based on the kinetics of ascorbate accumulation and the JIP test) with water-oxidizing complex, violaxanthin cycle, anaerobic stress associated with the creation of a reducing redox potential of the culture suspension. It was found that the key mechanism determining hydrogen production is the over-reduction of the plastoquinone pool. Other mechanisms are also realized under various conditions but do not show clear correlation with hydrogen production. The obtained results indicate that induction of stress through starvation of cultures is a convenient approach for studying hydrogen production by microalgae, but due to the low activity of PSII, it is impractical. New approaches are required to create industrial systems based on microalgae, allowing full realization of their photosynthetic potential.

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INTRODUCTION

Environmentally friendly energy production is a global concern. Despite the significant advances in "green energy" research, the proposed methods are still inefficient and economically unfeasible. Molecular hydrogen (H_2) is considered a promising energy carrier because it can be produced from water, and its combustion product is water. Certain groups of microalgae can produce hydrogen under light

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after a period of anaerobic adaptation. However, this process is inhibited by oxygen and is therefore short-lived. Upon illumination, the accumulated oxygen inactivates hydrogenase, and hydrogen production ceases [1-5]. In most studies on hydrogen production, the green microalga *Chlamydomonas reinhardtii* has been used as a model organism [6]. Long-term light-dependent hydrogen production by this microalga has been observed under photoheterotrophic conditions with sulfur [7], nitrogen [8], or phosphorus [9] deprivation.

We previously showed that under nitrogen deprivation, the photoautotrophic cultures of *C. reinhardtii* did not produce hydrogen, although they had reduced effective quantum yield of photosystem II, PSII (F_v/F_m') [10]. For hydrogen production, it was necessary to use a special light regime (reducing light intensity during the oxygen absorption phase), which led to over-reduction of the plastoquinone pool at the onset of anaerobiosis. Under sulfur deprivation, at the onset of anaerobiosis, the effective quantum yield decreased, and the plastoquinone pool became over-reduced with increase in the proportion of "closed" reaction centers (RC) [11]. It should be noted that in all experiments describing hydrogen production, there was a decrease in PSII activity. The literature data imply that several regulatory mechanisms could be involved in reducing PSII activity: accumulation of "closed" Q_B centers [7, 12], over-reduction of the plastoquinone pool (PQ) [13], appearance of the stably reduced forms of Q_A [14], state transitions [12], xanthophyll cycle [12, 15], degradation of D1 [16, 17], degradation of RbcL and CP43 [17], decoupling of the water-oxidizing complex and PSII [11, 18], photoinhibition (oxidative stress) [17]. At the same time, different authors point to one or two mechanisms of PSII inhibition [4]. It can be assumed that all these mechanisms are simultaneously involved in the suppression of PSII activity and are important for hydrogen production, and the authors studied different mechanisms separately. It is also possible that under conditions of sulfur or nitrogen deprivation (as well as under deprivation of other nutrients, except carbon), the decrease in PSII activity could be regulated by various mechanisms due to additional environmental factors determining difference in the rates of photosynthesis and respiration at the moment of transition of cultures to anaerobic conditions. As indirect confirmation of the latter assumption, it can be noted that photoautotrophic cultures under sulfur deprivation also did not always produce hydrogen, and at the onset of anaerobiosis, both preservation of potential PSII activity and sharp decrease in the effective quantum yield could be observed [19, 20].

The aim of this work was to examine correlation between the hydrogen production and the involved mechanisms of PSII regulation under nitrogen or sulfur deprivation in photoautotrophic cultures of *C. reinhardtii*. For this purpose, we used combination of methods based on chlorophyll fluorescence of microalgae cultures both under natural illumination and after dark adaptation, HPLC analysis of pigments and ascorbate, as well as gas chromatography to determine the amount of H_2. The obtained results indicate that inducing stress through starvation of cultures is a convenient approach for studying hydrogen production by microalgae, and its use reveals new details of the mechanisms of hydrogen production regulation.

MATERIALS AND METHODS

Study object and cultivation methods. Initial cultures of *C. reinhardtii Dang*, strain CC-124, were maintained on agar plates with standard Tris-acetate-phosphate (TAP) medium (pH 6.9) [21] at 28°C and illumination of 36 μmol photons $m^{-2}\cdot s^{-1}$. Single colonies were transferred to 10 ml of liquid TAP medium and incubated for 2 days under the same conditions. The cultures were then grown photoautotrophically on High-Salt Medium (HSM) [22] in 500-ml Erlenmeyer flasks, which were bubbled with a mixture of air and 2% CO_2 through membrane filters with pore size of 0.2 μm (Acro 37 TF, Gelman Sciences, Inc., USA) until the late exponential phase (usually three days under used conditions). For experiments, cultures were grown under photoautotrophic conditions in a photobioreactor and next used as inoculum in the same reactor, which reduced the stress caused by culture transfer. Control cultures were grown under optimal illumination without nutrient limitation.

C. reinhardtii was cultivated in a 1.5-liter photobioreactor consisting of coaxial glass cylinders with an internal stirrer, manufactured at the IBBP RAS [23]. Light path (thickness of the illuminated suspension layer) was 22 mm. Temperature (28°C) and pH (7.4) were automatically controlled by a microprocessor-based system and PC as described previously [11]. During cultivation, the cultures were aerated with a gas mixture (98% air + 2% CO_2 or 98% argon + 2% CO_2, 100 ml/min) through Acro 37 TF membrane filters with pore size of 0.2 μm (Gelman Sciences, Inc.). For illumination of cultures in the photobioreactor, cold white fluorescent lamps (Navigator NKL-4U, 30 W, 4000 K, China) were placed at the axis of glass cylinders. Light intensity on the surface of cultures was 169 μmol photons $m^{-2}\cdot s^{-1}$ of photosynthetically active radiation (PAR) (measured using a LI-250 recorder (LiCOR, USA) equipped with a quantum light sensor). The photobioreactor was connected physically and digitally to a JUNIOR-PAM fluorimeter

(Walz, Germany), as described previously [11], with movement of the culture suspension in the recirculation loop carried out by pulsed pneumatic pumps used in equipment for cultivating microorganisms, such as ANKUM (SKB BP RAS, USSR). The pulses significantly slowed down surface overgrowth in the cuvette. Before measurement, the pumps were turned off for 4 s, which excluded the movement of liquid in the measuring cuvette.

Obtaining starved cultures. To obtain sulfur-deprived cultures of *C. reinhardtii*, we used the dilution method: cultures were added to a sulfur-free medium, residual sulfur was consumed during growth, and the cultures gradually transitioned to sulfur deprivation [24]. To minimize stress during inoculation when transferring from a glass vessel to a photobioreactor, *C. reinhardtii* for inoculum was cultivated in a photobioreactor using HSM medium with doubled salt concentration (2HSM) to avoid uncontrolled limitation by any salt. It was previously shown that this medium did not inhibit growth of microalgae and allowed achieving higher chlorophyll concentrations. When the culture accumulated 50 mg/l of chlorophyll a and b (Chl (a + b)), part of the suspension was taken from the photobioreactor and remaining 300 ml of the suspension in the photobioreactor was diluted with 700 ml of 2HSM medium without sulfur (2*HSM − S) [11].

To obtain nitrogen-deprived cultures, we used a modified dilution method [10]. Nitrogen is a vital component of proteins and nucleic acids, so precise control of nitrogen deficiency during microalgae cultivation is crucial to prevent cell death. To select a suitable initial concentration of nitrogen in the medium, we studied dependence of the final concentration of Chl (a + b) in cultures on the initial concentration of NH_4Cl in the 2*HSM medium, as described previously [10]. Final concentration of Chl (a + b) increased with increasing initial concentration of NH_4Cl up to 0.25 g/l and remained constant (40 mg/l) with further increases in NH_4Cl concentration. This means that the initial (0.25 g/l) and higher concentrations of NH_4Cl do not lead to transition of cultures to the stationary phase. At NH_4Cl concentration of 0.125 g/l, final concentration of Chl (a + b) reached 21.5 mg/l, meaning that it was precisely exhaustion of nitrogen that led to transition of cultures to the stationary phase. This concentration of NH_4Cl in the medium was chosen for subsequent experiments. Under these conditions, the cultures began to grow under full nitrogen supply and gradually transitioned to nitrogen deprivation as it was consumed.

OJIP kinetic. The JIP test for a dark-adapted culture is a reliable and highly informative method reflecting all stages of electron transfer from the reaction center to photosystem I (PSI) [25, 26]. OJIP kinetics measurements were carried out using an AquaPen 110-C fluorimeter (Photon Systems Instruments, Czech Republic). Before measurement, the culture was dark-adapted for 15 min. In the case of an anaerobic (oxygen-free) culture in the photobioreactor, hermetically sealed tubes filled with argon were used for sampling. During sampling, the culture did not come into contact with air. Light pulse had duration of 1 s and intensity of 3000 μmol photons $m^{-2} \cdot s^{-1}$.

Below are the JIP test parameters determined in this work:

- F_v/F_m and $(F_m - F_0)/F_m$ – maximum photochemical quantum yield of PSII;
- $V_j = (F_j - F_0)/(F_m - F_0)$ – normalized variable fluorescence at the J stage (2 ms after light onset);
- $d_V/d_{t0} = 4(F_{300} - F_{50})/(F_m - F_0)$ – averaged initial slope of relative variable fluorescence, measured from 50 to 300 μs;
- ABS/RC – energy flux absorbed by one active RC of PSII, proportional to the apparent size of the antenna of active RCs of PSII capable of reducing QA;
- PIabs – performance index, an indicator of functional activity of PSII, related to the absorbed energy.

Ascorbate quantity measurements. To measure quantity of ascorbate with HPLC, the described previously protocol for sample preparation and mobile phases for ascorbate elution was employed [27]. For measurements, an Agilent 1100 HPLC system equipped with a 4.6×250 mm column (Waters Spherisorb ODS2, 5 μm, Supelco Inc., USA) was used. A 20-μl sample was loaded onto the column. For ascorbate elution, an isocratic mobile phase A was 50 mM KH_2PO_4 (pH 2.5, acidified with orthophosphoric acid) was used for 5 min at flow rate of 1 ml/min, followed by a short gradient of acetonitrile (mobile phase B) from 0% to 30% over the interval from 3.5 to 6 min to elute less polar components from the column. Percentage of mobile phase B was reduced to 0 over time between minutes 8 and 9, and the column was equilibrated with a mobile phase A for an additional 9 min. Total analysis time was 18 min. The analysis was carried out at room temperature. Signal (absorbance) was recorded in the range from 190 to 500 nm. The ascorbate peak appeared at approximately 5.1 min with maximum absorption at ~244 nm. As a standard, a serial dilution of ascorbate from 50 to 0.2 μM in extraction buffer containing tris(2-carboxyethyl) phosphine hydrochloride (TCEP) was used. TCEP is a reducing agent that prevents oxidation of ascorbate. Areas under the ascorbate peaks in the HPLC chromatograms were determined using software with linear approximation for constructing the calibration curve. Measurements for each sample were carried out in three biological replicates.

Carotenoid analysis. Pigment analysis of the samples was carried out using a Shimadzu HPLC system (Shimadzu, Japan) with a reverse-phase Agilent Zorbax SB-C18 column (4.6×250 mm, particle size 5 μm, Agilent, USA). To separate the pigment mixture, a solution A (23% ethyl acetate, 69% acetonitrile, and 8% water) and solution B (pure ethyl acetate) were used. The following solvent gradient was applied: for the first 5 min, solution A was pumped through the column. Then, from 5 to 25 min, solution A was replaced with a linear gradient of solution B (0-25%). Further, from 25 to 40 min, proportion of solution B was gradually increased from 25% to 100%. At the end of analysis, 100% solution B was passed through the column for 3 min. Flow rate of all solvents was 1 ml/min, and temperature was 25°C. Carotenoids were identified based on their absorption spectra and retention times on the column [28].

Results are presented as percentage content of violaxanthin and zeaxanthin, where 100% is the total amount of carotenoids (neoxanthin, violaxanthin, lutein, zeaxanthin, neolutein B, beta-carotene, alpha-carotene).

Other methods. Content of Chl (a + b) and Chl a/b ratio were determined spectrophotometrically (spectrophotometer U-VIS 1240 Mini, Shimadzu) after extraction with a 95% ethanol solution [29]. Extraction was carried out in the dark at room temperature for 5 min. Amount of starch in the cells was measured as glucose equivalents after enzymatic hydrolysis using a Glucose GOD FS kit (DiaSys, Germany) according to the method of Gfeller and Gibbs [30]. Final data on starch content are presented as glucose equivalents. Redox potential (eH) of the medium was measured as potential of a platinum electrode compared to an Ag/AgCl electrode (Mettler Toledo, USA). Percentage of H_2 in the gas from photobioreactor was analyzed using gas chromatography as described previously [19]. All experiments on cultivation of *C. reinhardtii* were repeated 2-4 times, and the figures show data from typical growth curves. Amount of H_2 produced was calculated taking into account gas flow rate (100 ml/min) and percentage of H_2 at the outlet using the following formula (1):

$$V(H_2) = H_2, \% \times V_{gas}\ (ml/min) \times 60/100\% \times V_{culture}. \quad (1)$$

Accumulation of H_2 was calculated as the sum of the corresponding time periods with the measured rates of H_2 production.

Statistical analysis. Each measurement was carried out in triplicate. For statistical processing Excel 2016 was used, and data were visualized with SigmaPlot 12. Data are presented as mean values and 95% confidence intervals.

RESULTS

Cultivation of *C. reinhardtii* in the atmosphere of air + CO_2 (control culture). As a control, we used photoautotrophic cultures of *C. reinhardtii* in a photobioreactor with the medium (2*HSM), aerated with a mixture of air + 2% CO_2. When cultivating *C. reinhardtii* under these conditions, the concentration of Chl (a + b) increased during the first 66 h, followed by the decrease (Fig. 1a). The maximum concentration of chlorophyll was 40 mg/l. It can be stated that after 66 h, the culture transitioned to the stationary phase due to depletion of some mineral component [11]. At the beginning of cultivation, pO_2 increased during the first 20 h. After 40-60 h of cultivation, pO_2 gradually decreased, indicating slowdown in the rate of photosynthetic oxygen production by the culture, which occurred before the transition to the stationary phase. Starch content in the cells and eH (Fig. 1, a and b) did not change during the entire cultivation period. Ascorbate content in the cells changed slightly and was minimal at the end of the exponential phase. Effective quantum yield of PSII (Y(II)) in the cultures initially was 0.73-0.77, gradually decreasing with this decrease accelerating at the onset of the stationary phase and reaching 0.5 at the end of cultivation. These changes are typical for photoautotrophic cultures and, in general, correspond to those described previously [10, 11, 31].

Analysis of the carotenoid content and of the Chl a/b ratio (Fig. 1d) was carried out. The obtained data confirm that in the control cultures, there is no activation of violaxanthin cycle and additional synthesis of antenna complexes throughout the entire cultivation period, including the late exponential phase, in which the cultures experienced light limitation.

Overall, the growth of *C. reinhardtii* under aerobic conditions represents a typical picture of photoautotrophic cultivation of microalgae without stress, but with transition from unlimited growth to light limitation followed by the depletion of some nutrient component in the stationary phase.

Cultivation of *C. reinhardtii* in the atmosphere of argon + CO_2 under nitrogen deprivation using two-stage light protocol. Decrease in light intensity from 169 to 30 μmol photons $m^{-2}\cdot s^{-1}$. Photoautotrophic cultures of *C. reinhardtii* did not produce hydrogen under nitrogen deprivation in our experiments [10]. For comparison, photoautotrophic cultures of the same microalga, starved for sulfur, produce a very small amount of hydrogen at constant light intensity of 110 μmol photons $m^{-2}\cdot s^{-1}$ if the inoculum was previously cultivated under illumination of 120 μmol photons $m^{-2}\cdot s^{-1}$ [19]. However, if at the early stage of oxygen consumption, the light intensity was switched from 110 to 20 μmol photons $m^{-2}\cdot s^{-1}$,

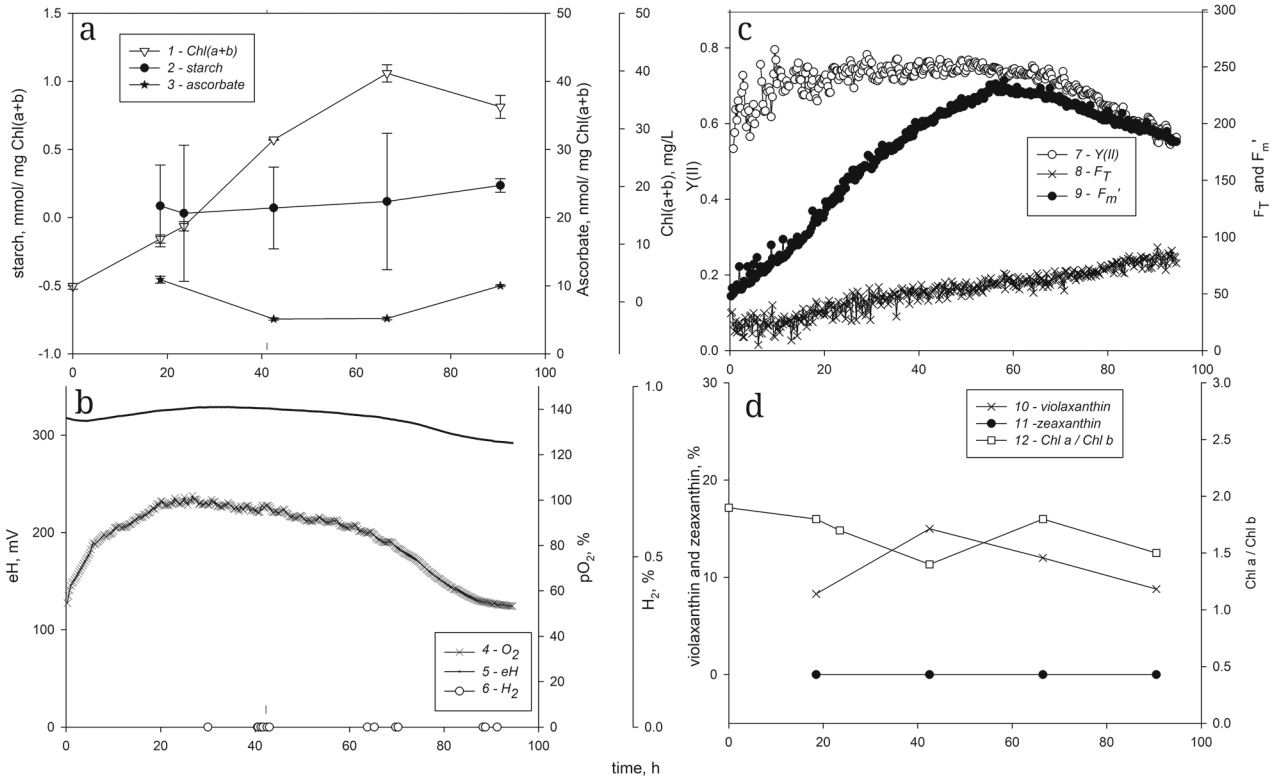

Fig. 1. Cultivation of *C. reinhardtii* in a photobioreactor in the gas phase of air + 2% CO2 (control). Light intensity 169 μmol photons $m^{-2} \cdot s^{-1}$. a) Chl (a + b) (*1*); starch (*2*), ascorbate (*3*). b) Partial pressure of oxygen, expressed as percentage of air saturation at 28°C (*4*); eH against Ag/AgCl electrode (*5*); H_2 content in the gas phase (*6*). c) Y(II) (*7*); F_T (*8*); F_m' (*9*). d) Ratio of Chl a/b (*12*) and percentage content of violaxanthin (*10*) and zeaxanthin (*11*) (100% – total amount of carotenoids).

hydrogen production significantly increased. Therefore, we applied a two-stage light protocol for the nitrogen-deprived cultures. For this, the culture of *C. reinhardtii* was inoculated as described previously [10] into the 2*HSM medium with 0.125 g/l NH_4Cl (Fig. 1). The culture smoothly transitioned from the unlimited growth for 43 h, to the stage of limitation and next to nitrogen deprivation, which generally corresponds to the data described previously [10]. After 43 h, when pO_2 in the culture began to decrease (early oxygen consumption stage in adaptation to nitrogen deprivation), the light intensity was reduced from 169 to 30 μmol photons $m^{-2} \cdot s^{-1}$. pO_2 decreased to zero within 60 min after changing the light intensity. When pO_2 reached zero, eH decreased from 300 mV and reached –60 mV after 30 min (Fig. 2). Two hours after changing the light intensity, the content of Chl (a + b) and starch began to decrease, as did the ascorbate content. Y(II) decreased from 0.74 to 0.4 (Fig. 2c, additional data are provided in the Online Resource 1), F_v/F_m decreased from 0.72 to 0.39. It is important to note that during these 2 h, the ascorbate content significantly decreased. These changes were accompanied by the decrease in PIabs. ABS/RC increased moderately, while other JIP test parameters were stable (additional data are provided in the Online Resource 1). H_2 was not detected at 43.0 and 43.25 h but appeared at 44 h in the gas phase (Fig. 2b). This indicates that the anaerobic stage of adaptation to nitrogen deprivation exists but is very short.

During subsequent incubation of the culture at the reduced light intensity, the content of Chl (a + b) and starch gradually decreased (Fig. 2a). The ascorbate content increased up to 116 h, followed by the decrease. The H_2 content in the gas phase reached maximum at 67 h. H_2 was produced at a gradually decreasing rate until the end of the experiment (Fig. 2b). Total amount of H_2 produced during the entire experiment was 122 ml per 1 liter of culture. Y(II) and F_v/F_m, after the significant decrease due to changing light intensity and establishment of anaerobiosis, gradually increased from 43 h to the end of the experiment. V_j, d_V/d_{t0}, and ABS/RC doubled after reduction in light intensity. PIabs reached minimum value at 66 h. After 137 h, there was a recovery of all JIP test parameters.

Analysis of the carotenoid content and of the Chl a/b ratio (Fig. 2d) was carried out. The obtained data confirm that there was no activation of the violaxanthin cycle and additional synthesis of antenna complexes in the cultures throughout the entire cultivation period.

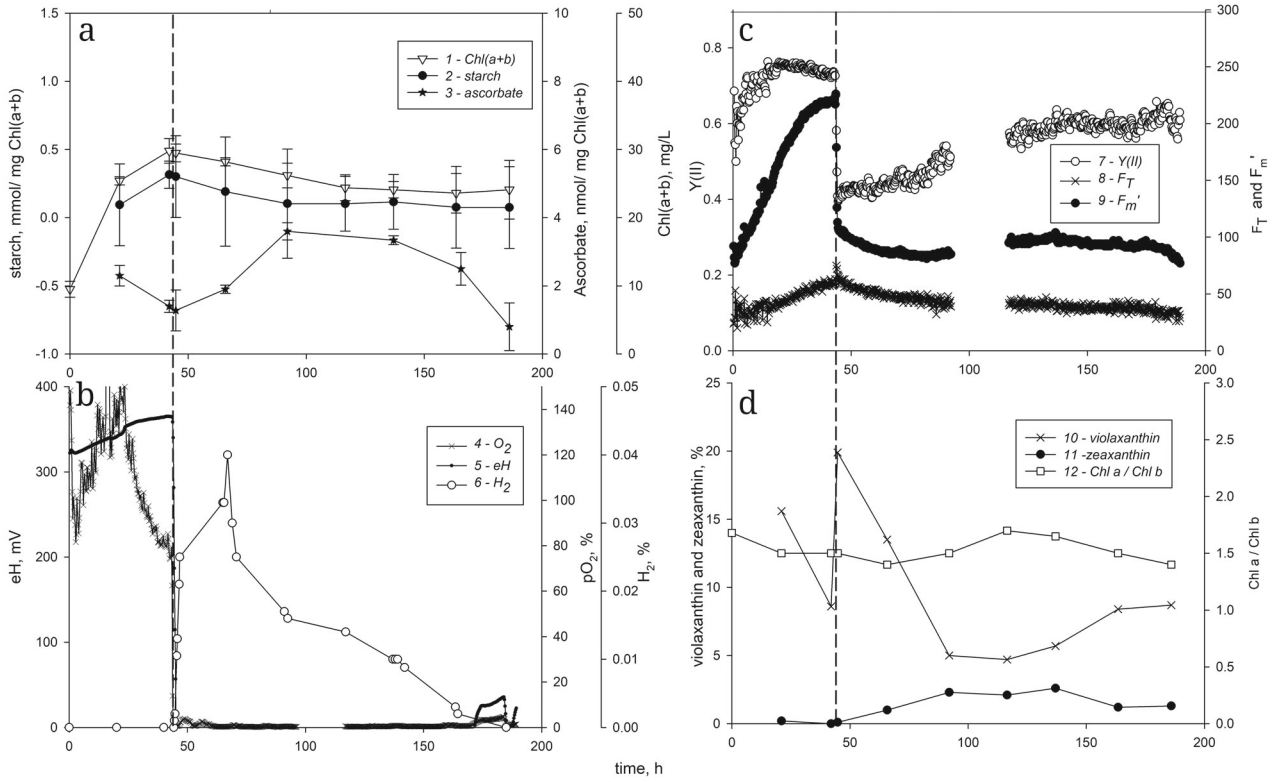

Fig. 2. Cultivation of *C. reinhardtii* in the photobioreactor in the gas phase of argon + 2% CO_2 under nitrogen deprivation using two-stage light protocol. Light intensity 167 μmol photons $m^{-2}\cdot s^{-1}$ up to 43.6 h, then 30 μmol photons $m^{-2}\cdot s^{-1}$ (vertical dotted line – change in light intensity). a) Chl (a + b) (*1*); starch (*2*), ascorbate (*3*). b) Partial pressure of oxygen, expressed as percentage of air saturation at 28°C (*4*); eH against Ag/AgCl electrode (*5*); H_2 content in the gas phase (*6*). c) Y(II) (7); F_T (*8*); F_m' (*9*). d) Ratio of Chl a/b (*12*) and percentage content of violaxanthin (*10*) and zeaxanthin (*11*) (100% – total amount of carotenoids). Gap in the measurements of F_T and F_m' is associated with a technical malfunction.

Cultivation of *C. reinhardtii* in the atmosphere of argon + CO_2 under nitrogen deprivation using two-stage light protocol. Increase in the light intensity from 169 to 300 μmol photons $m^{-2}\cdot s^{-1}$. In this experiment, the effect of high light intensity on PSII activity in the *C. reinhardtii* cultures under nitrogen deprivation was studied (Fig. 3). We were unable to set the light intensity to 300 μmol photons $m^{-2}\cdot s^{-1}$ from the beginning of the experiment, as at this light intensity, the culture at the time of inoculation, even at a Chl (a + b) concentration of about 12 mg/l, did not grow. Therefore, the two-stage light protocol was applied to the cultures: for the first 46 h of cultivation, the light intensity was 169 μmol photons $m^{-2}\cdot s^{-1}$, and after 46 h of cultivation (period of nitrogen starvation) it was increased to 300 μmol photons $m^{-2}\cdot s^{-1}$.

The culture smoothly transitioned from unlimited growth for 23 h, to the limitation stage and next to nitrogen deprivation. At 46 h of cultivation, the light intensity was increased to 300 μmol photons $m^{-2}\cdot s^{-1}$; by this time, nitrogen limitation was already observed. Chl (a + b), pO_2, ascorbate, and the effective quantum yield (Y) decreased. Starch accumulation occurred, lasting until the end of the experiment and reaching maximum values compared to all previously conducted experiments. Increasing the light intensity led to increase in the amount of ascorbate, with further decrease after 60 h. Maximum concentration of ascorbate was 5.5 nmol/mg Chl (a + b). eH remained stable throughout the cultivation, and although pO_2 decreased, it was not less than 10% of saturation, meaning that the transition to anaerobic conditions did not occur. Therefore, we were unable to detect hydrogen in the gas phase of the photobioreactor at any stage of the experiment.

All parameters obtained from the JIP test (additional data are provided in the Online Resource 1) did not differ from those obtained for the cultures grown under constant light intensity, except for the initial slope of the fluorescence curve (d_V/d_{t0} (M_0)), which showed increase after 65 h and correlated with the changes in the amount of ascorbate. These changes indicate decoupling of the water-oxidizing complex and PSII [31, 32].

Analysis of the carotenoid content and of the Chl a/b ratio (Fig. 3d) was carried out. The obtained data confirm that there was no activation of violaxanthin cycle and additional synthesis of antenna

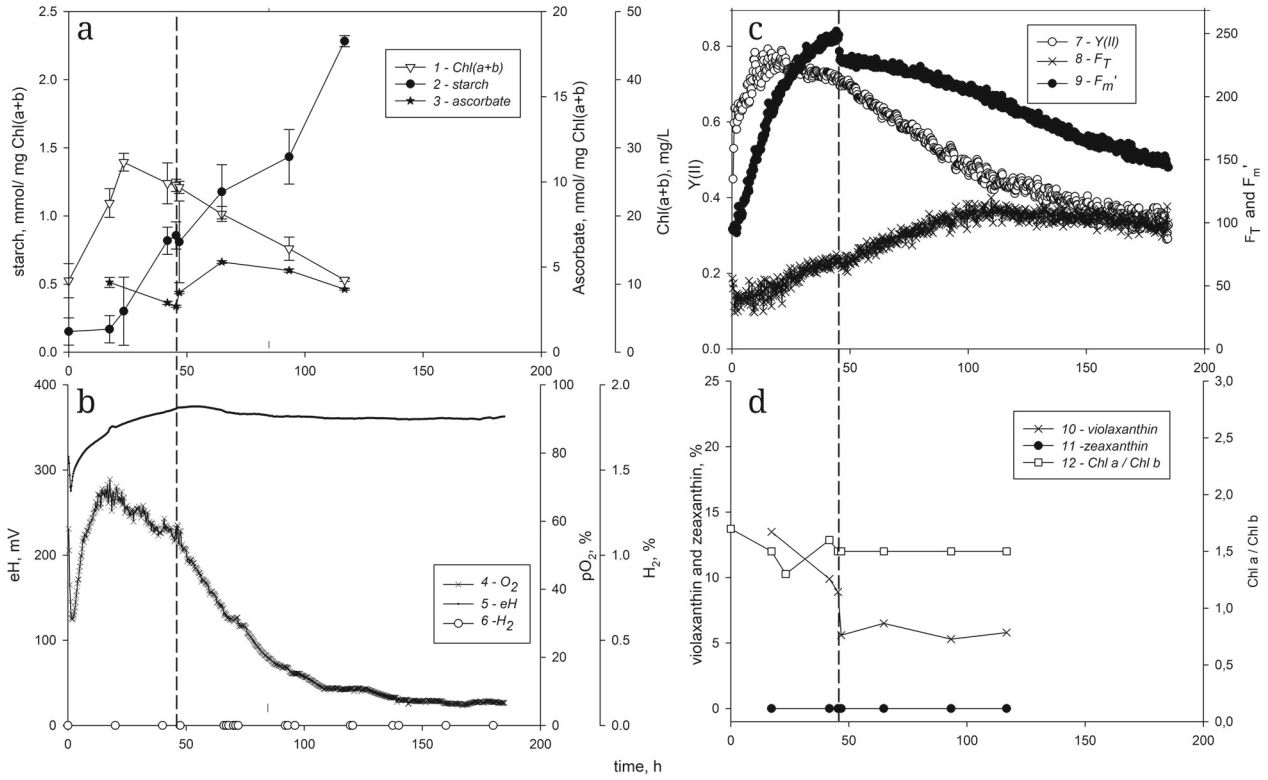

Fig. 3. Cultivation of *C. reinhardtii* in a photobioreactor in the gas phase of argon + 2% CO_2 under nitrogen deprivation using two-stage light protocol. Light intensity 167 μmol photons $m^{-2}\cdot s^{-1}$ up to 46 h, then 300 μmol photons $m^{-2}\cdot s^{-1}$ (vertical dotted line – change in light intensity). a) Chl (a + b) (*1*); starch (*2*), ascorbate (*3*). b) Partial pressure of oxygen, expressed as percentage of air saturation at 28°C (*4*); eH against Ag/AgCl electrode (*5*); H_2 content in the gas phase (*6*). c) Y(II) (7); F_T (*8*); F_m' (*9*). d) Ratio of Chl a/b (*12*) and percentage content of violaxanthin (*10*) and zeaxanthin (*11*) (100% – total amount of carotenoids).

complexes throughout the entire cultivation period in the cultures.

Cultivation of *C. reinhardtii* in the atmosphere of argon + CO_2 under sulfur deprivation. Light intensity 169 μmol photons $m^{-2}\cdot s^{-1}$. When cultivating *C. reinhardtii* in the atmosphere of argon + 2% CO_2, with the culture inoculated into the sulfur-free medium, concentration of Chl (a + b) increased up to 23 h (beginning of sulfur starvation) and was stable up to 41 h, after which it began to decrease (Fig. 4).

The culture smoothly transitioned from unlimited growth for 23 h to the stage of limitation and transition to sulfur deprivation, which generally corresponds to the data described previously [11]. pO_2 remained stable up to 23 h, after which it decreased (oxygen consumption stage), reaching zero (anaerobic and hydrogen production stage) by the 65th h. The redox potential was stable when pO_2 was stable, then decreased simultaneously with pO_2 and sharply dropped when pO_2 reached zero, followed by the increase in the late hydrogen production stage, i.e., under anaerobic conditions, the redox potential of the culture liquid was reducing. The ascorbate content increased throughout the experiment from 10.5 to 31.7 nmol/mg Chl (a + b). Hydrogen was detected in the gas phase at 66 h. H_2 content in the gas phase increased up to 93 h and then decreased. H_2 in the gas phase was detected even after 130 h of cultivation. Total hydrogen production during the entire incubation period was 170 ml/l. The effective quantum yield of PSII, Y(II), began to decrease after 60 h of cultivation, mainly due to decrease in Fm′.

All parameters obtained from the OJIP test (additional data are provided in the Online Resource 2) 23 h after inoculation in the atmosphere of argon + CO_2 without sulfur were very close to those for the control culture with similar concentration of Chl (a + b). Thus, at this time, the culture did not experience sulfur deprivation. At the 41.5-h point, Y, F_v/F_m, V_j, Sm, N did not change, while Pabs significantly decreased.

After 68 h of growth, when H_2 appeared in the gas phase, Y, F_v/F_m, Pabs decreased, while V_j, S_m, and N did not change significantly.

The results of pigment analysis (Fig. 4d) demonstrate that in this experiment, during adaptation to high light intensities, there is enzymatic interconversion between violaxanthin and zeaxanthin.

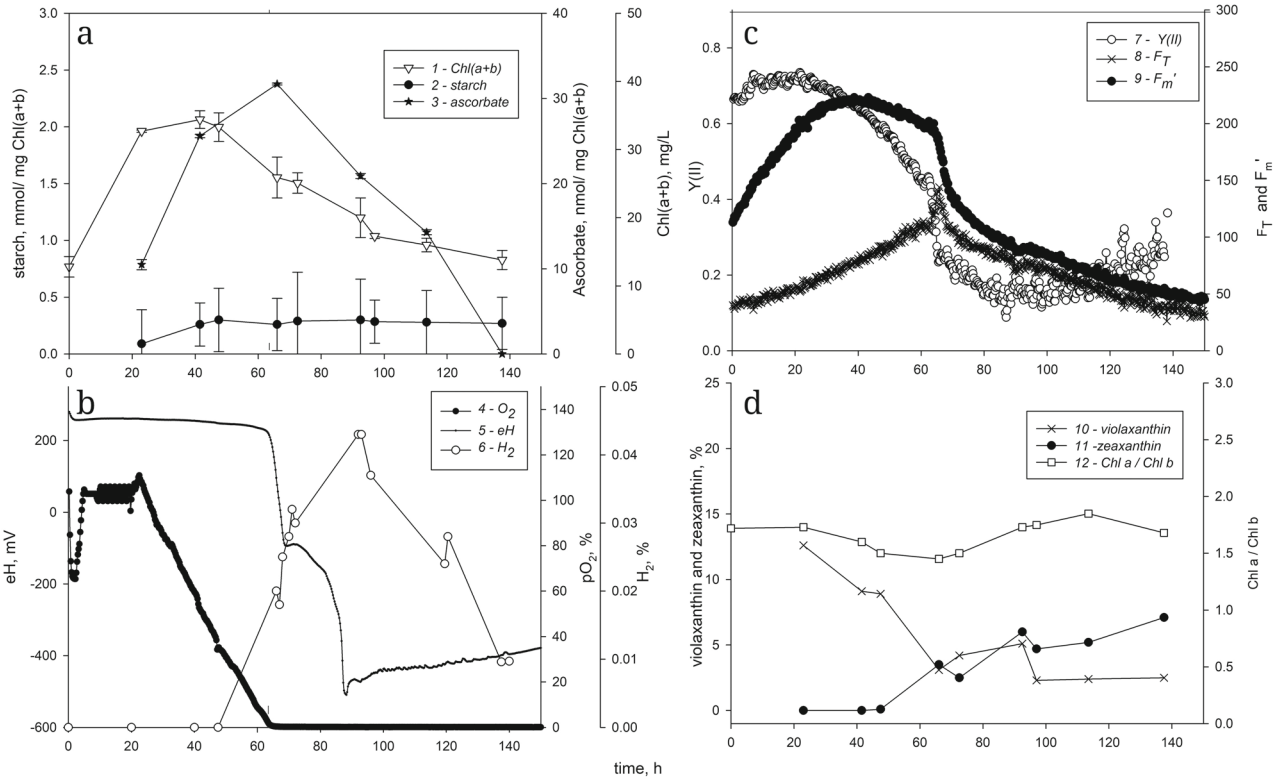

Fig. 4. Cultivation of *C. reinhardtii* in a photobioreactor in the gas phase of argon + 2% CO_2 under sulfur deprivation. Light intensity 167 μmol photons $m^{-2}\cdot s^{-1}$. a) Chl (a + b) (*1*); starch (*2*), ascorbate (*3*). b) Partial pressure of oxygen, expressed as percentage of air saturation at 28°C (*4*); eH against Ag/AgCl electrode (*5*); H_2 content in the gas phase (*6*). c) Y(II) (*7*); F_T (8); F_m' (*9*). d) Ratio of Chl a/b (*12*) and percentage content of violaxanthin (*10*) and zeaxanthin (*11*) (100% – total amount of carotenoids).

Cultivation of *C. reinhardtii* in the atmosphere of argon + CO_2 under sulfur deprivation using a two-stage light protocol. Decrease in light intensity from 169 to 30 μmol photons $m^{-2}\cdot s^{-1}$. When cultivating *C. reinhardtii* in the atmosphere of argon + 2% CO_2, with the culture inoculated into the sulfur-free medium, concentration of Chl (a + b) increased up to 40 h, after which it began to decrease (Fig. 5). The culture smoothly transitioned from the unlimited growth for 40 h to the stage of limitation and transition to sulfur deprivation. After 40 h, the light intensity was reduced to 30 μmol photons $m^{-2}\cdot s^{-1}$. pO_2 and the redox potential sharply dropped at the moment of reducing the light intensity. Sharp drop was also observed in the effective quantum yield of PSII, mainly due to the sharp decrease in F_m'. Starch, ascorbate, and the Chl a/b ratio remained stable throughout the cultivation. H_2 content in the gas phase increased up to 89 h and then decreased. Total hydrogen production during the entire incubation period was 84 ml/l.

Additionally, a pigment analysis was carried out to assess operation of the violaxanthin cycle. In this experiment (Fig. 5d), zeaxanthin was absent throughout the entire cultivation. The percentage content of violaxanthin decreased.

All parameters obtained from the JIP test (additional data are provided in the Online Resource 2) 40 h after inoculation in the atmosphere of argon + CO_2 without sulfur were very close to those for the cultures grown on complete medium, confirming the conclusion that, at this time the culture did not experience sulfur deprivation. After reducing the light intensity, decrease in F_v/F_m and PIabs and increase in V_j, d_V/d_{t0}, ABS/RC were observed. After 66 h of cultivation, V_j, d_V/d_{t0}, ABS/RC doubled, while PIabs decreased to almost zero.

Cultivation of *C. reinhardtii* in the atmosphere of argon + CO_2 under sulfur deprivation using a three-stage light protocol. Increase in the light intensity from 169 to 300 μmol photons $m^{-2}\cdot s^{-1}$. In this experiment, the effect of high light intensity on PSII activity in the cultures adapted to low light intensity was studied (Fig. 6). For this, a three-stage transition was applied, as cultures died when exposed to high light intensity immediately after inoculation at low concentration. On the first day after inoculation, the light intensity was 40 μmol photons $m^{-2}\cdot s^{-1}$; after 20 h, the light intensity was increased to 167 μmol photons $m^{-2}\cdot s^{-1}$, and after 46 h of cultivation (period of sulfur starvation), it was increased to 300 μmol photons $m^{-2}\cdot s^{-1}$.

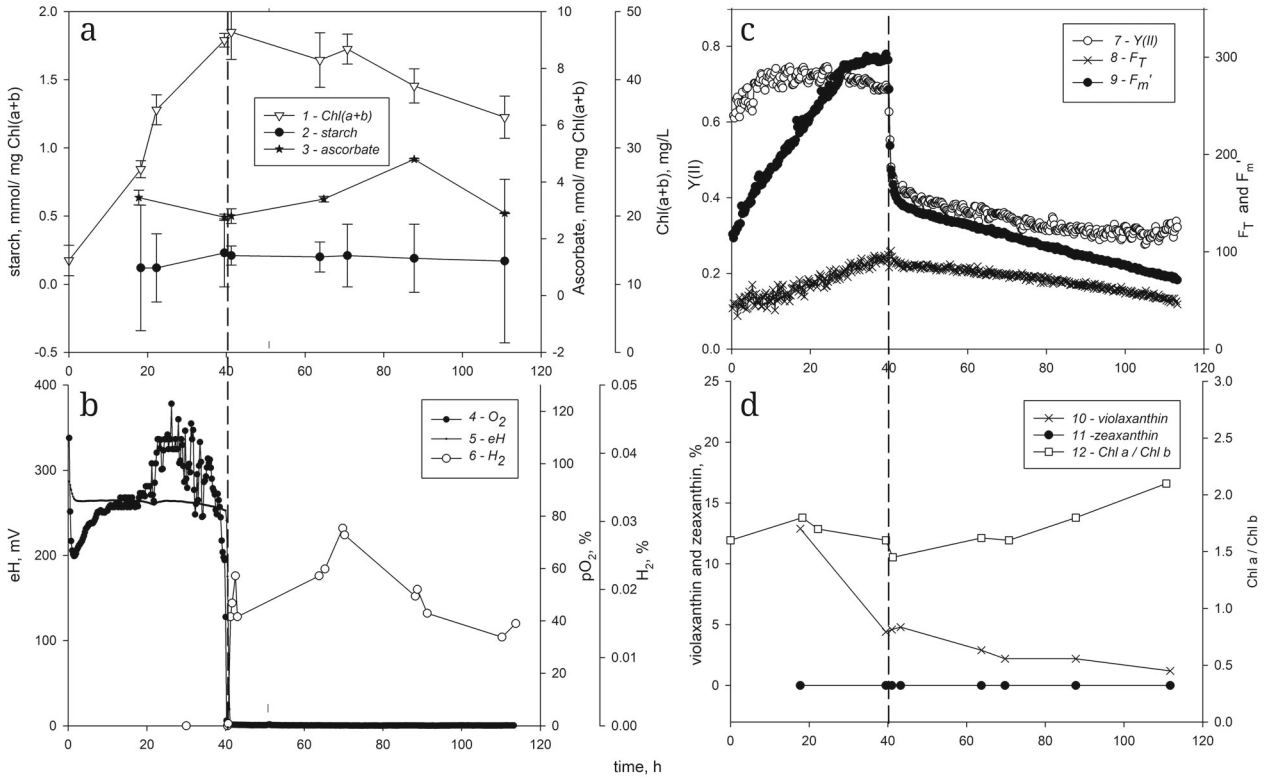

Fig. 5. Cultivation of *C. reinhardtii* in a photobioreactor in the gas phase of argon + 2% CO_2 under sulfur deprivation using two-stage light protocol. Light intensity 167 μmol photons $m^{-2}\cdot s^{-1}$ up to 40 h, then 30 μmol photons $m^{-2}\cdot s^{-1}$ (vertical dotted line – change in light intensity). a) Chl (a + b) (*1*); starch (*2*), ascorbate (*3*). b) Partial pressure of oxygen, expressed as percentage of air saturation at 28°C (4); eH against Ag/AgCl electrode (*5*); H_2 content in the gas phase (*6*). c) Y(II) (*7*); F_T (8); F_m' (*9*). d) Ratio of Chl a/b (*12*) and percentage content of violaxanthin (*10*) and zeaxanthin (*11*) (100% – total amount of carotenoids).

On the first day of cultivation, concentration of Chl (a + b) did not increase, and no culture growth was observed. At this time, the effective quantum yield of PSII was 0.750. After increasing the light intensity to 167 μmol photons $m^{-2}\cdot s^{-1}$ (21 h), sharp decrease in the effective quantum yield was observed, with rapid recovery to previous values. pO_2 and the amount of Chl (a + b) increased up to 40 h. Increase in the amount of ascorbate up to 5 nmol/mg Chl (a + b) was observed. The ratio of Chl a/b did not change, and the amount of starch remained stably low throughout the experiment.

After 46 h from the start of cultivation, the light intensity was increased to 300 μmol photons $m^{-2}\cdot s^{-1}$; at this moment, sharp increase in pO_2 was observed, followed by sharp decrease to 0. The amount of ascorbate increased twofold 20 min after increasing the light intensity and continued to increase throughout the remaining cultivation period. After the depletion of oxygen in the medium, the redox potential sharply decreased, indicating synthesis of the redox-active reduced compounds. The H_2 content in the gas phase increased up to 72 h and then decreased. Total hydrogen production during the entire incubation period was 101.6 ml/l.

All parameters obtained from the JIP test (additional data are provided in the Online Resource 2) did not change after increasing the light intensity and up to 46 h were very close to those for the cultures grown on complete medium, confirming the conclusion that, at this time the culture did not experience sulfur deprivation. After 70 h of cultivation, at the onset of anaerobiosis, V_j, d_V/d_{t0}, ABS/RC doubled, while PIabs decreased to almost zero.

The results of pigment analysis (Fig. 6d) demonstrate that in this experiment, during adaptation to high light intensities, there is an enzymatic interconversion between violaxanthin and zeaxanthin.

Thus, cultures adapted to low light intensities and transferred to very high light intensities under sulfur starvation regulate PSII activity by at least three mechanisms: over-reduction of the plastoquinone pool, decoupling of the water-oxidizing complex and PSII, as well as violaxanthin cycle.

DISCUSSION

In this work, we investigated the mechanisms of PSII activity regulation in the photoautotrophic

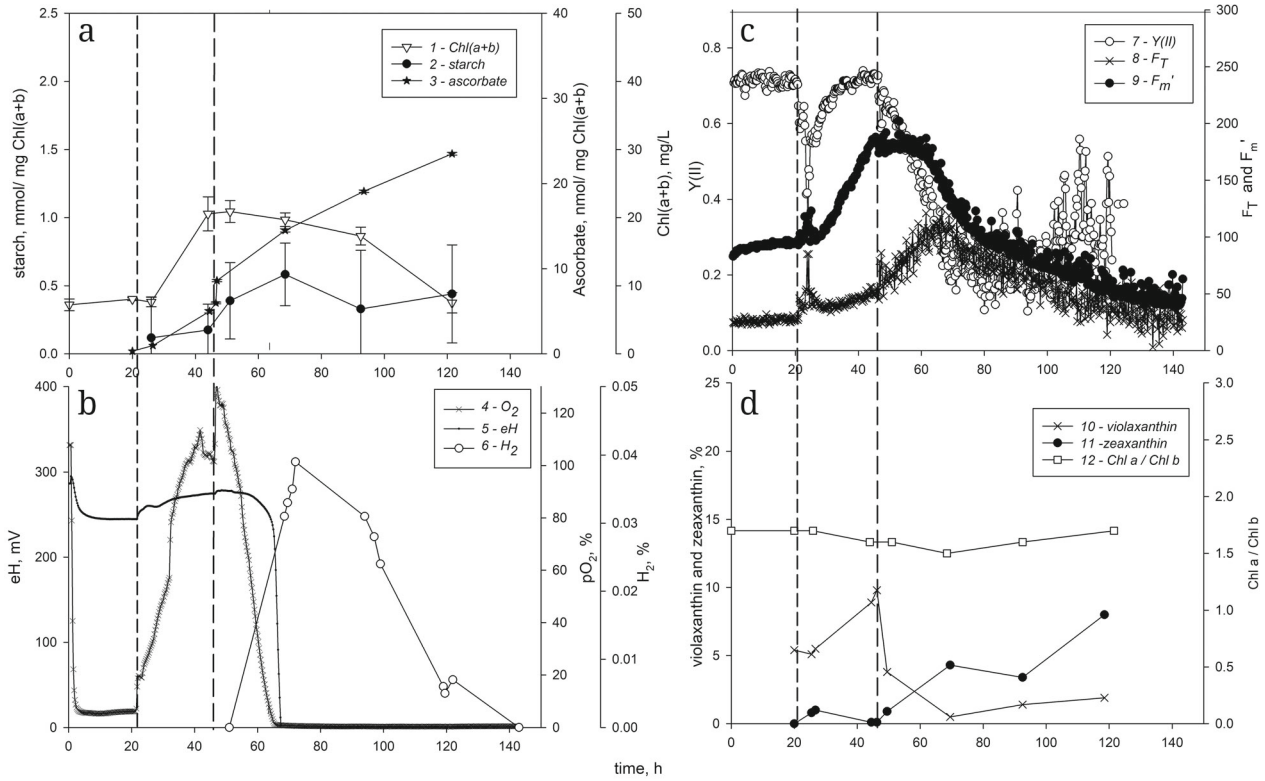

Fig. 6. Cultivation of *C. reinhardtii* in a photobioreactor in the gas phase of argon + 2% CO_2 under sulfur deprivation using three-stage light protocol. Light intensity 40 μmol photons $m^{-2}{\cdot}s^{-1}$ up to 20 h, 167 μmol photons $m^{-2}{\cdot}s^{-1}$ up to 46 h, next 300 μmol photons $m^{-2}{\cdot}s^{-1}$ (vertical dotted line – change in light intensity). a) Chl (a + b) (*1*); starch (*2*), ascorbate (*3*). b) Partial pressure of oxygen, expressed as percentage of air saturation at 28°C (*4*); eH against Ag/AgCl electrode (*5*); H_2 content in the gas phase (*6*). c) Y(II) (*7*); F_T (*8*); F_m' (*9*). d) Ratio of Chl a/b (*12*) and percentage content of violaxanthin (*10*) and zeaxanthin (*11*) (100% – total amount of carotenoids).

cultures of *C. reinhardtii* under nitrogen or sulfur deprivation under various light conditions and their relationship with hydrogen production.

Control cultures grown in the complete HSM medium at light intensity of 169 μmol photons $m^{-2}{\cdot}s^{-1}$ demonstrated typical photoautotrophic growth without signs of stress. The JIP test parameters indicated high efficiency of PSII, and the level of reduction of the plastoquinone pool remained low, as evidenced by the high ratio of the area above the OJIP curve to the total area and the stable level of F_0 [26]. Under these conditions, hydrogen was not produced.

When cultivated in the nitrogen-free medium with decrease in light intensity from 169 to 30 μmol photons $m^{-2}{\cdot}s^{-1}$ during the oxygen consumption period, we observed a significant increase in the reduction of the plastoquinone pool, previously observed for the sulfur-deprived cultures [19]. This was accompanied by the decrease in photosynthetic activity (Y(II), F_v/F_m) due to decrease in F_m' and increase in F_T, and hydrogen production (up to 122 ml/l). At the same time, as previously noted [13, 33], the increase in FT is due to the sharp increase in the number of active centers with reduced QA, caused by the sharp over-reduction of the plastoquinone pool. In contrast, in the case of increasing the light intensity to 300 μmol photons $m^{-2}{\cdot}s^{-1}$, over-reduction of the plastoquinone pool did not occur, despite the decrease in F_v/F_m, and hydrogen was not produced. These results are consistent with the data obtained in photoheterotrophic cultures [16, 24].

Similar patterns were observed for the culture grown in the sulfur-free medium. At constant light intensity of 169 μmol photons $m^{-2}{\cdot}s^{-1}$ and when light intensity increased to 300 μmol photons $m^{-2}{\cdot}s^{-1}$, there was a moderate over-reduction of the plastoquinone pool, observed as increase in FT, and hydrogen production (170 and 101.6 ml/l). In both cases, there was enzymatic interconversion between violaxanthin and zeaxanthin, and decoupling of the water-oxidizing complex (WOC) and PSII was observed for the cultures only at constant light intensity. Decreasing the light intensity to 30 μmol photons $m^{-2}{\cdot}s^{-1}$ led only to over-reduction of the plastoquinone pool and, consequently, to hydrogen production. With the sharp decrease in light intensity, the rate of photosynthesis was lower than the rate of respiration, and cultures very quickly transitioned to anaerobic conditions (where

this occurred). Anaerobic conditions led to inability to consume NADPH (especially under conditions where NADPH was not consumed in the Calvin cycle due to the sulfur or nitrogen starvation). As a result, the entire photosynthetic electron transport chain (ETC) became over-reduced, including the plastoquinone pool. This phenomenon was first discovered in 2001 [33].

Comparison of the obtained results with the literature data shows that the behavior of photoautotrophic and photoheterotrophic cultures of *C. reinhardtii* under sulfur and nitrogen starvation is largely similar [16, 24]. In both cases, there is a decrease in PSII activity manifested by the measured JIP test parameters – maximum (F_v/F_m) and effective (Y(II)) quantum yield, which is accompanied by accumulation of the reduced forms of the plastoquinone pool.

CONCLUSION

A key observation of our work is that hydrogen production was observed only in those cultures where there was a significant over-reduction of the plastoquinone pool, as evidenced by the sharp increase in FT. This was observed both under sulfur and nitrogen deprivation. In the cases where there was no rapid over-reduction of the plastoquinone pool associated with rapid transition of the cultures to anaerobic conditions, hydrogen was not produced, despite the decrease in photosynthetic activity.

Other mechanisms of PSII regulation studied by us, such as the violaxanthin cycle or increase in the proportion of PSII antenna complexes (decrease in the ratio of Chl a/b), apparently play a secondary role and do not have a determining effect on hydrogen production. Accumulation of ascorbate under starvation also did not correlate unambiguously with hydrogen production.

Thus, our data allow us to conclude that the key mechanism determining hydrogen production under sulfur or nitrogen deprivation in *C. reinhardtii* is over-reduction of the plastoquinone pool, which is achieved under certain light regimes against the background of suppressed photosynthesis. Other studied mechanisms play a lesser role. The obtained results expand our understanding of the regulation of photosynthetic apparatus under stress conditions and could be used to optimize the processes of hydrogen production by microalgae.

Abbreviations. PSII, photosystem II; Y(II), effective quantum yield of photosystem II.

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Contributions. V. I. Grechanik – conducting experiments, discussing results, discussing and editing the manuscript; M. A. Bolshakov – measuring ascorbate and analyzing pigment composition, discussing results; A. A. Tsygankov – concept, discussing results, writing the manuscript, discussing and editing the article.

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Ethics approval and consent to participate. This work does not contain any studies involving human and animal subjects.

Conflict of interest. The authors of this work declare that they have no conflicts of interest.

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