Հայաստանի Գիտությունների Ազգային Ակադեմիա
Национальная Академия Наук Армении
National Academy of Sciences of Armenia

Հայաստանի Կենսաբանական Հանդես
Биологический Журнал Армении
Biological Journal of Armenia

• Փորձարարական և տեսական հոդվածներ • Экспериментальные и теоретические статьи • • Experimental and theoretical articles •

Biolog. Journal of Armenia, 4 (74), 2022

DOI:10.54503/0366-5119-2022.74.4-6

GROWTH AND HYDROGEN PRODUCTION BY *Chlorella vulgaris* Pa-023 UNDER SULFUR AND NITROGEN DEPRIVATION

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The photodependent production of hydrogen (H_2) as a promising source of renewable energy is currently of great interest. Green algae carry out photoproduction of H_2 associated with electron transport during photosynthesis and catalyzed by [Fe]-hydrogenase, which is sensitive to oxygen and is inactivated by water photolysis. The issue of incompatibility between water photolysis and hydrogenase can be solved by creating deprivation of nutrients such as nitrogen and sulfur. The results have shown that H_2 generation by *Chlorella vulgaris* Pa-023 is stimulated 2.5 times by sulfur deprivation and 2.7 times by nitrogen deprivation compared to algae grown on a complete Tamiya medium. The use of a specific inhibitor of PS II, DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), demonstrated that during nutrient deprivation in algae operates a PS II-dependent pathway of H_2 generation. Thus, sulfur and nitrogen deprivation stimulates photoproduction of H_2 by *C. vulgaris*.

Chlorella vulgaris – hydrogen production – sulfur and nitrogen deprivation

Այժմ մեծ հետաքրքրություն է ներկայացնում լուսակախյալ ջրածնի (H_2)՝ որպես վերականգնվող էներգիայի խոստումնալից աղբյուրի արտադրությունը: H_2-ի ֆոտոարտադրությունը կանաչ ջրիմուռներում կապված է ֆոտոսինթեզի ընթացքում էլեկտրոնի տեղափոխման հետ և կատալիզվում է [Fe]-հիդրոգենազով, որը զգայուն է թթվածնի նկատմամբ և ապաակտիվանում է ջրի ֆոտոծեղքման արդյունքում: Ջրի ֆոտոծեղքման և հիդրոգենազի միջև անհամատեղելիությունը կարելի է հաղթահարել՝ ստեղծելով կենսածին տարրերի՝ ազոտի և ծծմբի, անբավարարություն: Արդյունքները ցույց են տվել, որ սննդանյութերի անբավարարությունը խթանել է H_2-ի ֆոտոարտադրությունը *Chlorella vulgaris* Pa-023-ում՝ ծծմբի դեպքում՝ 2.5 անգամ, իսկ ազոտի դեպքում՝ 2.7 անգամ, ստուգիչի՝ ամբողջական Տամիյա միջավայրում աճեցված ջրիմուռի հետ համեմատած: ՖՀ II-ի մենահատուկ արգելակչի 3-(3,4-երկքլորոֆենիլ)-1,1-երկմեթիլմիզանյութի կիրառումը ցույց է տվել, որ սննդանյութերի անբավարարության ժամանակ ջրիմուռում գործում է H_2-ի արտադրության ՖՀ II-ից կախյալ ուղին: Այսպիսով, թե՛ ծծմբի և թե՛ ազոտի սակավությունը խթանում է *C. vulgaris*-ում H_2-ի ֆոտոարտադրությունը:

Chlorella vulgaris – ջրածնի արտադրություն – ծծմբի և ազոտի անբավարարություն

В настоящее время большой интерес вызывает фотозависимое производство водорода (H_2) как перспективного источника возобновляемой энергии. Выделение H_2 в зеленых водорослях связано с фотосинтетическим транспортом электронов и катализируется [Fe]-гидрогеназой, которая чувствительна к кислороду и инактивируется фотолизом воды. Несовместимость между фотолизом воды и гидрогеназой можно преодолеть создав дефицит биогенных элементов, таких как азот и сера. Результаты показали, что при дефиците элементов фотовыделение H_2 водорослью *Chlorella vulgaris* Pa-023 стимулировалось: при не-

достатке серы в 2.5 раза, дефиците азота в 2.7 раза, по сравнению с контролем – микроводорослями, выращенными на полноценной среде Тамия. Применение ингибитора ФС II – 3-(3,4-дихлорфенил)-1,1-диметилмочевины показало, что при дефиците биогенных элементов в *C. vulgaris* действует ФС II-зависимый путь производства H_2. Таким образом, дефицит как серы, так и азота стимулирует фотовыделение H_2 в *C. vulgaris*.

Chlorella vulgaris – выделение водорода – дефицит серы и азота

Nowadays, green algae are used as raw materials in various sectors of the economy: food industry, pharmaceuticals, agriculture, cosmetology, and energetics as biofuel producers [6]. Green algae can produce hydrogen (H_2) as a promising source of renewable energy [2, 3, 5, 10]. H_2 generation in green algae is associated with electron transport during photosynthesis and is catalyzed by [Fe]-hydrogenase [2, 5, 10].

Presently, one of the important aspects of H_2 production is the selection of H_2-producing organisms and the optimization of conditions that ensure high H_2 yields. For example, the deficiency of certain elements is one of the ways to regulate carbon metabolism, which contributes to the creation of anaerobic conditions in algae and the long-term production of H_2 [1, 9, 12]. The deficiency of various elements can lead to inhibition of photosystem (PS) II in green algae, followed by oxygen assimilation during respiration, which is the main reason for the formation of anaerobic conditions in algae.

Researchers have shown that the green algae *Dunaliella salina* produces H_2 under different growth conditions [11]. The lack of some nutrients in the environment suppresses the activity of PS II of *D. salina* and, accordingly, leads to a decrease in the oxygen concentration in the cell, stimulating the release of H_2 [11]. Sulfur deprivation leads to the inhibition of PS II in other green algae *Chlamydomonas reinhardtii* with subsequent oxygen assimilation during respiration, which is the main reason for the formation of anaerobic conditions in algae [1, 2, 12]. Nutrient limitation, particularly nitrogen, can promote lipid accumulation in green algae [4].

In green algae, hydrogenase is responsible for the production of hydrogen. There are several sources of electron transport to hydrogenase, starch is the main source in the dark [5, 10]. Under these conditions, cells experience a severe energy shortage due to the simultaneous cessation of photo- and oxidative phosphorylation [5, 10]. During anaerobiosis, starch is broken down into fermentation end-products such as formate, acetate, ethanol, and carbon dioxide. When the algal culture is transferred from dark anaerobic conditions to light, the hydrogen yield increases by several orders of magnitude compared to dark conditions, but these changes are transient and completely extinguish within a few minutes. Under light conditions, electrons begin to be actively supplied to hydrogenase from the photosynthetic electron transport chain, which leads to the active work of PS II. Nutrient deficiency in algae inhibits PS II and water oxidation activity, but, since it has little effect on cellular respiration, it leads from an aerobic to anaerobic state [1, 10]. Nitrogen deprivation stimulated H_2 yield in green alga *Parachlorella kessleri* RA-002 isolated from Armenia [8]. Moreover, during nitrogen deprivation, PS II activity decreases up to 20 % contributing to the creation of anoxic conditions and H_2 production [8]. However, the mechanism of H_2 production by green algae under nutrient deprivation remains unclear.

In this work, the previously unexplored H_2 generation in sulfur and nitrogen-deprived *Chlorella vulgaris* Pa-023 isolated in Armenia has been investigated. To reveal the mechanism of H_2 production under these conditions, PS II inhibitor DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) was used.

Materials and methods. *C. vulgaris* Pa-023 (Microbial Depository Center, NAS, Yerevan, Armenia) was grown upon continuous illumination (2000 lux) (fig. 1A). This strain was cultivated in a standard Tamiya medium, under aerobic conditions at 25 ± 0.2^0C and pH 7.5 ± 0.02, upon continuous illumination [8]. Sodium acetate (1 g/L) was added to Tamiya medium as a carbon source. Aerobic conditions were maintained via shaking the algae culture on an orbital shaker (WideShake SHO-1D, DAIHAN Scientific, Korea) [7, 8]. The growth of algae was monitored by changes in the optical density (OD_{680}) using a Spectro UV-Vis Auto spectrophotometer (Labomed, USA). The growth rate was calculated as (ln OD_t – ln OD_0)/*t*, where OD_0 is the initial value of $OD_{680,}$ and OD_t is the value of OD after *t* days [8]. For H_2 production assay algae, cultivated aerobically, were harvested by centrifugation at 2500 rpm for 10 min, washed twice, and then resuspended in fresh Tamiya media (fig. 1B). The measurements of H_2 production were performed under anaerobic conditions as described [7, 8]. For the creation of a nitrogen-deprived medium, KNO_3 was omitted from Tamiya medium, and for sulfur-deprived conditions, all sulfates were replaced by chlorides [8]. To study the effect of PS II inhibitor, 30 μM DCMU was added to the medium after 24 h of cultivation under anaerobic conditions, after which the cells were maintained in the dark conditions for about 15 min [8]. The redox potential of *C. vulgaris* medium was measured using a couple of redox (platinum (Pt) and titanium-silicate (Ti-Si)) electrodes, as described [7]. The H_2 yield in *C. vulgaris* was calculated as described earlier [8]. The data are reported as mean ± SD and calculated based on three independent experiments.

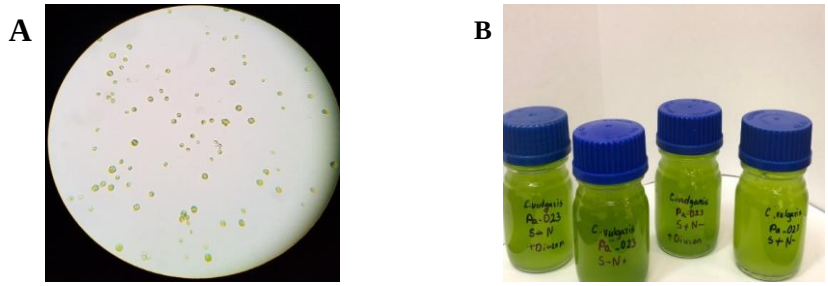

Fig. 1. (A) Light microscope images of *C. vulgaris* Pa-023 and (B) culture of algae, grown under sulfur- and nitrogen-deprived conditions.

Results and Discussion. *C. vulgaris* Pa-023 is a microscopic unicellular green alga isolated in Armenia (fig. 1A). *C. vulgaris* Pa-023 was grown under mixotrophic conditions under illumination with acetate as a carbon source. The growth rates of algae were monitored during anaerobic growth under nutrient deprivation (fig. 2). Sulfur- and nitrogen-deprivation led to a decrease in the specific growth rate in *C. vulgaris* by ~1.4 and ~1.2-fold, respectively, compared with the control cultivated in complete Tamiya medium (fig. 2). The addition of the PS II inhibitor, DCMU, resulted in the inhibition of algae growth rate.

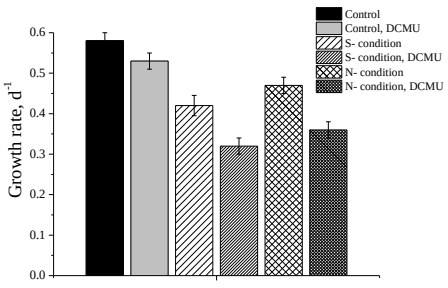

Fig. 2. The effect of sulfur and nitrogen deprivation on the growth rate of *C. vulgaris* Pa-023.

The redox potential is known to be an important factor for microbial growth. It is characterized as the ability of a biological system to oxidize or reduce different substrates [7, 8]. During the anaerobic growth of *C. vulgaris*, the redox potential decreases from positive values to low negative values [7, 8]. The decrease in the redox potential is associated with the formation of fermentation products, the production of amino acids, as well as the synthesis of proteins and other compounds, which is probably specific to the metabolic processes occurring during the growth of microorganisms under anaerobic conditions [7].

The redox potential of *C. vulgaris* control cells, grown in a complete Tamiya medium decreased up to –450 mV within 48 h, which indicates the reduction processes and H_2 production under anaerobic conditions (fig. 3). Sulfur- and nitrogen-deprivation enhanced the drop in the redox potential up to –520 and –550 mV, respectively (fig. 3). Such changes in the redox potential can be due to the formation of various products of algal metabolism, as well as reduced equivalents, e.g. synthesis of NADPH, which can have a significant impact on algal metabolism, since an increase in the availability of NADPH significantly changes the nature of the end products [9, 10].

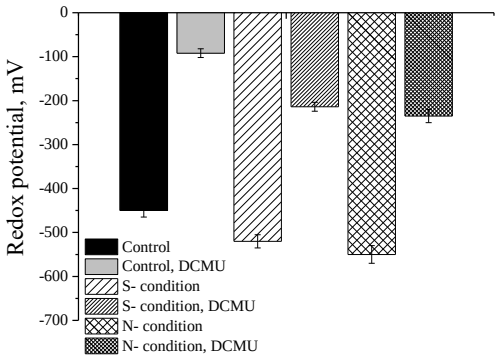

Fig. 3. The change in the redox potential in *C. vulgaris* Pa-023 grown under sulfur- and nitrogen-deprived conditions.

To synthesize ATP under anaerobic conditions and illumination, green algae carry out the so-called "anaerobic photosynthesis", which proceeds with the production of H_2. H_2 production in green algae happens as a result of "direct" photolysis of water [2, 5, 10]. H_2 production was recorded in *C. vulgaris* during anaerobic growth under nitrogen- and sulfur-deprived conditions (fig. 4). Stimulation of H_2 production in *C. vulgaris* under nitrogen and sulfur deprivation was observed (fig. 4). It was ~2.5 and ~2.7 times higher than the H_2 yield of algae grown in the presence of sulfur and nitrogen, respectively (fig. 4). In our previous research, nitrogen deprivation led to ~5 times increase of H_2 production by *P. kessleri* RA-002 compared to the control [8]. Green algae can break down H_2O and release O_2 with the participation of two photosystems that convert light energy into the chemical energy of ATP and NADPH [5, 10]. In nutrient-deprived cells, PS II provides 85 % of the electrons for hydrogenase, starch is not used up during H_2 production, and the remaining 15 % is obtained from other sources [2, 9].

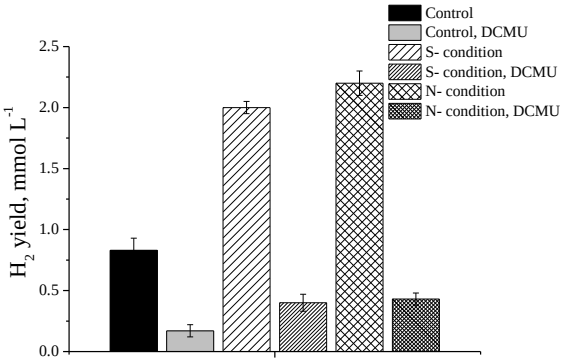

Fig. 4. Effect of sulfur and nitrogen deprivation on H_2 production by *C. vulgaris* Pa-023.

H_2 production in green algae occurs via both PS II-dependent and independent pathways [2, 3, 10]. To identify the H_2 generation route in *C. vulgaris* Pa-023, a specific inhibitor of PS II, DCMU, was used, which was added after 24 h of algae growth, when H_2 production was recorded. In the presence of DCMU, H_2 production significantly (~5 times) decreases in sulfur- and nitrogen-deprived cells, indicating that a PS II-dependent pathway is operating here (fig. 4).

Thus, the data obtained show that nitrogen and sulfur deprivation has great potential for application in biotechnology as a way of stimulation of H_2 yield in green microalgae.

Acknowledgment. Author is grateful to supervisor Dr. Lilit Gabrielyan for kind advice on this study and assistance in interpreting the results. This work was supported by the RA MESCS Science Committee and Belarusian Republican Foundation for Fundamental Research in the frames of the joint research project 21SC-BRFFR-1F012 (PI Lilit Gabrielyan).

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Received on 26.09.2022