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Biological Generation of Hydrogen

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Abstract—Microorganisms are capable of evolving hydrogen. Such microbiologic processes can be divided into dark anaerobic hydrogen evolution, light-dependent hydrogen evolution without oxygen evolution, and light-dependent hydrogen evolution accompanied oxygen evolution (biophitolysis). The review describes recent advances in biohydrogen production from whole-cell microorganisms.
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The high oil prices and negative climate impact of oil combustion products attract enhancing present interest in nontraditional energy sources. Hydrogen is an ecologically clean energy carrier for future energetics. At present hydrogen is industrially produced by steam reforming of methane, catalytic reforming of hydrocarbons, and electrolysis of water [1]. Hydrogen production from fossil fuels can be considered as the necessary transition step to hydrogen energetics [2]. Hydrogen production with use of solar, wind, and geothermal energy, as well the energy accumulated in wastes are the most environmentally safe technologies.

In view of the active research on hydrogen evolution, a special term was introduced—"biohydrogen"—to refer to a biologically produced hydrogen. In terms of the energy sources and electron donors used by microorganisms, microbiologic processes can be divided into dark anaerobic hydrogen evolution, light-dependent hydrogen evolution without oxygen evolution, and light-dependent hydrogen evolution accompanied oxygen evolution (biophytolysis). Hydrogen production with use of subcellular biologic structures is subject to separate consideration. This review describes principal recent advances in biohydrogen production from whole-cell microorganisms.

DARK HYDROGEN EVOLUTION

Microorganisms of different taxonomic groups can reduced protons on fermentation, thereby getting rid of excess reducer in cases where the medium contains no terminal electron acceptor (for example, oxygen, nitrate, nitrite, or sulfate). Even though there are diverse metabolic pathways leading to dark hydrogen evolution, useful terminal reactions are few in number and include decomposition of pyruvate, formate, acetaldehyde, and pyridine nucleotides ($NAD(P)H_2$), as well as hydrolysis of CO:

$$\text{Pyruvate} \longrightarrow \text{Acetyl-CoA} + CO_2 + H_2, \quad (1)$$

$$\text{Formate} \longrightarrow CO_2 + H_2, \quad (2)$$

$$\text{Acetaldehyde} \longrightarrow \text{Acetate} + H_2, \quad (3)$$

$$NAD(P)H_2 \longrightarrow NAD(P) + H_2, \quad (4)$$

$$CO + H_2O \longrightarrow H_2 + CO_2. \quad (5)$$

Reactions (1)–(4) are realized in microorganisms accomplishing different types of fermentation.

Reaction (1) is characteristic of strict anaerobic bacteria (for example, clostridia) which ferment carbohydrates with the participation of pyruvate : ferredoxine : oxidoreductase, ferredoxine (Fd), and Fd-dependent hydrogenase. Reaction (2) is accomplished by endobacteria with the assistance of the formate hydrogen lyase complex including formate dehydrogenase, electron carriers, and hydrogenase. The formate used for hydrogen production can be present in the culture medium or form from pyruvate due to the action of pyruvate formate lyase. Reaction (3) was described for a microorganism isolated from symbiotic association with a methane-forming bacteria [3]. Reaction (4) is characteristic of all microorganisms capable of synthesizing NAD(P)H-dependent hydrogenases of the *hoxYH* type [4].

A great number of microorganisms of different groups are capable of the anaerobic oxidation of CO to form hydrogen by reaction (5), using this reaction as an energy source [5]. The reaction can be of practical importance, especially for removal of CO from syngas, but it is beyond the scope of the present review.

At present we know great deal about metabolism pathways leading on fermentation to hydrogen formation, ferments involved in the process, and genes responsible for synthesis of the ferments. Some works appeared describing new metabolisms pathways. For example, is was recently shown that membrane vesicles from the hyperthermophilic archeon Pyrococcus furiosis on hydrogen production from reduced Fd can synthesize adenosine triphosphate (ATP) due to transmembrane potential, thus effecting the simplest type of respiration [6]. The reduction of Fd in this bacteria on glycolysis occurs not only by reaction (1), but also by oxidation of glyceraldehydrophosphate with reduction of Fd with an unusual enzyme glyceraldehydrophosphate : Fd oxidoreductase, rather than with reduction of NAD.

Carbohydrate fermentation produces, together with H_2, other compounds (ethanol, acetate, propionate, butanol, etc.). This is associated with the fact that the stoichiometric decomposition of glucose into 12 molecules of H_2 and 6 molecules of CO_2 cannot provide sufficient energy for bacteria to grow. Theoretical calculations show [7, 8] that to impart sufficient energy to bacteria and produce as much oxygen as possible, glucose should decompose by the following reaction:

$$C_6H_{12}O_6 \longrightarrow 2CH_3COO^- + 2HCO_3^- + 4H^+ + 4H_2, \quad (6)$$
$$\Delta G_0 = -206 \text{ kJ mol}^{-1}.$$

In experiments with different bacteria and their consortia, 0.5–4.0 mol H_2 mol^{-1} glucose are usually obtained [9], and, therewith, the highest yields were obtained with thermophilic anaerobic bacteria [10, 11]. It follows from Eq. (6) that fermentation releases as H_2 no more than 1/3 of energy accumulated in glucose. At the same time, upon complete decomposition of hydrocarbons into methane and carbon dioxide methane preserves up to 85% of the hydrocarbon energy [12]. Moreover, the natural enzymatic hydrogen production is always accompanied by hydrogen uptake to form methane, which is favored by energy [13].

In view of the low yield of fermentation and its attendant by-product formation (which does not allow dark hydrogen evolution to be referred as a waste-free technology), the production of hydrogen by carbohydrate fermentation has not until recently been considered as a practically feasible way to fuel production. In this connection little attention has been given to optimization of this process by the end of the XX century. In the publications dealing with hydrogen evolution from carbohydrates, most emphasis has been given to metabolic pathways of carbohydrate decomposition and reaction stoichiometry. At the same time, it is already more than a quarter century ago that processes with hydrogen evolution rates of up to 1.8–2.3 l H_2 h^{-1} l^{-1} suspension have been described [3].

Over the past years the interest in the dark hydrogen evolution by bacteria has grown (the projects funded in different countries are described in [14]). This is explained by the following reasons:

(1) If formerly all power stations were considered as centralized systems with a power of no more than 30 MW [15], then now, with the development of fuel cells, decentralized systems are becoming more and more attractive [14]. In the latter case, power stations can be located near sources of raw materials, thus much reducing expenses for transportation.

(2) Hydrogen and methane can be produced by consortia of microorganisms using various carbohydrate-containing wastes, as well as specially grown biomass.

(3) Carbon dioxide attendant in hydrogen evolution is emitted exclusively in the site of power production, which facilitates its subsequent utilization.

(4) The accumulation of knowledge and advances in genetic research allow one to intentionally control the cell metabolism.

(5) First evidence has appeared showing that the fermentation of cellulose-containing wastes to organic acids followed by light-dependent hydrogen evolution compares with methanogenesis in hydrogen productivity [13].

With the initiation of funding of research of dark hydrogen evolution, publications concerning the practical aspect of this process have appeared. The key questions to be solved before passing to development and engineering activities, include stability of the process, specific rate per bioreactor unit volume, efficiency of substrate consumption, and spectrum of renewable raw materials.

In real conditions, when unsterile substrates are used, the fermentation process may well transform into methanogenesis or, being inhibited by products, stop or switch to another fermentation process. Various approaches to selectively inhibiting methanogenic bacteria have were tested, based on physiologic features of these bacteria: inability to form spores, toxic action of oxygen, narrower growth pH range, and sensitivity to specific inhibitors (2-bromoethanesulfonic acid, iodopropane, and acetylene). The most widespread is heat treatment of soils and sludges used as seed material: The samples are heated for 15–120 min at 84–104°C [16–20]. It was also shown that

acid treatment of active sludge (pH = 3; 30 min) would be more effective than alkaline treatment (pH = 10; 30 min) [20]. Multifactor planning was used the effect of seed pretreatment conditions: treatment and culturing temperatures (37 and 55°C), additions of bromoethane sulfonate and acetylene, and type of inoculate (mesophilic and thermophilic samples) of the quantitative characteristics of hydrogen evolution [21]. As shown, acetylene pretreatment increased the yield of hydrogen and shortened the reaction lag period (time needed to redirect the metabolism of microorganisms and to initiate hydrogen evolution).

It should be noted that the above pretreatment methods are productive in laboratory conditions. Their use accelerates the evolution of hydrogen and increases its yield per mole substrate. However, the prospects of practical application of pretreatment seem questionable, since methanogenic bacteria contained in the substrate can with time switch the process in a real bioreactor to methanogenesis. Total pretreatment of raw material makes the hydrogen production process unprofitable. Therefore, research into choosing bioreactor pHs completely inhibitive of methanogenesis seem to hold greater promise. Oh et al. [22] showed that pH 5.5 provides stable hydrogen evolution from glucose within 234 days in unsterile conditions. Kyazze et al. [23] found that mixed suspension cultures steadily evolved hydrogen in mesophilic conditions at pH 5.2–5.3 [23]. Methanogenesis could also be prevented in hyperthermal conditions [24]. But here, too, bromoethane sulfonate was applied to initiate granulation in a UASB reactor (anaerobic reactor with microorganisms located in granules filling the whole volume).

An important aspect of stability research is to reveal inhibiting concentrations of substrates and metabolism products. First of all, the anaerobic hydrogen evolution is inhibited by hydrogen itself, which follows from the thermodynamics of the process. It is well khown that high concentrations of hydrogen not only slow down its evolution rate, but also results in formation of deeper reduction products than acetate. Van Niel et al. [25] studied hydrogen production by saccharose under the action of the extremely thermophilic bacteria *Caldicellulosiruptor saccharolyticus* to show that acetate ranked second in the inhibiting activity after an inert gas [25]. The same authors noted that hydrogen evolution ceased at a saccharose concentration of about 120 g l^{-1}. According to Kyazze [23], a mixed mesophilic culture reduced considerably the rate of hydrogen evolution and the efficiency of saccharose conversion even at 50 g l^{-1} substrate concentration [23]. Accumulation of products can not only affect the rate of hydrogen evolution, but also change completely the fermentation pathway. Thus, butyrate (4 g l^{-1}) not only terminated hydrogen evolution, but also initiated propionate synthesis; moreover, the butyrate concentration therewith doubled (from 9.83 to 18.9 g l^{-1}) [23].

Regardless of the fact that process stability studies are best performed in conditions as close to real as possible, we can note that a laboratory bioreactor fed with a synthetic medium containing saccharose steadily produced hydrogen for 3 years [26].

The rate of hydrogen evolution depends on the concentration of active biomass in the reactor and the mass-exchange characteristics of the latter. Important parameters are the concentration of carbohydrates in the feeding solution and its retention time on continuous feeding. The role of these parameters and start-up procedures were described in [27]. In the present review we will dwell on principal factors responsible for process productivity, putting emphasis on the most recent publications.

Reactors with immobilized bacteria or active sludge granules in optimal conditions faster evolved hydrogen than suspensions. Thus, the use of fibers as an immobilizing matrix provided long-term stable hydrogen evolution at a rate of about 1 l h^{-1} l^{-1} reactor and the glucose conversion efficiency of 1.1 mol H_2 mol^{-1} glucose [22]. Lee et al. [28] found a carrier promoting granulation in a mesophilic process. The reactor bottom was coated with charcoal (as small spheres), the reactor was inoculated with active sludge, and a medium was delivered continuously to obtain virtually formed granules in 100 h. The concentration of the biomass reached 26 g l^{-1}, and it was not washed out at a retention time of 0.5 h. In optimal conditions (retention time 0.5 h, saccharose concentration in the feeding medium 20 g l^{-1}), the rate of hydrogen evolution was 7.3 l H_2 h^{-1} l^{-1}, and, therewith, 1 mol of saccharose (consumption >90%) gave 3 mol of hydrogen. The referees suggested that by varying the height/diameter ratio of the reactor one can vary mass-exchange characteristics of the process and verified this suggestion in their other work [29]. An optimum height/diameter ratio was found at which the rate of hydrogen evolution was 6.9 l H_2 l^{-1} h^{-1}. Therewith, 1 mol of saccharose gave up to 3.5 mol of hydrogen, even though the optimum height/diameter ratios hydrogen evolution rate and saccharose conversion efficiency were not coincident. With stirred active sludge granules, the rate of hydrogen evolution was 9.3 l H_2 l^{-1} h^{-1} and the conversion efficiency was 4 mol H_2 mol^{-1} saccharose.

Wu et al. [30] tested immobilization of active

sludge on silicon. A UASB reactor was loaded with silicon-immobilized active sludge particles, and a saccharose-containing feeding solution was delivered at a high rate. Such particles were found to stimulate granulation of active sludge. As a result, the biomass concentration attained 35 g l^{-1} and did not fall even at a retention time of 15 l H_2 l^{-1} h^{-1}. Therewith, 1 mol of saccharose gave about 3.5 mol of hydrogen. The referees noted that increased retention time decreased not only the rate of hydrogen evolution, but also the number of different types of hydrogen-producing bacteria in granules.

To energize 5-kW fuel cells that are capable of providing complete energy supply for a single-family house [31, 32], the sufficient hydrogen delivery rate to the fuel cells can be of about 2900 l h^{-1}. Assuming that scaling-up does not decrease the rate of hydrogen evolution, to make use the process described in [30] a 200-l reactor will suffice, which is already practically feasible.

Along with optimization of the dark hydrogen evolution process, some other approaches to a more efficient conversion of substrates to hydrogen are available. The first is based on putting cultures in unbalanced feed conditions, when the cultures scarcely grow but continue substrate conversion to hydrogen [15]. Presumably, under these conditions hydrogen evolution not serve for generating power, but also for decreasing the concentration of soluble metabolism products. Even though first attempts to resort to this technique resulted in no essential changes in the stoichiometry of the process [33], further research is required for final conclusions. Another approach is based on metabolic engineering of bacteria [34, 35]. This approach envisages comprehensive analysis of fermentation types characteristic of the consortium and their directed alteration to improve the yield of hydrogen via suppression, introduction of new, and superexpression of available genes. However, this approach is to be refined methodically to be tested on practice.

Obviously, neither glucose nor saccharose are suitable substrates for practical hydrogen production. Much more preferred are easily decomposed cellulose-containing wastes (sawdust, cuttings, grass residues, etc.) which depolymerize to sugars. In this case, along with hydrogen production, utilization of wastes whose volume steadily grows with the progress of civilization becomes possible. There are two approaches to treatment of cellulose-containing wastes for hydrogen production: integration of cellulose decomposition and hydrogen production in a single bioreactor and preliminary hydrolysis of wastes to sugars (chemical or enzymatic) followed by transferring the hydrolysate to a bioreactor for hydrogen production. The integral processes involving cellulose-containing wastes are developed on pure cultures of microorganisms and their consortia. It is well known that clostridia contain a set of enzymes necessary for cellulose hydrolysis. It was shown that hydrogen can in principle be produced by pure or mixed cultures form various raw materials: crude starch [17, 18], microcrystalline cellulose [36], α-cellulose, milled filter paper, delignified wood fibers and cellobiose [37], as well as wastes of starch production from batata, containing starch, cellulose, and lignin [27]. However, further research is required for more effective hydrogen production from real wastes. Thus, the presently available data point to a high potential of dark hydrogen evolution as a practically important process. At the same time, considerable researcher's efforts are required not only for optimization of separate stages, but also for integration to a single technologic chain of preparation of raw materials, evolution of hydrogen, and removal of undesired by-products, primarily organic acids.

LIGHT-DEPENDENT HYDROGEN EVOLUTION

Processes of hydrogen evolution by microorganisms in the light are all underlain by photosynthesis. Among photosynthesizing microorganisms capable of evolving hydrogen most attention is attracted to microalgae, heterocyst cyanobacteria, and purple non-sulfur bacteria. Microalgae and cyanobacteria possess two photosystems and can decompose water to release oxygen. Bacteria with one photosystem (first of all purple and gree sulfur and non-sulfur bacteria) are incapable of evolving oxygen, and need stronger reduced, than water, electron donors to effect photosynthesis.

Microalgae do not evolve hydrogen under normal conditions, using energy accumulated on photosynthesis as ATP and NAD(P)H for their own metabolism. The reduction of NAD(P)H occurs via electron transfer from Fd reduced due to photosynthesis to NAD(P)H, under the actions of Fd : NAD(P)H oxidoreductase. Species synthesizing Fe-hydrogenase are able to light-dependent hydrogen evolution after preliminary dark anaerobic adaptation. In this case, reduced Fd can be oxidized with hydrogen evolution under the action of Fd-dependent Fe-hydrogenase:

$$Fd^{red} + 2H^+ \longrightarrow Fd^{ox} + H_2. \quad (7)$$

Microalgae, in particular *Chlamydomonas reinhardtii*, synthesize 2Fd-dependent hydrogenases hydA1 and hydA2 [38]. The latter two are Fe-hydrogenases, they are highly active and synthesized in

anaerobic conditions. The common properties of all Fe-hydrogenases is their fast inactivation in the presence of traces of oxygen.

Microalgae are considered to evolve hydrogen to throw off excess reducer in going from anaerobic dark conditions to the light [39]. Under dark conditions, no carbon dioxide fixation takes place in the Calvin cycle [major NAD(P)H consumption], and some time is required for this cycle to be initiated in the light. Photosynthesis to reduce Fd is initiated immediately after light appears. In this moment, cells contain an excess of NAD(P)H formed by dark anaerobic metabolism. Therefore, to throw off excess reducer, reduced Fd is oxidized by hydrogenase, evolving hydrogen. Simultaneously, oxygen is evolved (which allows this process to be called "direct water biophotolysis"). Thus, adapted microalgae evolve hydrogen until (in different experimental systems, from seconds to minutes) the concentration of evolved oxygen reaches a value at which complete inactivation of hydrogenase occurs.

Microalgae capable of water biophotolysis, as well as the biophysics and biochemistry of the process are described in detail in the reviews of Boichenko and co-authors [40, 41]. Here we only mention that the quantum yield of hydrogen evolution by microalgae in optimal conditions is close to the theoretical maximum [42], and the initial rates of the process attain 200 μmol H_2 h^{-1} mg^{-1} chlorophyll [40], which is close to the maximum rate of photosynthesis.

However, the toxic action of oxygen strongly restricts the duration of hydrogen evolution. In attempts to avoid this action, research on genetic modification of hydrogenases aimed at enhancing their resistance to oxygen and on separation of oxygen and hydrogen evolution processes in time is in progress.

There have been long-term efforts to enhance the resistance of hydrogenases by means of spontaneous mutagenesis and selection of useful mutants [43]. However, regardless of the extensive research, the resistance of hydrogenases in mutants is still insufficient for this approach to be considered successful. Presently much progress has been made in understanding the mechanisms of the synthesis, molecular structure, and mechanism of action of hydrogenases [4]. Molecular dynamics simulation shows that hydrogen can diffuse to the hydrogenase active center though a great number of molecular voids, whereas oxygen diffuses through two channels only [44]. On this basis, a site-directed mutagenesis of hydrogenase is suggested. The voids through which oxygen passes to the active center are suggested to be reduced by replacing certain amino acids [45]. However, this research is still in progress.

Oxygen and hydrogen evolution can be separated in time if microalgae are stressed by a deficiency of sulfur [46]. Under such conditions, C. reinhardtii in the light first evolved oxygen. With increasing incubation time, the activity of photosystem 2 decreased, and the rate of oxygen evolution became lower that the respiration rate, which resulted in anaerobiosis in the light, induction of hydrogenase synthesis and initiation of hydrogen evolution. This process was described in more detail in subsequent publications of other authors who described 5 phases of transfer of cultures from excess sulfur to its absence [47], behavioral features of photosystem 2 [48], effect of light intensity and pH [49, 50], and morphological and biochemical features of cultures under conditions of sulfur deficiency [51]. However, except for the pH optimal for growth [50], the mentioned papers all reported hydrogen production rates of about 0.10–0.15 l^{-1} of culture within 120–150 h. These values are lower than practically significant values by 3–4 orders of magnitude [31, 32].

Note that the "constituent parts" of the biochemical "machine" involved in water biophotolysis are already known. But the intricate nature of this process, along with a great number of constituent parts, is defined by a huge number of regulatory sites and metabolism branches competing for reduced Fd. To illustrate this statement, we present here data on the hydrogen evolution by a mutant of *C. reinhardtii* with a disturbed light-harvesting antenna to photosystem 1 and 2 [52]. This mutant was also characteristically unable to cyclic electron transport from the reduced Fd to plastoquinone pool (membrane electron carriers functioning between photosystems 1 and 2). This mutant evolved hydrogen an order of magnitude faster that the wild strain. This opens up new opportunities for improving the hydrogen yield of direct biophotolysis.

Thus, even though direct water biophotolysis is quite attractive, this method is still far from practical application, and principally new, breakthrough solutions are needed that can appear as a result of basic research.

Cyanobacteria or blue-green algae are procariots capable of photosynthesis with oxygen evolution. Representatives of these microorganisms possess hup and hox hydrogenases and various nitrogenases. Several hydrogen-activating enzymes induce different metabolism pathways leading to hydrogen evolution [53–55]. Even though the high energy "cost" [cf. reactions (7) and (8)] of thus produced hydrogen, the light-dependent hydrogen evolution by heterocyst

cyanobacteria under the action of nitrogenase is considered to hold the greatest promise (in what follows we will dwell on this metabolism pathway only):

$$Fd^{red} + N_2 + n\text{ATF} + 8H^+ \longrightarrow Fd^{ox} + NH_3 + n\text{ADF} + nP_i + H_2. \quad (8)$$

In these cyanobacteria, nitrogenase is largely localized in special cells, vis. heterocysts. Heterocysts are synthesized under deficiency of bound nitrogen. Their additional feature is the lack of photosystem 2, i.e. heterocysts are incapable of oxygen evolution. Thus, nitrogenase is sterically separated from oxygen which is inevitable present in the medim during photosynthesis; therewith, oxygen is formed in vegetative cells, while hydrogen, in heterocysts. Since these processes occur in different cells, they are intervened by a great number of intermediate steps, including saccharide diffusion from a vegetative cell to a heterocyst, and their net result is called "indirect water biophotolysis."

Under usual conditions, cyanobacteria do not evolve hydrogen in the course of nitrogen, because *hupSL* hydrogenase absorbs it effectively in an oxyhydrogen reaction [56]. Mutants containing no this enzyme evolve hydrogen in air, even though the rate of hydrogen evolution in the presence of molecular nitrogen is much higher:

$$Fd^{red} + n\text{ATP} + 8H^+ \longrightarrow Fd^{ox} + n\text{ATP} + nP_i + 4H_2. \quad (9)$$

The creation of mutants lacking one or both hydrogenases [57] initiated a series of research on the effect of synthesis of Mo- and V-containing nitrogenases [58] and O_2 and CO_2 [56], as well as assessment of real photobioreactors in laboratory [59] and open air [60]. It was shown that the rate of hydrogen evolution in real conditions (the experiments were performed in London in 1998 and 1999 summers) reached 0.01 $1\,h^{-1}\,l^{-1}$ photobioreactor. The efficiency of the conversion of the light energy into the combustion heat of biomass and hydrogen was 0.60–0,85%, and, therewith, biomass accumulated 85–97% of the total accumulated energy.

For a more efficient hydrogen production in the bright light, *A.variabilis* PK84 intensive cultures were applied under nitrogen deficiency conditions [61]. It was shown that at the cell concentration 48 mg Chl *a* l^{-1} [Chlorophyll *a* (Chl *a*)] is the principal enzyme involved in photosynthesis in cyanobacteria) and irradiance 352 $\mu E\,m^{-2}\,s^{-1}$, the rate of H_2 evolution was 0.022 $1\,l^{-1}\,h^{-1}$ with the efficiency of light energy bioconversion 1.2–1.4%. The resulting data point to the possibility of hydrogen production with a fairly high efficiency in the bright light, too.

The authors of some papers and review mention problems that limit applicability of cyanobacteria for solar energy conversion. The sensitivity of the process to oxygen and simultaneous oxygen and hydrogen evolution were also noted. Lines of further research to increase the rates of the process are suggested [31, 32, 53, 62]: search for new, more productive strains and modification or superexpression of nitrogenase or even its replacement by Fehydrogenase which reacts at a higher rate and thus can serve for a more efficient hydrogen production. Demands for developing principally new photobioreactors with a more uniform irradiation of cyanobacteria cultures and for intensifying research into immobilized cultures with the aim to increase the concentration of cells in the reactor are mentioned. This research work will undeniably indicate the ways to improved hydrogen productivity of cyanobacteria, but will not solve the problem of simultaneous oxygen and hydrogen evolution. At the same time, due to the intrinsic structural organization of heterocyst cyanobacteria, the oxygen and hydrogen evolution sites are already separated by a microscopic distance. Spatially-oriented immobilization of heterocyst cyanobacteria (for instance, by genetic engineering of special markers on heterocyst walls) on gas-selective membranes will allow this separation to be realized at macroscopic distances.

Summarizing we can admit that indirect water biophotolysis, regardless of the impressive recent advances in this field, still calls for multidisciplinary basic research.

Purple non-sulfur bacteria can evolve hydrogen under the action of hox-hydrogenase and nitrogenase. The highest hydrogen evolution rates were attained with nitrogenase in reaction (9). Therefore, we restrict our consideration to this process. Purple bacteria have only one photosystem. Along with reduced sulfur compounds, volatile fatty acids and their derivatives can donate electrons for photosynthesi.

At present factors controlling the rate of light-dependent hydrogen formation by purple bacteria are well understood [3, 63]. The process is favored by a provide deficiency of any nitrogen species. For high rates, the culture should be provided with mineral components and organic substrate, irradiated with light of saturating intensity, and put in anaerobic conditions with optimal temperature and pH. The typical rates of hydrogen production by different types of purple non-sulfur bacteria in optimal conditions, reported over the past 30 years, span the range 100–250 ml $H_2\,h^{-1}1\,g^{-1}$ dry biomass [63].

Processes competitive with hydrogen evolution are already known: synthesis of polyhydroxyalkanates and hydrogen recycling by hup-hydrogenase [12, 64]. Over the past years mutants with impaired hup-hydrogenase [64] and/or polyhydroxyalkanate synthesis were obtained [65, 66]. Comparative analysis of the rates of hydrogen production by mutants and the parent strain showed that double mutants evolve hydrogen much faster (up to 2.5-fold [66]). However, the above assessments of hydrogen evolution rates were perfomed in nonoptimal conditions, which makes these data difficult to compare with results of other authors. Probably, this is an explanation of the fact that, regardless of considerable efforts on the development of new mutants, their specific hydrogen evolution rates are close to those reported for natural strains in earlier works.

Attempts to intensify the process in laboratory conditions by increasing the concentration of cells in the photobioreactor failed due to the exponential decay of light intensity in the absorbing medium. Thus, according to author's estimates, at the purple bacteria cell concentration as low as 1 g l^{-1} (cf. the concentrations of dark cultures are up to 35 g l^{-1}, vide supra), the intensity of light decays by an order of magnitude after it has passed a 1-cm suspension layer. Therefore, the following culture layers will turn to be in an attenuated illumination zone or even in the dark. This fact entails rigid requirements for bioreactors with all photosynthesizing microorganisms. The most common experimental photobioreactors have suspension layer thicknesses of 1–5 cm [63, 67, 68]. Therewith, the rates of hydrogen evolution are, according to different authors, 0.08–0.26 l H_2 h^{-1} l^{-1} suspension.

The concentration of cells inside the reactor can also be increased by their immobilization on light-transmitting matrices with a high surface area/volume ratio [67, 68]. In this case, up to 12 g of cells (dry weight) can be immobilized in 1 l of matrix. Therewith, the rates of hydrogen evolution per unit volume increase considerably. Thus, *Rhodobacter sphaeroides* immobilized on a porous glass steadily evolved hydrogen at a rate of 1.1 l h^{-1} l^{-1} matrix for more than 1000 h; therewith, the maximum rates attained 3.8 l h^{-1} l^{-1} matrix and the conversion of organic acid attained 80% [69, 70].

The rate of hydrogen evolution per unit volume can also be increased by genetically modifying the photosynthetic apparatus simultaneously decreasing the amount of antenna pigment. In this case, one should expect increased saturating light intensity which for purple bacteria with a normal antenna composition is 1/20 the solar radiation intensity. These works have already been initiated [71, 72].

In view of high hydrogen production rates, purple bacteria can be considered candidates for hydrogen energetics. Therefore, search for low-cost substrates has intensified over the past years. Wastewater of milk industry [70], wastes of olive oil industry [73] and soybean processing [74], and even domestic wastewater have tested [75]. However, because of the fairly narrow spectrum of organic compounds suitable for purple bacteria, this method of hydrogen production resulted in no wastewater purification.

The by-products of dark hydrogen production include volatile fatty acids which are readily assimilated by purple bacteria, and, therefore, there are suggestions to combine dark hydrogen production by purple bacteria with light-dependent [14, 76]. In this case, the yield of hydrogen from cellulose-containing wastes can be increased to 11 mol/mol glucose, and, therewith, up to 4 mol of hydrogen can be produced a dark rector and up to 8 mol in a photobioreactor with purple bacteria from fatty acids obtained by fermentation.

These two processes feature higher hydrogen evolution rates compared with other biologic processes, and such an integral scheme may earlier than others pass from the basic research to practical developments. However, to this end, considerable efforts are required not only on optimization of separate processes, but also on their integration.

To conclude, the above-described biohydrogen production processes are all may turn practically useful for decentralized power-generating systems. Apparently, the integral method of biohydrogen production from readily degradable cellulose-containing wastes with the simultaneous use of solar energy seems to be closer that others to the development stage.

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