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REVIEWS

Hydrogen in Metabolism of Purple Bacteria and Prospects of Practical Application

A. A. Tsygankov[1] and A. N. Khusnutdinova

Institute of Basic Biological Problems Russian Academy of Sciences, Pushchino, Russia

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Abstract—Purple bacteria are able to use H_2 for photoautotrophic, photomixotrophic, and chemoautotrophic growth, exhibiting high metabolic lability. Depending on the type of metabolism, hydrogen may be consumed with release of energy and/or reductive equivalents. Purple bacteria may also release H_2 as a terminal electron acceptor or in the course of dinitrogen fixation. Thus, hydrogen metabolism in purple bacteria is diverse; these bacteria are often used as models for investigation of the metabolic traits and interrelation of the metabolic pathways involving molecular hydrogen. In this review, the present-day state of investigation of hydrogen metabolism in purple bacteria is reflected and its possible practical applications are discussed. Nitrogenase and hydrogenase, the major key enzymes of hydrogen metabolism, are discussed in brief. A generalized scheme of H_2 role in the metabolism of purple bacteria is presented. Experimental approaches for investigation of the rates of hydrogen production are discussed. Immobilized systems are noted as the most promising approach for development of model systems for hydrogen production.

Keywords: purple bacteria, hydrogen metabolism, H_2 production involving light energy, hydrogenase, nitrogenase

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INTRODUCTION

Purple bacteria are proteobacteria capable of anoxygenic photosynthesis. According to the features of their sulfur metabolism, purple bacteria are divided into two groups: sulfur and nonsulfur; the known sulfur bacteria belong to the class *Gammaproteobacteria*, whereas nonsulfur purple bacteria are found among alpha- and betaproteobacteria. Purple sulfur bacteria grow in fresh and saline water and soil in anaerobic habitats provided with sufficient insolation and considerable amounts of reduced sulfur compounds. Purple nonsulfur bacteria may be isolated from insolated habitats rich in organic compounds. Many purple sulfur bacteria are capable of photoautotrophic and photomixotrophic growth, as well as of growing in the darkness by respiration (at low oxygen levels) or by fermentation; the metabolism of purple nonsulfur bacteria is more labile. They can utilize a wide range of organic compounds to grow in photoheterotrophic and chemoheterotrophic conditions by respiration or fermentation. They can also grow in photoautotrophic conditions by utilizing H_2, reduced sulfur compounds, and even metals, as well as in chemoautotrophic microaerobic conditions by utilizing H_2.

Depending on the metabolic pathways employed by purple bacteria, H_2 may act as an electron donor and/or energy source, or as an electron acceptor. In the pathways involving H_2 uptake or release, its activation is mediated by different enzymes. In fact, several different reaction cascades producing or consuming H_2 can occur in a bacterial cell simultaneously. Apart from that, H_2 is also a byproduct of nitrogen fixation. Thus, hydrogen metabolism in purple bacteria is complex and versatile. For this reason, purple bacteria are an important subject of research concerning both the properties of hydrogen metabolism and the interactions among different metabolic pathways involving molecular hydrogen. Due to their high growth rates in nitrogen-fixing conditions, purple bacteria can produce high amounts of molecular hydrogen in the light. This ability makes purple bacteria promising candidates for practical application in the production of molecular hydrogen fuel employing sunlight as an alternative energy source.

In this review, we discuss the modern state in the research of hydrogen metabolism in purple bacteria and its potential practical applications.

THE ROLE OF MOLECULAR HYDROGEN IN THE METABOLISM OF PURPLE BACTERIA

It has been established that activation of hydrogen molecules within different metabolic pathways is mediated by different enzymes that are divided into two groups: hydrogenases and nitrogenases. Hydrogenases, depending on their metabolic function, can

[1] Corresponding author; e-mail: ttt-00@mail.ru

Table 1. Classification of [NiFe] hydrogenases

Group	Hydrogenase function	Purple bacteria possessing the genes encoding this type of hydrogenase
1	Membrane-bound H_2-consuming hydrogenases	*Rhodobacter capsulatus*; *Rhodobacter sphaeroides*; *Rhodopseudomonas palustris*; *Rhodospirillum rubrum*; *Rubrivivax gelatinosus*; *Rhodoferax ferrireducens*; *Phaeospirillum fulvum*; *Phaeospirillum molischianum*; *Rhodovulum* sp.; *Rhodomicrobium vannielii*; *Thiocapsa roseopersicina*; *Allochromatium vinosum*; *Thiorhodococcus drewsii; Thiocapsa marina*; *Thiocystis violascens; Lamprocystis purpurea*; *Thiohalocapsa* sp.; *Marichromatium purpuretum*; *Thiorhodovibrio* sp.; *Thioflavicoccus mobilis*; *Thioalcalivibrio* sp.
2a	Cyanobacterial membrane-bound H_2-consuming hydrogenases	–
2b	H_2 sensor hydrogenase	*Rb. capsulatus*; *Rb. sphaeroides*; *T. roseopersicina*; *Rf. ferrireducens*; *Rps. palustris*; *Rv. gelatinosus*; *Rm. udaipurense*; *Trhiorhodococcus* sp.; *Thioalkalivibrio* sp.; *Tr. drewsii*; *Rm. vannelii*; *T. marina*
3a	F_{420}-interacting hydrogenases	–
3b	Reversible NADPH-interacting hydrogenases	*Rb. capsulatus*; *Rhodovulum* sp.; *Thiorhodospira* sp.; *Thioalkalivibrio thiocyanoxidans*; *Ta. nitratireducens*; *L. purpurea*; *T. marina*; *Thiohalocapsa* sp.; *Tf. mobilis*; *Tc. violascens*; *Thiorhodococcus drewsii*; *Thiorhodovibrio* sp.
3c	MV-reducing hydrogenases	–
3d	Reversible NAD(P)-interacting hydrogenases	*Rf. ferrireducens*; *A. vinosum*; *T. roseopersicina*
4	Membrane-bound H_2-producing hydrogenases	*R. rubrum*; *Rv. gelatinosus*; *Rps. palustris*; *Rhodovulum* sp.; *Lc. purpurea*
5	Hydrogenases with high affinity to H_2	–

participate in H_2 consumption and/or production. Nitrogenases have so far been shown to participate only in hydrogen production. In our review, the properties of hydrogen metabolism in purple bacteria are described based on hydrogenase classification. This approach was chosen because hydrogenases of the same type originating from different bacteria catalyze H_2 activation within similar metabolic pathways, and also because purple bacteria do not constitute a phylogenetically distinct group.

Hydrogenase Classification

Based on the presence of metals in the active center of hydrogenase molecules, they are classified as [FeFe], [NiFe], and [Fe]-only hydrogenases (where the active center is not situated within the protein globule but is attached as a cofactor). Previously, [FeFe] hydrogenases and [Fe]-only hydrogenases were known as Fe-hydrogenases and metal-free hydrogenases, respectively. However, after the latter were found to possess an unusual active center in the form of an associated iron-containing cofactor [1], they came to be termed [Fe]-only hydrogenases, whereas a different name ([FeFe] hydrogenases) had to be proposed for the hydrogenases containing two iron groups in the active center located within the protein globule [2]. [NiFe] hydrogenases, which show the highest diversity of enzyme properties and gene sequences, are divided into five groups (Table 1). This classification is based on the presence of unique sequence fragments not found in other hydrogenase genes [3]. In particular, it is the difference between gene sequences that determines the distinction between groups 3b and 3d, although the corresponding enzymes perform similar functions (Table 1).

It should be noted that many purple bacteria are capable of hydrogen-dependent growth under photoautotrophic conditions, which certainly indicates that they possess hydrogenases. However, in a number of purple bacteria, hydrogenase genes have not been identified yet and therefore could not be included in Table 1.

The terms "consuming," "producing," and "reversible" used in Table 1 require certain comments. All hydrogenases catalyze a reversible reaction of H_2 activation. However, hydrogenases of these three groups behave differently in the tests measuring hydrogen release in the presence of reduced methyl viologen and hydrogen uptake in the presence of an oxidized electron acceptor (most commonly, benzyl viologen). Reversible hydrogenases exhibit similar activities in both tests; hydrogen-consuming hydrogenases are

more active in hydrogen uptake, while hydrogen-producing enzymes are more active in hydrogen release. This is related to the difference in the test conditions. The hydrogen release test is performed under reductive conditions (methyl viologen is nearly completely reduced, $Eh < -550$ mV), while the hydrogen uptake reaction occurs in more oxidative conditions ($Eh > -150$ mV). At the same time, it was shown that the activity of different hydrogenases depends on the redox potential of the medium [4, 5]: the range of redox potentials corresponding to the peak enzyme activity is 0–200 mV for hydrogen-consuming hydrogenases and $Eh < -300$ mV for hydrogen-producing enzymes, while reversible hydrogenases work, upon activation, within a wide range of redox potentials. The redox potential corresponding to the peak enzyme activity is an important parameter characterizing the function of a particular hydrogenase in the cell.

No [FeFe] hydrogenases have been yet identified in purple bacteria, and it is assumed that purple bacteria cannot synthesize this type of enzyme. However, the *hydC* gene found in the genomes of *Rhodopseudomonas* (*Rps.*) *palustris* BisA53 and *Rhodopsirillum* (*Rsp.*) *rubrum* was annotated as a structural [FeFe] hydrogenase gene [3, 6]. The gene from *Rsp. rubrum* was used for heterologous expression in *E. coli* in the presence of accessory genes from *Clostridium* (*C.*) *acetobutilicum* and successfully produced an active enzyme. Homologous expression experiments did not produce an active protein, probably due to the lack of the accessory protein genes in the *Rsp. rubrum* genome [6, 7]. Similar genes were found in purple nonsulfur bacteria *Phaeospirillum* (*Ph.*) *fulvum* (GenBank acc. no. EPY03056), *Rhodovulum* (*Rh.*) sp. (WP_008382437), and *Ph. molischianum* (WP_002727095).

The [FeFe] hydrogenase genes from *Rps. palustris* BisA53 and *Rsp. rubrum* were found to be phylogenetically related to the periplasmic [FeFe] hydrogenase from *Desulfovibrio* (*D.*) *vulgaris*, which, in contrast to most other [FeFe] hydrogenases, acts as a hydrogen-consuming hydrogenase maintaining the required redox state of the cells. At the same time, it was reported that the rate of hydrogen production from pyruvate in the dark by *Rsp. rubrum* increased twofold following transfection with a plasmid carrying a homologous *hydC* (structural [FeFe] hydrogenase gene) [8]. However, this phenomenon may be a consequence of alterations in the bacterial metabolism: additional energy required to synthesize more protein should be obtained by fermentation, which is associated with increased spending of reductive equivalents and H_2 release.

[Fe]-only hydrogenases (Hmd) of methanogenic bacteria participate in reduction of carbonate to methane; they catalyze methenyl-tetrahydromethanopterin reduction to methylen-tetrahydromethanopterin in the presence of H_2 [9]; these enzymes have not been described in purple bacteria.

Thus, it can be postulated that the only group of fully functional hydrogenases that can be synthesized by the purple bacteria studied in this respect are [NiFe] hydrogenases, which are involved in different metabolic pathways.

Purple Bacteria Possessing Group 1 [NiFe] Hydrogenases

Group 1 hydrogenases exhibit considerable diversity. Enzymes of this group are the key elements of the electron transport chain, transferring electrons to the quinone pool, often with direct involvement of cytochromes or heme-containing proteins. Most probably, this interaction is mediated by the hydrophobic C-terminal end of the small hydrogenase subunit. Group 1 hydrogenases contribute to the generation of the proton-driving force due to asymmetrical H_2 consumption relative to the membrane [10]. A characteristic feature of these enzymes is the twin arginine translocation motif at the N-terminal end of the small subunit [11].

The hydrogen-consuming membrane-bound HupSL hydrogenase is the most common group 1 enzyme found in purple bacteria. Since *hupSL* mutants of *Rhodobacter* (*Rb.*) *capsulatus* were incapable of photo- and chemoautotrophic growth using H_2, it is commonly accepted that HupSL activates H_2 for electron transfer to the ubiquinone pool [12]. Electron transfer is mediated by the *hupC* product (initially termed *hupM*), which also serves for the hydrogenase attachment to the membrane. The *hupC*-mutants of *Rb. capsulatus* were also incapable of photo- and chemoautotrophic growth utilizing H_2. At the same time, these cells produced HupSL hydrogenase, which catalyzed methylene blue reduction by H_2 consumption; the enzyme was located in the cytoplasm [13].

Apart from *Rb. capsulatus*, structural genes encoding the HupSL hydrogenase, as well as the corresponding enzyme activity, were described in *Rb. sphaeroides* [14], *Rsp. rubrum* [15], *Thiocapsa* (*T.*) *roseopersicina* BBS [16], and in *Rhubrivivax* (*Rv.*) *gelatinosus* [17]. The structural genes of the same hydrogenase were also detected in the genomes of *Rps. palustris* and *Rhodococcus* (*Rd.*) *ferrireducens* [3]. The data available for other purple bacteria suggest that the ability of *Rps. palustris* for hydrogen-dependent photoautotrophic growth is due to the presence of an active HupSL hydrogenase [18].

The same group also includes hydrogen-consuming membrane-bound hydrogenases of the HydSL type, which were found in purple sulfur bacteria *T. roseopersicina* [19] and *Allochromatium* (*A.*) *vinosum* [20], but not in nonsulfur bacteria. Based on the hydrogenase classification proposed in [14], the corresponding operon should be referred to as *hyn*,

although the old term is still used more commonly. In *T. roseopersiñina*, the structural *hydSL* genes do not form a cluster with accessory genes [21], which is unusual for hydrogenase operons; however, it was shown that hydrogenase assembly involved the products of the *hyp* group genes, as well as specific endopeptidase encoded by *hynD* [22]. The genes encoding the large and the small hydrogenase subunits are separated by *isp1*/*isp2*, which are presumably involved in sulfur metabolism. This notion is based on the *isp1* and *isp2* homology to the genes encoding sulfur metabolism enzymes [23], as well as on the fact that, in vivo, the HydSL hydrogenase of *T. roseopersicina* participates only in reduction of elementary sulfur to hydrogen sulfide in the presence of hydrogen [24]. HydSL is a membrane-bound enzyme, as suggested by its predominant localization [25], as well as by the structure of the C-terminal end of the HydS subunit [26].

Analysis of the hydrogenase gene sequences deposited in the GenBank database indicated that group 1 hydrogenases were also present in purple nonsulfur bacteria *Ph. fulvum* (WP_021130964), *Ph. molischianum* (WP_002725307), *Rhodovulum* sp. (WP_008386860), *Rhodomicrobium* (*Rm.*) *vannielii* (WP_013418645), and in purple sulfur bacteria *Thiorhodococcus* (*Tr.*) *drewsii* (WP_007039718), *T. marina* (WP_007190983), *Thiocystis* (*Tc.*) *violascens* (WP_014779996), *Lamprocystis* (*Lc.*) *purpurea* (WP_020502968), *Thiohalocapsa* (*Thc.*) sp. (WP_023413708), *Marichromatium* (*M.*) *purpuretum* (AHF04392), *Thiorhodovibrio* (*Thr.*) sp. (WP_009149949), *Thioflavicoccus* (*Tf.*) *mobilis* (WP_015282042), and *Thioalcalivibrio* (*Ta.*) sp. (WP_019642772).

Group 1 hydrogenases are extremely heterogeneous, but all of them are oriented into the periplasmic space, i.e., they are more affected by oxygen than all other hydrogenases. Classification of hydrogenases according to the motifs responsible for iron–sulfur coordination clusters of the small subunits and to the redox potential of the electron acceptors reveals that group 1 hydrogenases have been undergoing evolutionary selection for higher oxygen tolerance [27]. The most important evolutionary achievement of group 1 hydrogenases was the acquisition of a 3Fe–4S cluster coordinated by six cysteine residues and located proximally to the active center [28]. The membrane-bound hydrogenase from *Ralstonia* (*R.*) *eutropha* provides a characteristic example [29]. Such a cluster has a noncubic structure with increased distances between the Fe atoms. In the course of H_2 oxidation it acts as an electron acceptor, and in the presence of oxygen it donates electrons for O_2 reduction to water [30]. The six-cysteine iron–sulfur cluster motif is fairly widespread [27]; it has been described in small hydrogenase subunits of purple sulfur and nonsulfur bacteria *Rv. gelatinosus* (WP_014429771), *Rb. capsulatus* (WP_013066511), *Rb. sphaeroides* (WP_011910314), *Tr. drewsii* (WP_007039717), *Thioalkalivibrio* sp. (WP_019642773), *Rm. udaipurense* (KAI95445), *Rm. vannielii* (WP_013418644), *Rps. palustris* (WP_011156495), *Tc. violascens* (WP_014779997), *Lc. purpurea* (WP_020502969).

Purple Bacteria Possessing Group 2 [NiFe] Hydrogenases

Characteristic features of group 2 [NiFe] hydrogenases are the C-terminal end of the large subunit retained after the enzyme assembly and the lack of the signal peptide on the N-terminal end of the small subunit; as a consequence, these enzymes are located in the cytoplasm or are weakly attached to the inner surface of the cytoplasmic membrane [3].

In purple bacteria, group 2 [NiFe] hydrogenases are represented by subtype 2b sensor hydrogenases. The structural *hupUV* genes were detected in *Rb. capsulatus* [31], *Rb. sphaeroides* [3], *Rd. ferrireducens* and *Rps. palustris* [3]. Analysis of the GenBank database of protein sequences for the presence of the L1 and L2 motifs characteristic of subgroup 2b [3] showed that sensor hydrogenase genes were encoded in the genomes of *T. marina* (WP_007194465), *Rv. gelatinosus* (WP_014429792), *Thiorhodococcus* sp. (EXJ13868), *Thioalkalivibrio* sp. (WP_019642787) *Tr. drewsii* (WP_007041639), *Rm. udaipurense* (KAI95462), *Rm. vannelii* (WP_013418661).

The principal function of these hydrogenases is related to regulating the synthesis of other hydrogenases rather than to bacterial metabolism as such. In the presence of hydrogen, the sensory hydrogenase triggers phosphorylation of hydrogenase operon transcription regulator. The purple sulfur bacterium *T. roseopersicina* was found to possess H_2-sensor hydrogenase genes [21], which did not seem to affect the transcription of other hydrogenase genes [32].

An interesting feature of sensor hydrogenases is their low activity and increased resistance to oxygen-induced inactivation, which is usually explained by the presence of bulky amino acids (i.e., those with large side chains) in its gas channel [33]. This notion was supported by the results of site-directed mutagenesis experiments in *Rb. capsulatus*, when a substitution of two bulky amino acids of the gas channel by smaller ones widened the gas channel and led the enzyme's active center to irreversible oxidation by oxygen [34].

Purple Bacteria Possessing Group 3 [NiFe] Hydrogenases

Reversible group 3 hydrogenases found in purple bacteria belong to subgroups 3d and 3b according to the classification proposed by Vignais and Billoud [3]. Subgroup 3d includes reversible NAD(H)-dependent multisubunit enzymes loosely attached to the cell membrane or dissolved in the cytoplasm. The enzyme comprises five subunits; two of them (HoxYH) are

structural hydrogenase proteins, while the other three (HoxEFU) represent the diaphorase unit interacting with NAD(H) and carrying FMN and Fe–S binding sites. These hydrogenases bear certain similarity to the NADH–ubiquinone oxidoreductase (respiratory chain complex 1) [3]. This homology invites a discussion on the possible evolutionary origin of complex 1 from a hydrogenase ancestor [3]. Apart from *T. roseopersicina* [35], the *hoxEFUYH* genes and the corresponding hydrogenase activity were detected in *A. vinosum* [20]. Presumably, the physiological function of Hox hydrogenases is maintaining the favorable redox status of the cells [36]. Most information is available on the reactions catalyzed by Hox hydrogenase of *T. roseopersicina* [35, 37]. This enzyme is involved in anaerobic hydrogen production in the dark in the presence of pyruvate or glucose, in CO_2-induced light-dependent H_2 uptake, in CO_2-independent photoproduction of H_2 in the presence of thiosulfate excess, as well as in recycling of nitrogenase-produced H_2 [24, 37]. The structural *hoxYH* genes were also detected in the genome of *Rf. ferrireducens* [3]. A search for the L1 and L2 motifs of subgroup 3d [3] in the protein sequences of the GenBank database identified HoxH subunits in *Lc. purpurea* (WP_020504783), *T. marina* (WP_007191642), *Rhodovulum* sp. (WP_008384205), *Thiohalocapsa* (*Tha.*) sp. (WP_023411848), *Tf. mobilis* (WP_015281226), *Tc. violascens* (WP_014777645), *Tr. drewsii* (WP_007042613), *Thiorhodovibrio* (*Trv.*) sp. (WP_009150973), and *Rb. capsulatus* (WP_023918080).

Group 3b includes archaean NADP-dependent sulfhydrogenases. In *Pyrococcus* (*P.*) *furiosis*, they were shown to reduce elementary sulfur to hydrogen sulfide, utilizing NADPH as an electron donor [38]. Group 3b hydrogenases are encoded in the genomes of *Thiorhodospira* (*Trs.*) sp. (WP_006786889), *Tha. thiocyanoxidans* (AHE97962), and *Tha. nitratireducens* DSM 14787 (YP_007217306).

Purple Bacteria Possessing Group 4 [NiFe] Hydrogenases

Hydrogenase group 4 includes multimeric enzymes composed of at least six subunits. Their main physiological function is reduction of the water protons to discharge the excess of reductive equivalents produced by anaerobic oxidation of the low-potential C-1 organic compounds, such as CO or formate. These so-called CO-inducible hydrogenases (CooLH) were detected in *Rsp. rubrum, Rv. gelatinosus* [39, 40], and *Rps. palustris* [41]; their genes were also found in genomes of *Rhodovulum* sp. (WP_008388614) and *Lc. purpurea* (WP_020505780). Together with CO-dehydrogenases (CODH) they catalyze CO oxidation to CO_2 and H_2. The CooF subunit described in *Rsp. rubrum* is presumably responsible for electron transfer between CODH and CooLH. Group 4 hydrogenase complexes comprise two transmembrane subunits and four hydrophilic subunits. Analysis of the corresponding coding sequences revealed their homology to NADH–ubiquinone oxidoreductase subunits (respiratory chain complex 1), which indicated that CO oxidation was probably coupled to H_2 release and generation of proton-driving force on the membrane [42].

It should be noted that a fifth group of hydrogenases was identified recently [43]. A characteristic feature of these enzymes is their high affinity to hydrogen, which provides the microorganisms with an advantageous ability to utilize atmospheric hydrogen [44]. Although group 5 hydrogenases were found in some proteobacteria, e.g., in *R. eutropha*, no purple bacteria are currently known to possess such enzymes.

Hydrogenase Assembly

Hydrogenases described in purple bacteria are composed of several subunits. They possess an active center containing iron and nickel atoms associated with the ligands, as well as iron–sulfur clusters. Such complex structures have to be assembled by means of a special procedure mediated by accessory proteins. Most commonly, genes encoding subunits of hydrogen-consuming hydrogenases form clusters with accessory protein genes, and sometimes also with those that encode the regulatory proteins affecting transcription of the structural genes. The mechanisms of hydrogenase assembly in purple bacteria have not been studied, but the data obtained for other bacteria [45, 46] suggest that this process involves at least seven accessory proteins that participate in the active center assembly: attachment of Ni, Fe, CO, and CN ligands, and cleavage of the C-terminal part of a large subunit. Accessory genes are usually designated as *hypA–F*. In cells possessing several hydrogenases, Hyp proteins can participate in assembly of more than one enzyme. Endopeptidases that cleave the C-terminus of the large subunit at the final stage of assembly, as well as proteins involved in hydrogenase translocation within the cell, may be specific to particular hydrogenases. The assembly process was studied in most detail for hydrogenase 3 of *E. coli*, but other hydrogenases in other organisms may be assembled in more complex ways involving other proteins [45]. For instance, the HupSL hydrogenase operon of *Rb. capsulatus* includes the *hupGHJK* genes, the products of which presumably participate in the assembly of the small HupS subunit [3].

An important role in hydrogenase assembly belongs to the system of Ni transportation into the cell. In *E. coli*, it is the NikABCDE–NikR protein system, where NikR is the Ni-binding transcriptional regulator of NikABCD operon, which encodes an ATP-binding Ni transporter. This operon is under dynamic regulation by nickel and oxygen (via FNR regulation) [47]. The HypA–HypB complex is

responsible for Ni incorporation into the hydrogenase active center, with HypB presumably serving for Ni storage [48].

In the active center, iron atoms are coordinated by CO and CN ligands. The putative CN ligand precursor is carbamoyl phosphate. Its attachment requires at least two accessory proteins: HypF, a carbamoyl transferase, and HypE, which transfers the carbamoyl group onto the COOH terminus of a cysteine residue, dehydrating it to thiocyanate, followed by further transferring of the ligand to the iron of the active center via HypC and HypD complex, which is responsible for iron delivery into the active center [45]. CO ligands are synthesized using a different pathway, which has not been fully studied in any bacterium.

There are at least three systems that may participate in the assembly of Fe–S clusters of small subunit: Nif, Isc, and Suf. The Isc system is found in eubacteria and eukaryotes, whereas the Nif and Suf systems were described in bacteria, archaea, and plants (plastids) [49]. All systems include homologous components. Sulfur is usually donated by cysteine residues, but the source of iron is thus far unknown. The distal and proximal iron–sulfur clusters are often close to the surface of small subunits and are more exposed to oxygen; it was reported that they are protected from oxygen by chaperon-like proteins HoxQ and HoxO commonly found in microorganisms possessing O_2-tolerant group 1 hydrogenases [50]. Obligate anaerobes, such as *Clostridia*, lack such proteins [51]. The assembly of the 4Fe–3S cluster coordinated with six cysteine residues and responsible for oxygen tolerance of group 1 hydrogenases in *R. eutropha* requires an auxiliary protein HoxR homologous to HyaE of *E. coli* [52].

Regulation of Hydrogenase Synthesis

In microorganisms whose metabolism invariably involves H_2, hydrogenases are expressed constitutively [53]. However, in most cases their expression depends on the environmental conditions.

[NiFe] hydrogenases are found in microorganisms inhabiting very diverse environments and adapting to various ambient conditions. Some of them can possess more than one hydrogenase, with their synthesis most commonly controlled differently. The control of hydrogenase synthesis enables a rapid and efficient response to changing environmental conditions, in particular, to changes in energy demands. The regulation occurs at the gene transcription level and is usually performed by a two-component system. In response to a specific signal recognized by a sensor protein, a histidine kinase associated with it undergoes autophosphorylation at a conserved histidine residue. Following that, an asparagine residue of a transcription regulation factor activating or suppressing gene expression is transphosphorylated [54]. There are several factors that regulate hydrogenase synthesis.

Molecular hydrogen is a factor that induces expression of *hupSL* hydrogenases in *Rb. capsulatus, Rb. sphaeroides*, and *Rps. palustris.* The relevant system comprises an H_2-sensor hydrogenase (HupUV/HoxBC) and a two-component signal transduction system including a histidine kinase (HupT/HoxJ) and a response regulator HupR/HoxA. The functioning of this regulatory system was studied in detail in *Rb. capsulatus* [34, 55] and *Rps. palustris* [56]. In both these microorganisms, regulatory cascade includes the same components: the presence of hydrogen is detected by H_2-sensor hydrogenase (HupUV/HoxBC) and the signal is transmitted to the histidine kinase (HupT/HoxJ). The signal transduction occurs by phosphate group transfer from the histidine kinase onto the transcription factor (HupR/HoxA).

The system's efficiency in regulating the synthesis of hydrogen-consuming hydrogenases varies among different purple bacteria. For instance, in *Rps. palustris*, synthesis of membrane-bound hydrogenases is completely repressed in the absence of H_2 [56]. *Rb. sphaeroides* also exhibited hardly any hydrogenase activity in H_2-free medium regardless of the presence of other regulatory factors [57]. At the same time, *Rb. capsulatus* can synthesize the membrane-bound hydrogenase HupSL in the absence of hydrogen [58], although the highest hydrogenase activity was observed in cells grown in the presence of H_2 [59]. Addition of hydrogen activates hydrogenase expression via the sensor hydrogenase and the two-component regulation system [60]. Although *T. roseopersicina* possesses all components of the H_2-dependent regulatory system (HupUV, HupT, and HupR), the synthesis of HupSL hydrogenase is hydrogen-independent. Moreover, it was shown that this bacterium cannot synthesize HupUV transcripts [32].

Organic compounds, such as malate, lactate, or acetate repress the synthesis of the membrane-bound hydrogen-consuming hydrogenase HupSL in *Rb. capsulatus* [61] and *Rb. sphaeroides* [59], but the molecular mechanism underlying this effect remains unidentified.

Molecular oxygen suppresses hydrogenase transcription, which usually occurs under strictly anaerobic or microaerobic conditions. The presence of oxygen is detected by means of common regulatory proteins of the CRP/Fnr family. In *E. coli*, the anaerobic Fnr factor is a cytoplasmic O_2-sensitive regulator comprising a sensor and a DNA-binding domain. Transcription factors of the Fnr group share a similar structure: the N-terminal domain is responsible for detecting oxygen signaling and the C-terminal DNA-binding domain contains a helix-turn-helix (HTH) DNA-recognition motif. The Fnr factors activate expression of genes involved in anaerobic respiration while repressing transcription of genes associated with aerobic metabolism [62]. Fnr factor dimer binds to the

Fnr consensus sequence TTGAT-N4-ATCAA. Its activity is associated with [4Fe–4S] clusters, which are converted into more stable [2Fe–2S] clusters in the presence of O_2 [63].

Although repression of hydrogenase synthesis by molecular oxygen is a widespread phenomenon described in many purple bacteria [64], there is little data on the corresponding regulatory systems. The purple sulfur bacterium *T. roseopersicina* was found to possess an Fnr homologue, FnrT. It regulates the expression of the thermostable HydSL hydrogenase by binding to two sites upstream from the gene promoter: the basic gene expression is determined by proximal binding site, while distal site modulates the expression level [65].

CO-dependent expression of the CooLH hydrogenase. In *Rsp. rubrum*, CO-dependent growth is determined by a CO oxidation system encoded by the *coo* genes organized in two CO-regulated transcription units. Expression of the *coo* regulon composed of *cooS*, which encodes an oxygen-sensitive CO dehydrogenase, and *cooLH*, which encodes a CO-inducible hydrogenase, is regulated by the CooA transcription factor [66]. CooA is a homodimer, in which each monomer is CO-sensitive in anaerobic conditions [67]; it contains a b-type heme. CO binding stabilizes the dimer and enables it to bind the target DNA sequence, thus activating gene transcription.

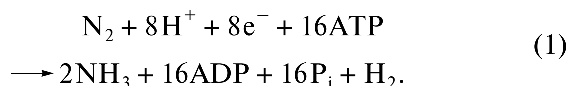
Regulation by the redox potential. Hydrogen oxidation is coupled with electron transfer along the electron transport chain. Thus, the hydrogenase functioning is incorporated into the oxidative metabolism of the cell.

Rb. capsulatus and *Rb. sphaeroides* possess a two-component regulation system comprising a redox potential sensor (RegA/Prra) and a protein kinase (RegB/PrrB). The RegAB system affects expression of the genes involved in photosynthesis, CO_2 fixation, and nitrogen fixation, as well as those encoding DMSO reductases, hydrogenases, and oxidases of different types [68, 69]. The common property of all RegAB-regulated genes is that all of them are affected by the redox potential of the cell. The N-terminal region of RegA contains a typical acceptor domain linked to a short C-terminal effector domain with an HTH motif [70]. RegB is a typical membrane-associated protein whose six hydrophobic regions form six potential integration domains required for signal perception in vivo. The kinase and phosphatase activities of RegB are required for normal regulation of RegA-dependent promoters in vivo [71]. A highly conserved redox-activated cysteine residue is responsible for signal transduction by RegB. Under oxidative conditions, intramolecular disulfide bonds transform an active kinase dimer into an inactive tetramer. In *Rb. capsulatus*, it was shown that RegA negatively affects HupSL expression by binding to *phupS*, promoter of structural *hupSL* genes [69].

Nitrogenases

Nitrogenases are metalloprotein complexes responsible for biological fixation of molecular nitrogen. According to the metal composition of the active center, nitrogenases can be divided into three types: molybdenum-containing Mo-nitrogenases (encoded by the *nif* operon), vanadium-containing V-nitrogenases (*vnf*), and Fe-nitrogenases that contain neither Mo nor V (*anf*). Nitrogenases of each type are highly conserved across different bacterial species. Mo-nitrogenases are the most widespread and the best-studied nitrogenase type in bacteria [72–74]. They are two-component metalloenzyme complexes encoded by the *nifHDK* genes, whose products are termed γ-, α-, and β-proteins, respectively. A Mo-nitrogenase complex is composed of a heterotetrameric nitrogenase (Mo–Fe protein, $\alpha_2\beta_2$) and a homodimeric nitrogenase reductase (Fe-protein, γ_2). Each αβ subunit of the Mo–Fe protein contains a Fe–Mo cofactor (Fe_7S_9Mo homocitrate) located within the α subunit and a P-cluster (Fe_8S_7) lying at the border between the α and β proteins. The Fe–Mo cofactor serves as a binding center for molecular nitrogen. The Fe-protein possesses one [4Fe-4S] cluster located between the subunits and two nucleotide binding sites; it performs ATP-dependent electron transfer onto the nitrogenase.

Under physiological conditions, Mo-nitrogenase catalyzes the reaction of nitrogen fixation, which can be written as follows:



$$N_2 + 8H^+ + 8e^- + 16ATP \longrightarrow 2NH_3 + 16ADP + 16P_i + H_2. \quad (1)$$

It should be pointed out that intact cells produce one hydrogen molecule per one dinitrogen molecule fixed only under optimal conditions. When nitrogen supply is limited, hydrogen is produced in a higher stoichiometric proportion. For instance, under conditions of nitrogen excess (58–95 Pa in the gaseous phase), nitrogen-fixing purple bacteria *Rb. capsulatus* produced 0.8 to 0.9 hydrogen molecules per a molecule of fixed nitrogen, whereas nitrogen limitation led to release of up to five hydrogen molecules per one nitrogen molecule [75]. In the absence of dinitrogen, hydrogen production rate was approximately equal to the nitrogen fixation rate (measured by acetylene reduction rate) [64]. This fact underlies all attempts to employ nitrogen-fixing purple nonsulfur bacteria in systems of solar energy transformation.

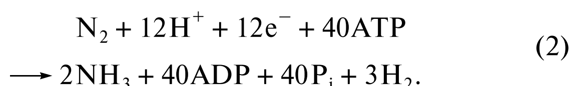
Nitrogenases are highly sensitive to molecular oxygen: nitrogenase-containing cell-free extracts lose nitrogenase activity after several seconds of exposure to the air. Therefore, although purple bacteria are capable of chemoheterotrophic growth in the presence of oxygen, they can fix nitrogen only under microaerobic conditions when the rate of oxygen diffusion into the cell is lower than the rate of respiration [76].

Apart from Mo-nitrogenases, some purple bacteria were found to possess nitrogenases that do not contain molybdenum in the active center. In particular, *Rps. palustris* can synthesize nitrogenases of two other types: vanadium-containing nitrogenase and nitrogenase that contains neither molybdenum nor vanadium [77]. Similarly to *Azotobacter* (*Az.*) *chroococcum* and *Az. vinelandii* [78], *Rps. palustris* synthesizes V-nitrogenase (vnfHDGK) in response to deficiency of fixed nitrogen and molybdenum, when Mo-nitrogenase is not produced. In environments that contain vanadium but not molybdenum, V-nitrogenase is synthesized, while the third nitrogenase is produced only in environments lacking both molybdenum and vanadium [77]. It should be noted that *Rps. palustris* is the only purple bacterium currently known to synthesize two alternative nitrogenases. It was also found that *Rb. capsulatus* [79, 80] and *Rsp. rubrum* [81] can synthesize a nitrogenase that contains neither molybdenum nor vanadium. The structural genes encoding alternative nitrogenases have been cloned and sequenced in *Rps. palustris, Rb. capsulatus*, and *Rsp. rubrum*. There is currently no information as to whether other purple nonsulfur bacteria possess alternative nitrogenase genes.

The structural genes *vnfD* and *vnfK* encoding the V–Fe protein in *Rps. palustris* are highly homologous to the corresponding genes of *Az. chroococcum* and *Az. vinelandii* [77]. In contrast to Mo–Fe proteins of Mo-nitrogenases, the V–Fe protein of V-nitrogenases comprises, in addition to α and β subunits, two δ subunits (encoded by *vnfG*); i.e., it is a heterohexamer [72].

Fe-Nitrogenases, which contain neither molybdenum nor vanadium, are also heterohexamers. It was shown that amino acid residues responsible for binding the Fe–Mo cofactor and the P-cluster in Mo–Fe nitrogenases are also present in alternative nitrogenases [72].

There have been no studies analyzing the stoichiometry of V- and Fe-nitrogenase activity in isolated enzyme preparations from purple bacteria. It was only observed that the ratio of hydrogen production and acetylene reduction rates was higher in those cultures of purple bacteria that produced V-nitrogenase and not Mo-nitrogenase [80, 82]. Based on the data obtained for purified V-nitrogenase specimens isolated from *Az. vinelandii*, it was assumed that the reaction of nitrogen fixation catalyzed by this enzyme occurs as follows [72]:



$$N_2 + 12H^+ + 12e^- + 40ATP \longrightarrow 2NH_3 + 40ADP + 40P_i + 3H_2. \quad (2)$$

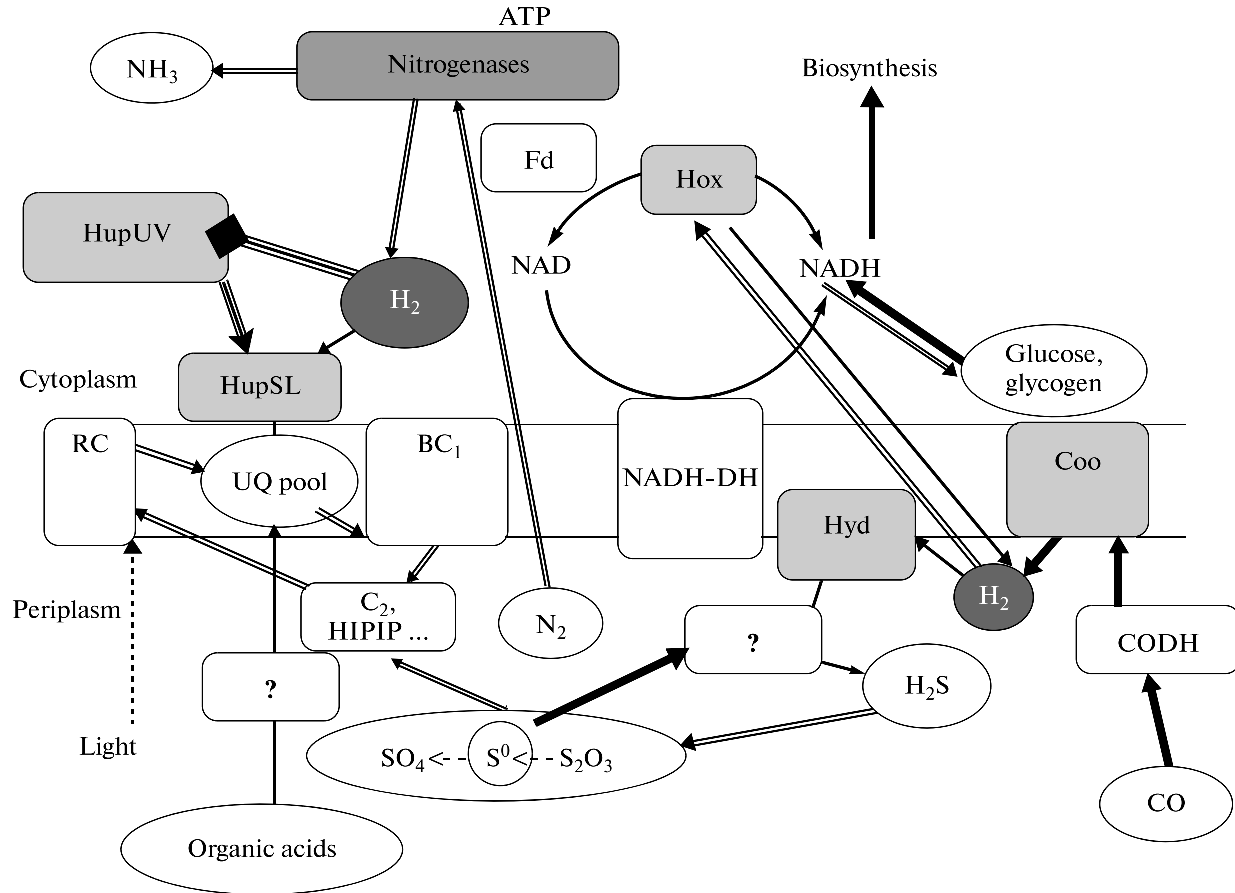
At the same time, V-nitrogenase is characterized by a high K_m for N_2, which may explain its low energy efficiency. For the Fe-nitrogenase of *Rb. capsulatus*, it was shown that the cultures growing at high partial N_2 pressures produced approximately one H_2 molecule per each N_2 molecule [75], whereas at lower nitrogen content in the gaseous phase this proportion could be as high as 50. Although for Fe-nitrogenase-containing cells and pure enzyme hydrogen production rates were higher than nitrogen fixation rates, a model was proposed to describe its functioning according to equation (1), which showed a satisfactory agreement with the experimental data [83].

For the same bacterial culture, specific nitrogen fixation rates were the highest when the cells produced Mo–Fe nitrogenase, somewhat lower for V–Fe nitrogenase, and the lowest for Fe nitrogenase. At the same time, the rates of hydrogen production by the cells containing Mo–Fe and V–Fe nitrogenases were similar and higher than those of Fe nitrogenase cells [72].

The synthesis and maturation of the nitrogenase enzyme complex in purple bacteria involves a large number of auxiliary gene products and is regulated in an extremely intricate manner [84, 85]. For this reason, description of the regulatory pathways controlling nitrogenase synthesis and maturation constitutes a separate issue lying beyond the scope of the present review. We shall only point out that the synthesis of Mo-nitrogenases is molybdenum-dependent and is repressed by oxygen excess and fixed nitrogen. In environments that lack molybdenum but contain vanadium, V-nitrogenase is synthesized, and only in the absence of both these metals do bacterial cells produce Fe-nitrogenase.

To sum up the information on hydrogen-metabolizing enzymes, we propose a general scheme of hydrogen metabolism in purple nonsulfur bacteria, which combines the known pathways of hydrogen uptake and release (figure). In addition to specialized enzymes involved only in hydrogen uptake (HupSL hydrogenase) or hydrogen production (nitrogenases), it includes those hydrogenases (Hox) that can catalyze the reaction in different directions, depending on the oxidative conditions. At the same time, photoautotrophic and chemoautotrophic growth may be supported both by HupSL (which is the case for all purple nonsulfur bacteria) and HoxYH hydrogenases. As for purple bacteria that possess both of these enzymes (e.g., *T. roseopersicina* and *A. vinosum*), it is not quite clear which hydrogenase acts as the key enzyme in given growth conditions. Light-dependent hydrogen production can be catalyzed both by nitrogenases and by the HoxYH hydrogenase. However, the rate of hydrogen production due to nitrogenase activity is considerably higher, and this process is considered the one important for practical applications. The possibility of hydrogen recycling by either HupSL or HoxYH hydrogenase reduces the overall hydrogen production rate by purple bacteria possessing these enzymes.

Hydrogen production by CooLH hydrogenase in the course of CO consumption in the dark is also considered a process of potential practical significance.



Generalized scheme of H_2-dependent metabolic pathways in purple bacteria. Blocks showing H_2 molecules and hydrogenases (Hup, Hox, Hyd, and Coo) are shaded, as well as Mo-, V-, and Fe-nitrogenases (shown as a single block, because a cell always synthesizes only one nitrogenase). Open arrows indicate the reactions occurring under phototrophic conditions; broad filled arrows show the reactions under chemotrophic conditions; thin arrows show light-independent reactions, and broad arrows with a stripe indicate functional signaling detecting H_2 presence and induction of HupSL synthesis. Hox hydrogenase is membrane-bound, but it is shown outside the membrane for the sake of convenience of the representation. Abbreviations: RC, reaction center; C_2, HIPIP..., soluble electron transporters between the bc complex and RC; Fd, ferredoxin; CODH, CO dehydrogenase. Question marks indicate the enzyme interacting with HydSL in the course of elementary sulfur reduction and the enzyme transferring electrons from organic acids to the ubiquinone pool.

This reaction is not associated with storage of light energy and for this reason is not included in our review.

PRACTICAL SIGNIFICANCE OF HYDROGEN PRODUCTION BY PURPLE BACTERIA

The recent upsurge of fossil fuel prices has renewed the worldwide interest in alternative energy sources. Among those, molecular hydrogen attracts increased researchers' attention as an ecologically pure source of energy. Burning of hydrogen produces only water and does not pollute the environment. Along with conventional methods of hydrogen production (e.g., hydrocarbon conversion or electrolysis), the prospects of employing photosynthesizing microorganisms are also considered. As it was described above, purple bacteria perform light-dependent hydrogen production. In this section, we analyze the main factors that can influence this process and present the available experimental data concerning the rates of hydrogen production by purple bacteria, with particular consideration of possible causes leading to low observed rates; we also discuss the problem of electron donors required for hydrogen production, the possibilities of using mixed cultures, and compare the published hydrogen production rates to the values relevant for practical needs.

Factors Affecting Hydrogen Production Rates in Purple Bacteria

The rate of light-dependent hydrogen production by purple bacteria is determined by many factors. Since this process involves nitrogenase activity, it requires derepression of this enzyme, which occurs in the course of nitrogen fixation. However, it was shown that nitrogenase activity of purple bacteria was the highest under conditions of nitrogen deficiency, including both fixed and molecular nitrogen. The

effect of nitrogen limitation was most clearly demonstrated on continuous chemostat cultures with nitrogen limitation. For instance, *Rsp. rubrum* [86] and *Rb. capsulatus* [58, 59, 87, 88], growing both with ammonium and with molecular nitrogen, nitrogenase activity (and hydrogen production rate) increased with decreasing flow rate (increasing nitrogen limitation). At flow rates lower than 15% of that corresponding to the maximal growth rate, hydrogen production rates began decreasing.

It should be noted that steady-state continuous cultures are in a state of equilibrium. In batch cultures, the nitrogen source (in the liquid phase) is initially in an excess, and no hydrogen is released. Nitrogen source depletion triggers nitrogenase synthesis, and hydrogen production begins. For this reason, hydrogen production by purple bacteria is studied using media with decreased nitrogen content, to ensure that growth arrest happens due to nitrogen depletion. The composition of media is controlled by the proportion between the nitrogen and the carbon sources. At C/N ratios higher than 7.5, the medium is first depleted in nitrogen, and the amount of the electron donor (which also serves as a carbon source) is sufficient for subsequent hydrogen production [88].

Since hydrogen production by purple bacteria is light-dependent, an important factor is culture illumination. It was shown that, in diluted cultures, the saturating light intensity for hydrogen production by different bacteria ranged from 10 to 60 W m^{-2} [89, 90]. In *Rb. capsulatus*, this value coincided with the saturating light intensity for growth [91]. For cultures with cell concentrations above 1 g L^{-1}, the saturating light intensity was considerably higher and strongly depended on the illumination method and the shape of the photobioreactor, which was related to the self-shading within the cultures. It is a common phenomenon that hydrogen production rates decrease in the cultures with high cell concentrations. A possible reason is that a high gradient of light intensity within the photobioreactor switches the culture growth mode from nitrogen limitation to dual limitation with nitrogen and light, as it was shown for *Rb. capsulatus* [92].

The rate of hydrogen production by purple bacteria also depends on the cultivation temperature. Many hydrogen-producing species of purple bacteria have a growth temperature optimum in the range of 30–35°C [88]. It is noted, that the temperature optimum is the same for both hydrogen production and culture growth [89]. However, in ambient conditions, a photobioreactor is often heated above these limits, and thermostatic control is required to maintain the optimal temperature. A targeted generation of *Rb. capsulatus* mutant strain that can tolerate temperatures up to 43°C was also described [93].

An important factor is pH of the medium. The optimal pH values required for growth and for hydrogen production were also found to coincide; usually, they were in the range of 6.5–8.5 [91]. A known exception is *Rps. acidophila*, which is capable of growth and hydrogen production (although at a low rate) in the pH range of 4.5–7.0 with an optimum at pH 6.0 [94]. Some data suggest that purple bacteria can adapt to ambient pH. For instance, it was found that, in *Rb. capsulatus* cultures grown at pH 7.0, the pH optimum for hydrogen production was close to 7.0. At the same time, in the cultures grown at pH 8.0, the pH optimum for hydrogen production was shifted to more alkaline values [82]. Interestingly, the pH range suitable for growth of the cultures exhibiting Mo-nitrogenase activity was narrower than for those with an alternative nitrogenase containing neither molybdenum nor vanadium.

Organic acids are the best electron donors supporting the growth and hydrogen production by purple bacteria. This is an important characteristic used to identify the species of purple bacteria. At the same time, the consumption of different organic acids can vary. For instance, the highest rates of hydrogen production by *Rb. capsulatus* were observed in presence of the same organic acid that was used for preliminary cultivation; these rates were nearly identical for lactate, propionate, acetate, and butyrate [95]. However, suspensions of the cells preliminarily grown on lactate exhibited the maximal hydrogen production rate only in the presence of lactate. In presence of acetate, propionate, or butyrate, the same suspensions produced hydrogen at rates two times lower. Interestingly, the saturation constant for hydrogen production from acetate depended on previous history of cultivation. Cultures grown under nitrogen limitation achieved the maximal hydrogen production rate at acetate concentrations of 0.5 mM and above (the saturation constant was significantly lower than 0.2 mM), while the same cultures that had been in the stationary phase for over 24 h required at least 4 mM acetate for saturation of hydrogen production (the saturation constant was approximately 0.5 mM).

To maintain the maximal rate of hydrogen production by purple bacteria, they must also be provided with other macro- and micronutrients, so as to avoid dual limitation or culture limitation with factors other than nitrogen.

Experimentally Determined Rates of Hydrogen Production by Purple Bacteria

Suspension cultures. Comparison of hydrogen production rates by purple bacteria obtained by different authors is often difficult. First of all, these studies employ different cultures, different media, different cultivation conditions, and different electron donors. Moreover, different units of measurement are often used. In particular, hydrogen production rate can be expressed per unit of biomass, while cell concentrations may vary from 0.1 to 3 g dry biomass per 1 L. Moreover, biomass concentration can be expressed

with reference to dry biomass, protein, or bacteriochlorophyll. Alternatively, hydrogen production rate may be calculated per unit of volume. Such measurements are more important to characterize the photobioreactor than the bacterial culture. When assessing the practical importance, the authors sometimes express hydrogen production rates per unit of illuminated surface to evaluate the possible surface occupied by a photobioreactor. Nevertheless, conversion of different units of measurements is often complicated and requires additional data, which are not always provided by the authors, e.g., the volume, the thickness, and the shape of the vessel used.

Specific hydrogen production rates per unit of biomass and the effects of environmental factors are most commonly assessed in suspension batch cultures. According to the reviews summarizing these data, hydrogen production rates range from 2 to 130 mL h^{-1} g^{-1} of dry biomass [90, 96, 97]. Such a strong variation is due not only to the differences described above. Not infrequently, the observed production rates are lower than possible, because the cultivation conditions are not optimal. The major possible reasons are the following:

Firstly, hydrogen production rates depend on illumination. During the growth of a batch culture, the cell concentration constantly increases, diminishing the average irradiance of the cells. It is, however, known that insufficient irradiance of bacterial cultures can reduce the observed hydrogen production rates to zero as a result of dual limitation by light and nitrogen source [92].

Secondly, consumption of organic acids by purple bacteria alkalinizes the medium. In some cases, by the moment of nitrogen depletion, pH values may reach 8.5–9.0, which strongly suppresses the nitrogenase activity in bacterial cultures.

Batch cultures are inappropriate models for studying the effects of cell concentration on specific hydrogen production rates calculated per unit of biomass or per unit of volume. These effects can be analyzed in continuous cultures. In *Rb. capsulatus* grown under ammonium limitation in the chemostat at optimal flow rates, actual specific hydrogen production rate as a function of biomass concentration (measured directly in photobioreactor) began to decrease at the concentrations exceeding 0.4 g L^{-1}, although the potential rate (measured in separate samples of the culture) remained constant. Consequently, the hydrogen production rate per 1 L of reactor volume increased nonlinearly and started to decrease at cell concentrations of 1.2 g L^{-1} and above. The apparent reason was that cultures with higher cell concentrations switched from the ammonium-limited growth mode to the mode of dual limitation by light and ammonium [92]. Thus, in a photobioreactor with a 13-mm-thick illuminated layer, the highest hydrogen production rate of 57 mL h^{-1} per 1 L of reactor volume was observed at the cell concentration of 1.2 g L^{-1}. A photobioreactor with a 20 mm-thick illuminated layer showed a similar hydrogen production rate (65 mL h^{-1} per 1 L of reactor volume) at *Rsp. rubrum* concentration of 3 g L^{-1} [86]. In a 5 mm-thick layer, the rate of hydrogen production by *Rsp. rubrum* reached 160 mL h^{-1} per 1 L of reactor volume [98].

Thus, the highest rates of volume-specific hydrogen production in suspension cultures are observed at 1–3 g L^{-1} of cell concentration and a further increase of cell concentration results in light limitation of the culture.

Analysis of maximal hydrogen production rates per 1 g of dry biomass obtained in suspension cultures of different strains and by different authors shows that there has hardly been any progress in the last three decades (Table 2). This fact indicates that the optimal conditions for light-dependent hydrogen production have already been determined, but no new strains characterized with higher hydrogen production rates have been discovered or produced by mutagenesis. However, a recent work described hydrogen production by a multiple *Rb. sphaeroides* mutant that lacked HupSL hydrogenase, did not synthesize polyhydroxybutyrate (a process which competes with hydrogen production for the reducer), had a decreased pigment content, and could synthesize nitrogenase in the presence of ammonium [107]. Although the observed hydrogen production rate was not very high (approximately 70 mL h^{-1} per 1 L suspension), this mutant could probably produce hydrogen at much higher rates, as suggested by the light intensity level used in this work, 10 W m^{-2}. This level is very close to limiting light intensities for wild-type strains, whereas strains with decreased pigment content have been known to produce hydrogen at considerably higher light intensities [108]. Therefore, this mutant strain can be expected to show considerably higher hydrogen production rates after the optimal conditions are found.

The available data also suggest that the use of suspension cultures is inefficient for practical applications, since the currently existing photobioreactors cannot provide them with sufficient illumination at concentrations higher than 3 g L^{-1}. Significantly higher cell concentrations can be attained by using immobilized cultures.

Immobilized cultures. Immobilized cells are widely employed in research. Immobilization techniques used for photosynthetic microorganisms may be divided into artificial immobilization and autoimmobilization techniques [109].

Artificial immobilization implies spatial fixation of the cells using different approaches: ion adsorption, inclusion in different types of gels, or covalent linking. The methods of artificial immobilization in the thin layers deserve special attention. They include inclusion in a sol-gel, covering with latex, and alginate films.

Table 2. Specific rates of hydrogen production by different purple bacteria. The data are listed in chronological order

Strain	Organic electron donor	Hydrogen production rate, mL h^{-1} g^{-1} dry biomass	Reference
Rb. capsulatus B10	Pyruvate	130	[98]
Rb. capsulatus	Malate	176	[99]
Rsp. rubrum S1	Lactate	160	[86]
Rb. sphaeroides RV	Lactate	262	[100]
Rsp. rubrum, hup$^-$ mutant	Lactate	41	[101]
Rb. sphaeroides RV	Lactate	75	[102]
Rb. capsulatus hup$^-$ mutant	Lactate	135	[103]
Rps. palustris	Acetate	45	[104]
Rb. capsulatus B10	Lactate	100	[87]
Rb. sphaeroides RV	Lactate	115	[105]
Rb. sphaeroides SCJ	Butyrate	173	[106]
Rb. sphaeroides HPCA	Succinate	70	[107]

Cultures of purple bacteria can be immobilized using different translucent gels, such as agar [110], carrageenan [111], alginate, polyvinyl alcohol [112], or latex [113]. One of the advantages of culture immobilization in a gel is that growing bacteria are not released into the medium; this allows flow-through to be used to purify the medium from organic compounds with simultaneous hydrogen production. Moreover, under such conditions the cells are better protected from short-term actions of adverse factors (e.g., pH or the presence of oxygen), since the rate of their flow through the gel is limited by the rate of diffusion. At the same time, reactor upscaling may be problematic not only because it would be difficult to provide cells with light, but also because an increase in linear dimension would reduce the rate of delivery of organic electron donors by increasing the distances for diffusion. In addition, if the cell concentration in the gel increases significantly, specific hydrogen production rates may be limited by substrate diffusion even on the laboratory scale. By immobilizing cells in latex, it is possible to produce layers as thin as 10 μm [113]. This process may prove to be useful in practical applications as the layers are free from any diffusion-related restrictions.

Autoimmobilization is based on the cells' ability to form biofilms [109]. Autoimmobilization, or biofilm formation, occurs in three principal stages: an individual cell's anchoring to the surface, colony-producing growth with exopolysaccharide synthesis, and biofilm formation and maturation as such. The phenomenon of biofilm formation can be a source of problems in medicine (e.g., when biofilms of pathogenic bacteria form within the human body) and technology (biofilms in vessels and pipes can cause corrosion and water deterioration). However, this property of bacteria can be used for their immobilization. Moreover, biofilms produced by the metabolic activity of bacteria in question are obviously more stable than individual cells, since this environment provides protection from harmful factors. The longest stage in biofilm formation is the anchoring of individual cells. For this reason, autoimmobilzation of phototrophic microorganisms involves a prolonged (for 2–4 weeks) incubation of the matrix in a cell suspension [114].

To overcome the restrictions associated with diffusion and to provide the cells with sufficient illumination, purple bacteria were immobilized on a matrix of 0.5 mm-thin porous glass with 10 μm pores [115]. Thus, the cells were in direct contact with the medium. To accelerate immobilization, negatively charged porous glass was activated using (3-(2-aminoethyl)aminopropyl)trimethoxysilan. It was found that, following incubation in a suspension of microalgae, cyanobacteria, or purple bacteria, 2–38% of the surface of activated glass was covered by cells [116]. Another study employed porous glass with 40–100 and 160–200 μm pores [117]. In both cases, stable hydrogen production lasted for up to 100 days.

The use of a translucent matrix has an additional advantage in comparison to the gels uniformly filled with cells. Such plates may be illuminated not only from the front, which implies illumination of a thin layer, but also from the edge. In this case, light passes not through an absorbing gel with cells, but through the matrix and, due to its complex geometry, light can reach its remote regions without significant loss of intensity. For instance, it was shown that for frontal illumination of a 0.5 mm-thick plate of porous glass carrying 11.2 g L^{-1} immobilized cells, the saturating irradiance was 20–40 W m^{-2}, whereas with lateral illumination (the layer thickness was 50 mm, i.e., 100 times greater), there was no total saturation even at 5000 W m^{-2} [118]. At the same time, hydrogen production rate achieved with lateral illumination was

Table 3. Rates of hydrogen production by immobilized purple bacteria

Strain	Conditions	Immobilization method	Hydrogen production rate, mL h^{-1} ml^{-1} carrier	Reference
Rps. palustris 42OL	Semi-continuous process; malate, glutamate; over 30 days	Agar gel	44	[109]
Rsp. rubrum PCC7061	Batch culture; malate, glutamate; 15 klx s; 80 h	Agar gel on a porous filter	565	[121]
Rsp. rubrum	Continuous flow; lactate, glutamate; 1300 W m^{-2}; over 110 days	Agar beads	57.3	[122]
Rb. sphaeroides RV	Continuous; succinate, glutamate; 300 W m^{-2}; short term	Activated microporous glass	560	[114]
Rb. sphaeroides GL1	Continuous; lactate, glutamate; 120 W m^{-2}; over 20 days	Foamed polyurethane	210	[120]
Rb. sphaeroides GL1	Continuous; lactate, glutamate; 120 W m^{-2}; over 30 days	Activated microporous glass	1780	[115]
Rb. sphaeroides GL1	Continuous; lactate, glutamate; 300 W m^{-2}; 1 day	Activated microporous glass	3800	[115]
Rps. palustris CGA009	Batch; acetate; 360 μE m^{-2} s^{-1}; 2 days	Latex plates	1840	[112]
Rb. sphaeroides GL1	Continuous; lactate, or products of starch fermentation	Fiberglass cloth, autoimmobilization	150	[119]

~80% of the rate obtained with frontal illumination. This observation suggests the possibility of upscaling photobioreactors with the cells of purple bacteria immobilized on porous glass.

A major shortcoming of porous glass is its high cost. There have been studies describing the use of fiberglass tissue, a very cheap material, as a matrix for immobilization of microalgae [114] and cyanobacteria [119]. Fiberglass tissue is also translucent and has a well-developed surface available for immobilization. Glass fibers of the tissue may be considered as light guides, so lateral illumination also might provide the cells with light at a large distance from the illuminated surface. However, no direct measurements have been performed for this type of illumination.

Glass activation with (3-(2-aminoethyl)aminopropyl)trimethoxysilan increases the cost of the process. A detailed analysis of different activation techniques used for immobilization of purple bacteria on fiberglass tissue compared the effects of preliminary treatment with different agents: sulfuric acid, sulfuric acid with subsequent (3-(2-aminoethyl)aminopropyl)trimethoxysilan activation, sulfuric acid with subsequent alkali treatment, and sulfuric acid with subsequent treatment with hydrophobic silane designed for automobile glass screens [120]. It was shown that a two-step treatment with sulfuric acid and alkali accelerated the colonization of the fiberglass tissue surface by purple bacteria. The hydrogen production rates reached 150 mL h^{-1} per 1 L reactor volume after 4–5 days of adaptation.

The use of foamed polyurethane as a translucent matrix for immobilization of purple bacteria was also reported [121]. This substance is chemically inert and has a developed surface, and its plasticity enables it to be molded in any complex shape without special treatment; meanwhile autoimmobilization of purple bacteria occurred in approximately 10 days without any pretreatment.

Volume-specific rates of hydrogen production were much higher for immobilized cultures of purple bacteria than that of suspension cultures (Tables 2 and 3). The apparent reason for that is that the cell concentrations reached in immobilized cultures were significantly higher than in the suspension cultures (e.g., 11.3 g L^{-1} in the beginning of the experiment [115]). Thus, the use of the cell immobilization makes it possible to provide illumination for much more concentrated cultures of purple bacteria.

Comparison of Potential and Practically Relevant Rates of Hydrogen Production

There are two principal criteria used to evaluate the practical significance of light-dependent hydrogen production: the specific production rate and the efficiency of light energy transformation. Since photoheterotrophic growth of purple bacteria involves consumption of both light energy and high-energy organic compounds—whose contributions to the whole process are inseparable—the efficiency of light energy transformation cannot be calculated individually. Nevertheless, it is a common practice to calculate the

ratio between the energy stored in the produced hydrogen and the energy of the consumed light and to define this ratio as the efficiency of light energy consumption. This parameter might be useful to describe the extent of the light use in light-dependent hydrogen production by purple bacteria in comparison to other processes and in the conditions where organic compounds are of no industrial importance. However, in its physical meaning it is not efficiency and requires a different term. Purple bacteria are fairly efficient in utilizing the energy of organic compounds and light. Some data suggest that up to 30% of energy consumed by purple bacteria from lactate and light is stored as biomass [91]. Our further discussion will employ the criterion of specific hydrogen production rates.

The specific rate that could be practical was estimated by Levin et al. [124, 125]. The authors considered the use of a hydrogen-producing bioreactor in a distributed energy network where each house-holder has personal hydrogen-producing bioreactor. In this scenario the energy supply system would have fuel cells producing electricity by hydrogen oxidation with atmospheric oxygen. The calculations were based on mean values of annual electric energy consumption per family (obtained in Canada) for the cases where electric energy is used for all needs except for heating (12971 kWh) and where electricity is the sole source of energy (19606 kWh). Such demands can be satisfied using fuel cells with a capacity of 1.5 and 2.5 kW, respectively. To supply peak load demands, 5 kW fuel cells are required. To feed the fuel cells with such capacities, taking into account the efficiency of the fuel cell themselves, they should be supplied with hydrogen at the rates of approximately 870, 1450, and 2900 L h^{-1}, respectively [124, 125]. In addition, the total volume of the hydrogen generator (e.g., photobioreactor) should not exceed 100 L, so that the whole autonomous energy supply system would not occupy too much space and would be attractive for the ultimate consumer. Thus, the hydrogen production rate required to satisfy all the energy demands except for heating was estimated as at least 8.7 L h^{-1} per 1 L of reactor volume minimum; the values obtained under consideration of all demands and potential peak loads were 14.5 and 29 L h^{-1} per 1 L of reactor volume, respectively.

A previous study analyzing the mass and energy balance of bacterial cells showed that the potential hydrogen production rate depended directly on the growth rate of the bacteria [126]. In particular, under the conditions of nitrogen deficit, *Rb. capsulatus* with its duplication time of 2.24 h is potentially capable of producing approximately 0.16 L h^{-1} H_2 per 1 g biomass. This estimate was based on the case of a bacterial culture that grows in optimal conditions and can produce hydrogen under steady-state conditions, i.e., indefinitely long. A study in a continuous culture of *Rb. capsulatus* experimentally determined the rate of hydrogen production by these bacteria as approximately 0.1 L h^{-1} g^{-1} biomass [87]; this value was close to the theoretical estimates cited above. While even higher hydrogen production rates have been reported (see, e.g., [89]), these values were obtained in short-term experiments. Keep in mind that to attain even the lowest hydrogen production rate required (8.7 L h^{-1} per 1 L photobioreactor) the necessary biomass concentration should be approximately 55 g per 1 L [126].

It can be expected that it is possible that bacterial strains with growth rates higher than those already described might exist. The use of such strains could enable a proportional decrease in the biomass concentration necessary to maintain the required hydrogen production rate in the photobioreactor. This notion does not seem altogether improbable, since an already known purple sulfur bacterium *Ectothiorhodospira shaposhnikovii* has a duplication time of only 1.5 h [127]. Unfortunately, its nitrogenase activity is very low, and it grows very slowly under conditions of nitrogen fixation.

Thus, keeping in mind points listed above, in order to achieve specific hydrogen production rates relevant for practical applications, it would be necessary to either find a technical means to provide bacterial cultures with concentrations of 55 g L^{-1} with light and essential nutrients and/or to find or to create a bacterium with a higher growth rate.

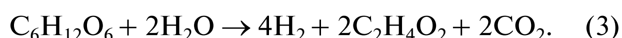
Electron Donors of Practical Significance

The source of electron donors used in photosynthesis is another important factor that determines, along with the hydrogen production rate, the practical applicability of the process. Purple bacteria possess a single photosystem, and require electron donors more reduced than that of water. Pure organic acids commonly used as electron donors in laboratory practice are obviously inappropriate for industrial energy production due to the cost considerations. However, it is possible to utilize organic waste. Moreover, it was reported that purple bacteria were used to produce hydrogen from industrial waste obtained, for instance, in the production of succinate, ethanol fuel [128], soy protein [129], in the dairy industry [116], etc. In many cases, the experiments were successful in producing hydrogen. However, purple bacteria are unable to utilize all compounds present in the waste. As a result, the culture liquid remaining after H_2 production would still need treatment prior to discharge, which is associated with additional costs. For this reason, a separate branch of research is currently under way with the search for an appropriate, economically efficient source of electron donors for hydrogen production by purple bacteria.

A promising approach is to combine sewage treatment by fermentation by a chemotrophic microbial consortium with production of H_2 and organic acids,

and further utilization of these organic acids with simultaneous hydrogen production by purple nonsulfur bacteria [130]. The consortium and purple bacteria can be grown in a mixed culture. There have been publications describing the experiments on simultaneous hydrogen-producing utilization of different substrates by an anaerobic bacterium and a purple bacterium, e.g., *Clostridium* sp. and *Rb. sphaeroides* [131], *Lactobacillus* sp. and *Rb. sphaeroides* [132] using glucose; *Lactobacillus* sp. and *Rd. marinum* using of algal biomass [133], *Clostridium* sp. and *Rhodobacter* sp. using of starch [134], and a phototrophic microbial community utilizing an acetate- and butyrate-containing synthetic medium [136]. It was found that the latter community, similarly to predefined bacterial mixtures, could produce hydrogen and was composed mainly of *Rb. capsulatus* and *Bacillus/Clostridium* spp.

A further possibility is two-stage cultivation. In its best-developed form, this approach appeared in the description of the European Hyvolution project [136]. In a microbial consortium maintained at acidic pH values, methanogenic archaea will be repressed, and fermentation will take place. As a result of fermentation, the available polymers and sugars will be degraded into hydrogen and organic acids, and the liquid culture containing mainly volatile fatty acids can be redirected (after removing stage I microorganisms) from the reactor with the chemotrophic consortium into a photobioreactor containing purple bacteria. If the fermentation liquid obtained in stage I contains little nitrogen and does not contain compounds inhibiting purple bacteria, then hydrogen can also be produced in stage II. In stage I, the highest hydrogen yield can be achieved by homoacetate fermentation of carbohydrates:



$$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2C_2H_4O_2 + 2CO_2. \quad (3)$$

If in stage II acetate is consumed by purple bacteria to produce H_2, further 8 mole H_2 can be obtained per 1 mole glucose:

$$C_2H_4O_2 + 2H_2O \rightarrow 4H_2 + 2CO_2. \quad (4)$$

Theoretically, 1 mole glucose could produce up to 12 mole H_2. Cost estimates show that it would be economically feasible to obtain 8 mole H_2 per 1 mol glucose [136].

Although the concept of such integrated systems is not new (see, for instance, [137]), the approval of the Hyvolution project inspired a powerful flow of publications about different aspects of this system.

A number of questions must be answered before the use of purple bacteria at stage II in the integrated system. In particular, it should be determined how cultures of purple bacteria behave when several organic acids are present simultaneously, what the saturating concentrations of individual organic acids are, what the highest concentrations that do not inhibit growth and hydrogen release by purple bacteria are, how hydrogen production is affected by potential by-products of fermentation, such as alcohols or acetone, and which inhibitors of methanogenesis could be applied at stage I without detriment to purple bacteria. There was absolutely no information about the behavior of purple bacteria in mixed cultures with other chemotrophs.

It was shown that, in a medium containing a mixture of organic acids, *Rb. capsulatus* cells consumed first C2 and C3 organic acids, and only after their depletion did they start to consume C4 acids [95]. The same work analyzed the effects of possible fermentation products that could inhibit the growth of purple bacteria: ethanol, methanol, and butanol. In nitrogen-fixing cultures of *Rb. capsulatus*, addition of 50 mM butanol caused a twofold decrease in hydrogen release rate; methanol and ethanol caused the same effect at much higher concentrations (1 M).

A further problem complicating the realization of an integrated system of this kind is that, for a great variety of carbohydrate-containing waste, light-independent H_2 production occurs most efficiently at pH 5.2–5.8 (see [96] for a review). On the other hand, the pH optimum for hydrogen production by different species of purple bacteria lies in the neutral or weakly alkaline values (6.5–8.5, see [90] for review). To minimize the cost of the integrated system by coupling the two stages without a need for pretreatment of the fermentation liquid, it is important for both reactors to function at the same pH. The only two known species of purple bacteria that grow at pH values below 6.0 are *Rps. acidofila* [76] and *Rhodoblastus* (*Rds.*) *sphagnicola* [138]. Unfortunately, *Rps. acidofila* grows at a low rate, while *Rds. sphagnicola* grows at low concentrations of organic acids, which makes them inappropriate as candidate H_2 producers. Therefore, a search for rapidly growing strains of purple bacteria with pH optimum for growth and nitrogen fixing in the range of 5.5–6.0 is an important task.

Experiments showed that culture liquid that was obtained after fermentation of 3% starch, freed from bacteria, and pH-adjusted to 7.0 still could not be used for H_2 production by purple bacteria without preliminary dilution, primarily because of the high concentration of organic acids [139]. This fact suggests that it is necessary to search for a novel strain (or to create a mutant strain) of purple bacteria, which would tolerate high concentrations of organic acids.

Thus, the electron donor problem can be solved successfully. Moreover, if hydrogen production is coupled to sewage treatment, the limitations concerning the reactor volume can be made less stringent, since the volume of conventional sewage disposal unit for a single-family house is 3–5 m^3. Nevertheless, the solution will require large-scale investigations aimed not only at the selection of carbohydrate-containing waste and optimization of the dark and the light-dependent stages, but also at an optimization of the whole system

as an integrated process in order to increase the overall efficiency of the system and not of its individual stages.

Interactions of Purple Bacteria with Other Bacteria

Considering possible applications of purple bacteria for hydrogen production on an industrial scale, especially using wastewater or fermentation products thereof, it is natural to pose a question concerning the possible influence of concomitant microorganisms on hydrogen production by purple bacteria, since it should not be expected that a pure culture of purple bacteria can be maintained for a long time in a functioning photobioreactor. Although a culture of purple bacteria in combination with carbohydrate-consuming chemotrophic bacteria was observed to improve the extent of carbohydrate processing and to increase the hydrogen production rate [130], interactions of purple bacteria with other chemotrophic microorganisms require special investigation. An important group of chemotrophic bacteria that can be present in wastewaters and fermentation products are sulfate-reducing bacteria. It was previously shown that sulfate-reducing bacteria had a negative effect on purple bacteria [140]. A later work was devoted specifically to interactions between sulfate-reducing and purple bacteria [141]. It was shown that sulfate-reducing bacteria did not inhibit the growth of the purple bacterium *Rb. sphaeroides*. However, in mixed cultures, hydrogen production decreased dramatically. Comparative analysis confirmed that sulfide production by sulfate-reducing bacteria induced the transition of molybdenum present in the medium into insoluble forms, which made it inaccessible for purple bacteria and hindered nitrogenase synthesis. Apparently, a possible way to overcome the inhibition of hydrogen production could be to significantly increase the concentration of molybdenum. However, this solution is appropriate only for the systems where sulfide is produced in small amounts.

By present, there have been hardly any studies analyzing interactions of purple bacteria with other species of chemotrophic bacteria.

DIRECTIONS OF FURTHER RESEARCH

Based on the above analysis of possible practical applications of purple bacteria as hydrogen producers, we can outline the directions of further research that could improve the prospects of industrial hydrogen production from organic waste using the solar energy.

First of all, it is necessary to comprehensively analyze the theoretical rates along all metabolic pathways involved in hydrogen metabolism in purple bacteria in order to identify the reactions potentially limiting the hydrogen production rates. Such studies have already been launched, and flow balance-based models were obtained for *Rb. sphaeroides* [142].

Another important task is to create mutant strains in which the nonvital pathways competing with hydrogen production for reductive equivalents would be blocked. Such research is also under way. In particular, it was shown that knock-out of the *hupSL* hydrogenase genes diminishes the recycling of the nitrogenase-produced hydrogen [103, 143, 144]. It was also found that polyhydroxybutyrate accumulation interferes with hydrogen production, and double mutants negative by hydrogenase- and polyxydroxybutyrate synthase were obtained [145]. Another study of a mutant strain with multiple genetic modifications was described in the previous section.

Since the stable hydrogen production rate depends on the bacterium's growth rate under conditions of nitrogen fixation, it is highly desirable to search for the strains of purple bacteria characterized by higher hydrogen production rates than those currently in use. The following are the criteria a potential strain should meet so that it could be employed in an integrated two-stage system described above:

(1) The optimal pH for growth should lie in the range of 5.5–6.0;

(2) The duplication time should not exceed 2 h;

(3) The bacterium should be able to tolerate (at least for a short time) elevated concentrations of organic acids (up to 100–200 mM), including butyrate and isobutyrate;

(4) It is desirable that the bacterium's photosynthetic apparatus could tolerate the full sunlight without destruction.

Although studies reporting new bacterial strains that have not been studied previously for hydrogen production appear regularly [146–148], we do not know of any publications describing a systematic search in this direction. Therefore, it can be hoped that, should such a search be performed, it could identify a bacterium with required properties.

(5) Taking into account that the volume-specific hydrogen production rate in a photobioreactor depends not only on the specific hydrogen production rate per cell but also on the concentration of bacterial cells in the reactor, it is necessary to find the way to increase the cell concentration in the photobioreactor without limiting the cultures by light. Along with the possible development of novel technical solutions, a promising approach could be to create mutant strains with impaired synthesis of the peripheral antenna, which would decrease the bacteriochlorophill concentration in the cell. Thus, light could penetrate to greater depths; at the same time, if the amount of nitrogenase per cell remains the same, the cell-specific hydrogen production rate would also be constant, but more cells located at greater depths would be able to contribute to hydrogen production. Such mutant strains have already been reported [108, 149], but the authors were unable to demonstrate convincingly their advantages in comparison to the parent strains. Appar-

ently, such experiments should be performed in continuous cultures to analyze the relationship between hydrogen production rates and cell concentrations.

In summary, we would like to point out that a significant body of information has already been accumulated concerning the metabolic pathways of purple bacteria that involve H_2. Extensive research is aimed at the description of novel strains, new culture conditions and electron donors, and at the creation of mutant strains; an ever growing number of studies deal with actual functioning of two-stage systems. This makes us hope that the time will come when hydrogen production using the solar energy with simultaneous treatment of at least some waste will be an ordinary element of life support systems, and not a challenging and interesting problem in both research and engeneering.

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