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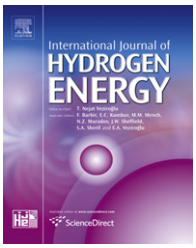

Review

Towards a super H_2 producer: Improvements in photofermentative biohydrogen production by genetic manipulations

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ABSTRACT

Photofermentative hydrogen production by purple non-sulfur bacteria is a potential candidate among biological hydrogen production methods. Hydrogen is produced under anaerobic conditions in light using different organic substrates as carbon source. The hydrogen evolution occurs mainly through the catalytic activity of the nitrogenases under non-repressive concentrations of ammonia. However, total hydrogen production is constrained due to several reasons in purple non-sulfur (PNS) bacteria, such as consumption of hydrogen by uptake hydrogenase, inefficient hydrogen production capacity of nitrogenase, limited electron flow to the nitrogenase, sensitivity of nitrogenase towards ammonia, etc. Hence, PNS bacteria need to be manipulated genetically to overcome these limitations and to make the process practically feasible. This review focuses on various approaches for the genetic improvement of biohydrogen production by PNS bacteria.

1. Introduction

The fossil fuels are limited and being consumed rapidly. Therefore, new alternative energy sources are to be investigated. Hydrogen as an energy carrier could be produced from renewable and sustainable energy sources and it could safely be used due to following reasons [1];

1. Hydrogen could be produced from sustainable energy sources such as biomass, solar energy, hydropower, wind power, etc.
2. The wide range use of hydrogen will omit the environmental impacts of fossil-based fuels due to the emissions.
3. The storage and distribution of hydrogen could be done safely.
4. Hydrogen can be used as a fuel for a wide range of end-use applications such as in the transportation and household uses.
5. Since hydrogen production would be location independent and the fact that each country has its own primary energy source, it would assist in energy diversity and security.

Today, although the hydrogen gas was mostly produced through the non-biological means (chemical ways) such as steam reforming, biological hydrogen production methods are still being investigated and being improved. The chemical methods which rely on fossil fuels should be replaced with biological hydrogen production processes which are mostly operated at ambient temperatures and pressures, thus less

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energy intensive [2]. Biological hydrogen production processes can be classified as follows:

1. Hydrogen production by dark fermentation
2. Photobiological hydrogen production
2.1. Photoautotrophic hydrogen production
2.2. Photoheterotrophic hydrogen production
3. Two-stage hydrogen production

Hydrogen, the candidate for the worldwide future alternative energy carrier, can be produced through photofermentation by photosynthetic bacteria, such as *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, *Rhodospirullum rubrum* and *Rhodopseudomonas palustris* [3–8]. Under anaerobic conditions, these bacteria are able to use simple organic acids such as malate, acetate and lactate as energy and electron sources.

The photofermentative hydrogen production process is catalyzed by the enzymes of microorganisms. Therefore, it was suggested that the overall efficiency of the process fundamentally depends on the amount or activity of these enzymes [9]. However, studies showed that, not only the activity and amount of the enzymes but also the other metabolic events such as electron flow to the enzymes determine the overall efficiency of the process (Fig. 1) [10].

In PNS bacteria during photofermentation, the electrons that are liberated from the organic compounds are delivered to the electron carriers in the membrane. In the meantime, the protons are pumped out creating proton motive force which is used to produce energy in the form of ATP. The nitrogenase enzyme, which is responsible for the hydrogen formation in PNS bacteria takes up electrons from ferredoxin/flavadoxin (Fd/Fn) and reduces protons to molecular hydrogen. Indeed, under ammonia-limited circumstances, these electrons are used by the nitrogenase enzyme to reduce molecular nitrogen into ammonium using ATPs. In the absence of any substrate such as N_2 nitrogenase acts as ATP-dependent hydrogenase and all the electrons are utilized for H_2 production. The hydrogen production property of nitrogenase is an inherent character of the enzyme [11,12]. For each electron to pass from nitrogenase, two ATPs are used. Since two electrons are needed for one H_2, four ATPs are consumed by nitrogenase:

$$2H^+ + 2e^- + 4ATP \rightarrow H_2 + 4ADP + 4P_i \quad (1.1)$$

Since oxygen is not produced during the anoxygenic photosynthesis, nitrogenase is not inactivated. But, the disadvantage of the nitrogenase-based hydrogen production over hydrogenase-based hydrogen production is that it requires extra energy in the form of ATP. However, the hydrogenases do not need extra energy. Despite this, capturing energy from the sun and electrons from the feedstocks or waste streams, photoheterotrophic hydrogen production process becomes feasible.

The biohydrogen production processes carried out by photosynthetic PNS bacteria confer several advantages over other biohydrogen production processes [13].

1. Since PNS bacteria perform anoxygenic photosynthesis, it provides a benefit for the photoheterotrophic H_2 production systems. Moreover, the oxygen problem in other biohydrogen production processes such as in microalgae-based hydrogen production process; the hydrogen production was recovered by sulfur deprivation [14].
2. Since the hydrogen production in PNS bacteria occurred mainly through the catalytic activity of nitrogenase, the H_2 production occurs even under the atmosphere of 100% hydrogen gas. The nitrogenase enzyme is not inhibited by the certain pressures of hydrogen gas unlike hydrogenase enzyme.

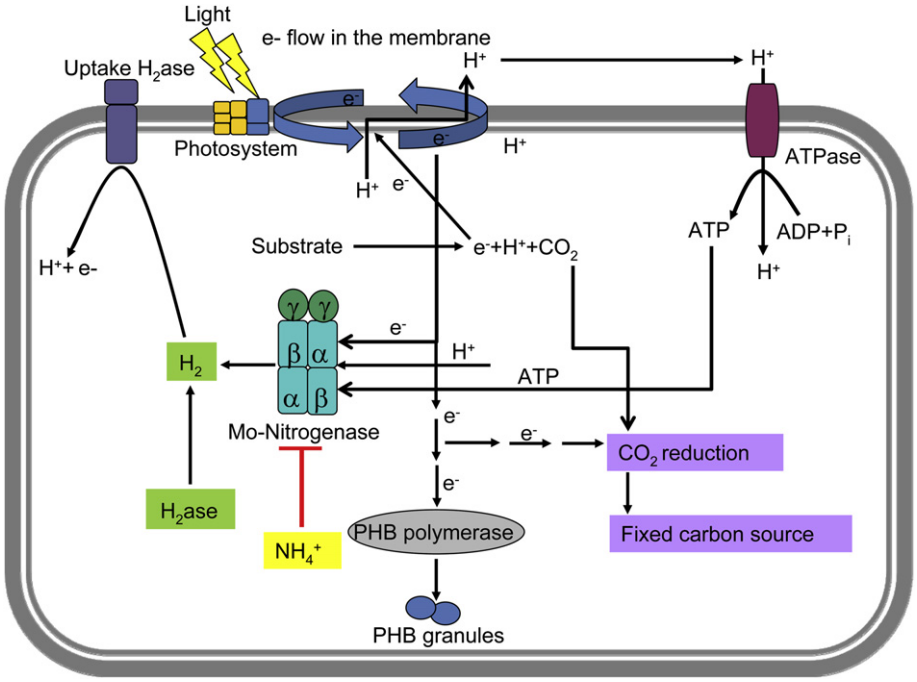

Fig. 1 – The general view of H_2 related pathways in a PNS bacterium.

Table 1 – Examples of growth modes observed in PNS bacteria.

Growth mode	Carbon source	Electron source	Remarks on growth mode	PNS bacteria and reference
Photolithotroph	CO_2	H_2	High amount of ribulose bisphosphate carboxylase/oxygenase (Rubisco) is needed in the cells.	*R. rubrum* S1 [31], *R. sphaeroides* [32]
Photoheterotroph	Organic carbon	Organic carbon	Good growth is observed and H_2 production occurs in the absence of ammonia under anaerobic conditions. CBB (Calvin–Benson–Bassham) pathway still functions to have a redox poise in the cell using CO_2 as an electron acceptor to remove excess reducing equivalents.	*R. sphaeroides* [4,32,33], *R. capsulatus* [5], *R. rubrum* [31]
Anaerobic respiration	Organic carbon	Organic carbon	In the presence of electron acceptor such as dimethyl sulfoxide (DMSO) or trimethylamine-N-oxide (TMAO) using fructose, lactate, acetate, succinate. Better growth observed when compared to fermentation.	*R. capsulatus* [34], *R. rubrum* [34]
Aerobic respiration	Organic carbon	Organic carbon	Better growth observed in the presence of O_2 using fructose or succinate when compared to anaerobic respiration and fermentation.	*R. capsulatus* [34], *R. rubrum* [34]
Fermentation (Anaerobic, dark)	Organic carbon	Organic carbon	In the absence of external electron acceptor. Lactate, acetate, succinate, H_2, and CO_2 are the major products in the case when the carbon source is fructose. Poor growth is observed.	*R. capsulatus* [34], *R. rubrum* [34]

3. The PNS bacteria can use a wide variety of organic compounds like sugars, fatty acids or waste streams of different factories [4–8].
4. As mentioned in the next section, the PNS bacteria show great metabolic versatility. Therefore, this ability makes the bacteria cope with different physiological conditions.
5. Applicable sequenced genomes and broad genetic techniques for the PNS bacteria made them amenable to genetic manipulations for improved hydrogen production [15–18].

2. The metabolism of purple non-sulfur bacteria (PNSB)

Among photosynthetic bacteria, purple non-sulfur (PNS) bacteria comprise a very diverse group of microorganisms with respect to their carbon, nitrogen and energy metabolism. The members of this group of bacteria show a wide range of metabolic activities upon varying environmental conditions. Therefore, in many studies, these bacteria provided the opportunity to investigate basic metabolic events such as nitrogen fixation, carbon fixation, anoxygenic photosynthesis and membrane bioenergetics [19–25]. PNS bacteria were initially told as ''non-sulfur'' because they were thought not to use hydrogen sulfide as an electron donor while growing photoautotrophically. However, in addition to molecular hydrogen, PNS bacteria can use sulfide as an electron donor but not at high concentrations like sulfur bacteria [20]. The famous genera of PNS bacteria are *Rhodospirillum*, *Rhodopseudomonas* and *Rhodobacter*.

Unlike the cyanobacteria, green algae and plants, PNS bacteria carry out anoxygenic photosynthesis. The photosynthetic apparatus contains only one photosystem which is not powerful enough to split water and produce oxygen which represses the activity of hydrogenases and nitrogenases [20,26]. Therefore, this property of PNS bacteria creates actually the advantage for the photoheterotrophic H_2 production. In *R. sphaeroides* which is growing chemoheterotrophically, the growth is supported by aerobic respiration and it has a typical gram-negative cell envelope. However, when the anaerobic conditions are set, the cytoplasmic membrane (CM) differentiates into specialized domains through invaginations which comprise the photosynthetic intracytoplasmic membrane system (ICM). The ICM specifically contains all of the membrane components required for the light reactions of photosynthesis. In other words, the ICM houses the photosynthetic apparatus containing bacteriochlorophylls and carotenoids [27–30].

PNS bacteria are very diverse in their carbon metabolism (Table 1) [31–34]. Although the preferred mode of growth is photoheterotrophy among PNS bacteria, most of them could also grow photoautotrophically [20,26]. They can use wide range of organic substances such as fatty acids, sugars, amino acids and alcohols during photoheterotrophic mode of growth [6–8,20,26,35–37]. The organic substances provide carbon and electrons for the cell. During photoautotrophic mode of growth, PNS bacteria use CO_2 as carbon source. H_2 or low levels of H_2S serve as electron sources since the light energy is not sufficient enough for the reduction of CO_2.

Various types of carbon sources such as acetate, lactate, malate and glucose can be used by PNS bacteria for hydrogen production. The theoretical maximum conversion of these substrates to hydrogen is as follows:

(Acetate) $C_2H_4O_2 + 2H_2O \rightarrow 2CO_2 + 4H_2$ (2.1)

(Lactate) $C_3H_6O_3 + 3H_2O \rightarrow 3CO_2 + 6H_2$ (2.2)

(Malate) $C_4H_6O_5 + 3H_2O \rightarrow 4CO_2 + 6H_2$ (2.3)

(Glucose) $C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 12H_2$ (2.4)

However, few of the substrates have considerable potential to be used for hydrogen production purposes. While malate and lactate have been efficiently converted to hydrogen, acetate and glucose were not as efficient as malate and lactate [35,37,38]. As an example, wild type *R. sphaeroides* ATCC 17023 was reported to produce 2.31 ml H_2/ml culture using 30 mM of lactate, 2.00 ml H_2/ml culture using 30 mM of malate, 1.62 ml H_2/ml culture using 30 mM of glucose and no hydrogen using 30 mM of acetate [38]. This is probably due to the substrate assimilation pathway of the bacteria and resulting redox status of the cell. In other words, the cell may not produce enough reducing equivalents to drive the hydrogen production process after substrate assimilation. Lactate (upon oxidation to pyruvate) and malate can easily enter the tricarboxylic acid cycle (TCA cycle) and meet the energy need of the cell for hydrogen production [35]. However, the substrates such as acetate entering the TCA cycle at the level of acetyl-CoA need to use side pathways where C_4 acids like malate and succinate are synthesized. Glyoxylate, citramalate and ethylmalonyl-CoA pathways are known pathways to be employed in different PNS bacteria to utilize acetate [39–41]. Thus, depending upon the pathway, the redox balance of each substrate differs from each other influencing the hydrogen production capacity.

3. Genetics of PNS bacteria

Here, the genetics of PNS bacteria will be overviewed. The gene transfer systems and genomes of the representatives of PNS bacteria will be investigated.

The first genetic exchange system for a photosynthetic bacterium dates back to 1974 when *Rhodopseudomonas capsulata* (*R. capsulatus*) has been genetically transformed [42]. The genetic exchange is mediated by a particle named as gene transfer agent (GTA) which is produced by strains of *R. capsulatus* [43]. The GTA particle resembles a small tailed bacteriophage but it has no plaque-forming activity and it packages 4.5 kb linear double-stranded DNA randomly [44,45]. The GTA-based gene transfer method helped to discover and map the genes for photosynthesis [46] and nitrogen fixation [47] in *R. capsulatus*. The genome of *R. capsulatus* has been completely sequenced by Haselkorn et al. in 2001 [48]. It has a single 3.5 Mb chromosome and a circular plasmid of 134 kb. It has a high GC content (68%) and 3709 open reading frames were recognized. One of the interesting features was that the chromosome contained numerous cryptic phage genomes. However, this should not be surprising since genetic exchange system of *R. capsulatus* largely depends on GTA.

The genomic composition of *R. sphaeroides* 2.4.1 is well studied and the whole genome of this bacterium was completely sequenced and annotated [15]. *R. sphaeroides* 2.4.1 was initially shown to possess two chromosomes, chromosome I (2973 kb) and chromosome II (911 kb) and five extra chromosomal replicons pRS241a (113.6 kb), pRS241b (104 kb), pRS241c (100 kb), pRS241d (99 kb), pRS241e (42 kb). The rich genome composition of *R. sphaeroides* reflects its metabolic complexity as well. The gene transfer to *R. sphaeroides* was reported in several ways although the most efficient and widely applied technique is the conjugation or diparental mating [16,49,50].

Today, many genomes of PNS bacteria have been sequenced which contributes significantly to the site-directed genetic manipulations. These include *R. sphaeroides R. capsulatus, R. palustris*, *Rhodospirillum centenum*, *Rhodospirillum rubrum* and all of them are available from the genome sequence databases (www.ncbi.nlm.nih.gov, http://genome.jgi-psf.org/). In addition to the well-known genetic techniques for PNS bacteria, the improvements in genome sequencing made genetic engineering of these bacteria more feasible [51].

4. Enzymes involved in H_2 production

Biohydrogen production is mediated by the enzymes in PNS bacteria. The two enzymes that play role in hydrogen production are nitrogenases and hydrogenases. In the following sections, these enzymes will be investigated in detail.

4.1. *Nitrogenases*

The function of nitrogenases is basically to contribute to the nitrogen cycle on earth by converting molecular nitrogen to ammonia which is then useable for other organisms. There exist three genetically distinct types of nitrogenase systems (nif, vnf, anf) [52,53]. All three nitrogenase systems consist of two dissociable component metalloproteins, component 1, dinitrogenase (MoFe protein, VFe protein, FeFe protein) and component 2, dinitrogenase reductase (Fe protein). Although component 2 has an identical structure in all nitrogenase systems, component 1 of the alternative nitrogenases differs from the conventional tetrameric MoFe protein in that it has a hexameric structure ($\alpha 2\beta 2\gamma 2$) containing a small 14 kDa γ subunit in addition to α and β subunits [52]. The most widely characterized system is the Mo-containing nitrogenase and studies indicate that all diazotrophs have Mo-nitrogenase, which is encoded by *nifHDK* genes [52–54]. From the genome sequence it is known that *R. sphaeroides* has only molybdenum nitrogenase (Mo-nitrogenase) [15], however, some purple non-sulfur phototrophs such as *R. capsulatus* and *R. rubrum* have additional alternative nitrogenase, an iron nitrogenase (Fe nitrogenase) encoded by *anfHDGK* genes [55,56]. And, post-translational control of nitrogenase activity has been detected in both *R. rubrum* [57,58] and *R. capsulatus* [59]. This control includes the ADP-ribosylation of dinitrogenase reductase upon exposure to darkness or addition of fixed N_2 source such as ammonia. The nitrogenase activity restores when the light is available or the fixed nitrogen source is removed. The other PNS bacterium, *R. palustris* CGA009, has genes for three different nitrogenases, therefore it appears to be a good catalyst for hydrogen production [60]. A striking property of alternative nitrogenases is that some of them have higher

nitrogenase and hydrogen production activity. The iron nitrogenase of *R. capsulatus* produced circa sixfold and that of *R. palustris* produced circa fivefold more hydrogen than their molybdenum nitrogenases [53,61,62]. Therefore, it is possible to use these alternative nitrogenases as a catalyst for the hydrogen production in the future.

The hydrogen production during nitrogen fixation is an inherent characteristic of the nitrogenase enzyme and in the absence of any substrates such as N_2, nitrogenase acts as ATP-dependent hydrogenase and all the electrons are utilized for H_2 production [11]. Thus, the inherent hydrogenase activity of nitrogenase enzyme was proposed to be a "safety valve" for the adjustment of reducing power of the cell [22,63]. *In vitro* hydrogen production experiment using *Azotobacter vinelandii* OP even in the nitrogen pressure of 50 atm showed that at least 25% of the electron throughput is used to reduce protons to molecular hydrogen [64]. Moreover, it was stated that the transfer of an electron through nitrogenase was accompanied by the hydrolysis of 2 ATPs. Therefore, for one H_2 to be produced by the nitrogenase, four ATPs are required. These results suggest that nitrogen fixation reaction competes with the hydrogen production, therefore, in the hydrogen production experiments; there should not be considerable amount of free nitrogen.

4.2. *Hydrogenases*

The other key enzyme in the H_2 metabolism is the hydrogenase. Hydrogenases are structurally and functionally diverse group of metalloproteins and they catalyze the simplest chemical reaction [65]:

$$2H^+ + 2e^- \rightarrow H_2 \quad (4.1)$$

They are classified roughly according to their preferences for H_2 production or consumption as "uptake", "bidirectional" or "H_2-evolving" hydrogenases. However, the unification of the function of all hydrogenases could be misleading but rather the direction of the enzyme could be attributed to the redox potential of the components in the cell and to the K_m values of hydrogenases for H_2 [65–68]. It may act as H_2 uptake enzyme in the presence of H_2 and an electron acceptor or it may evolve H_2 by reducing protons from water in the presence of an electron donor [65–67]. In PNS bacteria, the uptake hydrogenases catalyze the conversion of molecular hydrogen into proton and electron, therefore, H_2 acts as electron donor. Then, the reducing equivalents could potentially be used for either regeneration of energy or CO_2 reduction in the photoautotrophic mode of growth.

Previously, the classification of hydrogenases was based on the identity of specific electron donors and acceptors, namely, NAD, cytochromes, coenzyme F420, or ferredoxins. However, recently three major groups of hydrogenases are distinguished according to their metal atoms at their active site: one Ni and one Fe atom (in [NiFe]-hydrogenases), two Fe atoms (in [FeFe]-hydrogenases), no Fe–S cluster and no Ni (Hmd enzyme, metal free hydrogenase or [Fe]-hydrogenases) [65,66,68,69]. [NiFe]-hydrogenases being the most numerous and best studied class of hydrogenases of bacteria consist of the large subunit (α-subunit, ~60 kDa) containing the bimetallic active site and the small subunit (β-subunit, ~30 kDa) containing the Fe–S clusters [65]. Membrane-bound uptake hydrogenases (i.e., hupSL and hynSL), hydrogen sensors (i.e., hupUV), NADP-reducing (i.e., HydDA), bidirectional NADP/NAD-reducing (i.e., hoxYH) and energy-converting membrane-associated H_2-evolving hydrogenases are the subgroups of [NiFe]-hydrogenases [13,65,66,70]. Some phototrophic bacteria including *R. capsulatus* and *R. sphaeroides* have only membrane-bound uptake hydrogenase and sensor hydrogenases [65,66]. The function of uptake hydrogenase in these bacteria is to split hydrogen into protons and electrons which are then transferred to the quinine pool regenerating proton motive force [13,65,69]. Photoautotrophic mode of growth in which H_2 acts as electron donor was observed in *R. rubrum*, *R. palustris* and *R. capsulatus* which had the best growth among PNS bacteria under photoautotrophic conditions [13]. The [FeFe]-hydrogenases are generally monomeric and consist of the catalytic subunit only unlike [NiFe]-hydrogenases which are composed of at least two subunits. And, they are generally known to produce H_2 unlike the [NiFe]-hydrogenases. *R. rubrum ATCC 11170*, a PNS bacterium, was shown to have multiple hydrogenase activities [71] and according to the phylogenic classification of these enzymes, they are all [NiFe]-hydrogenases [65]. One of them was classified as membrane-bound uptake hydrogenase preferring to oxidize H_2 while the other two were H_2-evolving, energy-conserving, membrane-associated hydrogenases. Among H_2-evolving hydrogenases of *R. rubrum*, CO-induced hydrogenase (CooLH) has more potential towards hydrogen production rather than the other H_2-evolving hydrogenase of the bacterium (pyruvate-formate-hydrogen lyase system) [65,71]. The Hmd enzyme is a different type of hydrogenase and discovered in some methanogens functioning as H_2-forming methylenetetrahydromethanopterin dehydrogenase (Hmd) [65,72]. The Hmd enzymes are now known to contain a mononuclear iron center that has not yet been completely defined, however, significant progress has been made in understanding the assembly of the [NiFe] and [FeFe]-hydrogenase active sites and accessory proteins [65,66,68,70,73].

5. Approaches for enhanced hydrogen production

The efficiency of photobiological hydrogen production catalyzed by the nitrogenases in PNS bacteria is inherently limited. In addition to the inherent characteristics of nitrogenases, the total hydrogen production is constrained due to several reasons such as consumption of hydrogen by uptake hydrogenase, limited electron flow to the nitrogenase due to the presence of alternative electron sinks such as production of poly-3-hydroxybutyrate (PHB) or CO_2 fixation, inhibition of the nitrogenase from ammonium, light requirement of the nitrogenase, etc. (Fig. 1). Therefore, these limitations need to be overcome by several genetic manipulation approaches. In the following sections the approaches for the enhancement of the H_2 production will be investigated in details.

5.1. *Elimination of uptake hydrogenase*

In nature, many roles have been attributed to the uptake hydrogenases. In the context of biohydrogen production, the

physiological function of uptake hydrogenase in most PNS bacteria is to catalyze the conversion of molecular hydrogen to electrons and protons decreasing the efficiency of H_2 production [13]. As an example, the hydrogen consumption activity of *R capsulatus* hydrogenase is quite pronounced such that the ratio of H_2 uptake to H_2 evolution activity is 100 [13]. The hydrogen respiration let the cells recapture the electrons from molecular hydrogen and deliver it to the electron carriers in the membrane while making proton motive force which is then used to produce ATP. In this way, the uptake hydrogenase helps to maintain a redox poise or redox balance [13,69]. The uptake hydrogenases have roles in bioremediation of chlorinated compounds and toxic heavy metals too [13]. Moreover, uptake hydrogenase and H_2 respiration have been shown to be essential for the virulence of the pathogenic bacteria [74]. Since uptake hydrogenase decreases the efficiency of H_2 production, it was targeted to be eliminated in many PNS bacteria either by antibiotic resistance gene insertion into the *hup* genes or by deletion of *hup* genes (Table 2) [5,36,37,75–78]. It was shown that the inactivation of uptake hydrogenase resulted in significant increase in total hydrogen production in these bacteria.

5.2. *Heterologous expression of hydrogen evolving hydrogenases in PNS bacteria*

Light is essential for efficient photobiological hydrogen production by PNS bacteria but it is discouraging to know the fact that continuous H_2 production in the outdoor using sunlight is impossible or rather inefficient during night. Therefore, one promising approach is to relieve the dependency of PNS bacteria from light for hydrogen production. Thus, continuous hydrogen production under light and dark conditions would be possible and certainly improve the efficiency of biological hydrogen production process. In a study done by Kim et al. (2008), constitutive hydrogen evolution under both photoheterotrophic and dark fermentative conditions by recombinant *R. sphaeroides* was reported (Table 2) [79]. To achieve this, a cosmid vector containing genes for pyruvate-formate lyase (PFL), formate-hydrogen lyase (FHL) complex, Fe-only hydrogenase (HydC) together with their accessory genes was mobilized into *R. sphaeroides* KCTC 12085. The recombinant *R. sphaeroides* gained the ability to produce H_2 through the concerted actions of FHL and Fe-only hydrogenase of *R. rubrum* and nitrogenase of its own during day and night. Similarly, we have observed that simultaneous expression of accessory genes is critical for a functional heterologous gene expression (unpublished data).

5.3. *Inhibition of PHB synthesis*

The production of Polyhydroxyalkanoic acids (PHAs) especially the poly-3-hydroxybutyric acid in PNS bacteria is another way of disposing excess reducing equivalents [38]. PHAs are commonly produced by the PNS bacteria as intracellular storage material and the polymer is accumulated in the case of high carbon to nitrogen ratio [38,80]. PNS bacteria have substantial potential for the production of PHB such that *R. sphaeroides* strains could produce PHB between 60 and 80% of their cell dry weight while *R. rubrum* could produce 45% of its cell dry weight [80,81]. From these results, it is clear that the bacteria spend most of its energy for the production of PHB especially when grown on acetate as carbon source [80,81]. Therefore, the PHB production pathway of several PNS bacteria was targeted to be blocked so that all the reducing power of the cells are allocated for the H_2 production (Table 2) [38,75,76].

The PNS bacteria such as *R. sphaeroides* and *R. rubrum* were reported to produce more PHB polymers when the carbon source was acetate which is the main product of thermophilic dark fermentation [38,80–82]. In addition, the four representatives of PNS bacteria *R. sphaeroides, R. capsulatus, R. palustris* and *R. rubrum* were shown to possess different acetate assimilation pathways (Fig. 2) [83]. The proposed acetate assimilation pathways are ethylmalonyl-CoA pathway for *R. sphaeroides* [39,40], citramalate cycle for *R. capsulatus*, glyoxylate cycle for *R. palustris* and an unknown pathway for *R. rubrum* [41]. In relation to biohydrogen production and redox poise of the cell when the carbon source is the acetate, the ethylmalonyl-CoA pathway has some disadvantages. First, the pathway requires ATP and reducing power in the form of $NADPH_2$, which thereby diminishes energy and the electron output of the overall process (Fig. 2). Second, the acetate assimilation pathway shares common elements with the polyhydroxybutyrate biosynthetic route. That is, the initial

Table 2 – The hydrogen production potentials of some genetically modified PNS bacteria and their parental strains.

Microorganism	Type of modification	H_2 production by modified strain	H_2 production by parent strain	Carbon source	Reference
R. sphaeroides GK1	Hup$^-$	2.85 l H_2/l culture	2.36 l H_2/l culture	Malate, 15 mM	[36]
R. capsulatus ST410	Hup$^-$	3.3 l H_2/l culture	2.1 l H_2/l culture	Malate, 30 mM	[77]
R. rubrum K4A	Hup$^-$	7.3 l H_2/l culture	2.2 l H_2/l culture	Lactate, 50 mM	[78]
R. capsulatus KZ1	Qox$^-$	0.79 l H_2/l culture	0.94 l H_2/l culture	Malate, 15 mM	[5]
R. sphaeroides PN1a	PHA-synthase$^-$	0.45 l H_2/l culture	No production	Acetate, 30 mM	[38]
R. palustris CGA571	nifA mutant (Q209P)	104 μmol H_2/mg protein[a]	36.5 μmol H_2/mg protein	Acetate, 20 mM	[84]
R. sphaeroides KCTC 12085	Contains PFL, FHL and HydC from *R. rubrum*	4 mol H_2/mol of glucose[b]	2 mol H_2/mol of glucose	Glucose, 30 mM	[79]

Abbreviations; Hup: uptake hydrogenase, Qox: quinol oxidase, PHA-synthase: polyhydroxyalkanoic acid synthase, nifA: transcriptional activator of nitrogenase, PFL: pyruvate-formate lyase, FHL: formate-hydrogen lyase complex, HydC: Fe-only hydrogenase of *R. rubrum*.

a The hydrogen was produced in the presence of ammonia.

b The hydrogen production was carried out under photoheterotrophic conditions.

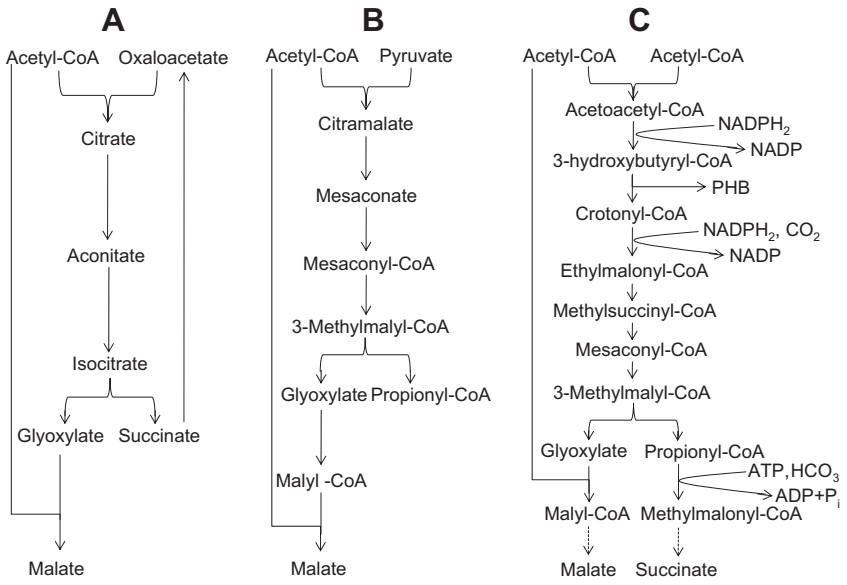

Fig. 2 – Known acetate assimilation pathways operating in PNS bacteria: glyoxylate cycle (A), citramalate cycle (B), and ethylmalonyl-CoA pathway. Adapted from [40,83].

steps of both pathways are the same and they branch only at the PHB polymerization/crotonyl-CoA formation steps. Therefore, simultaneous PHB synthesis occurs during acetate assimilation which in turn decreases the efficiency of hydrogen production. In *R. capsulatus*, the acetate assimilation goes through a special pathway, named as citramalate cycle (Fig. 2). The pathway is similar, but more complex than the glyoxylate cycle. The process is independent of CO_2 fixation and PHB biosynthesis, which might explain the better performance of *R. capsulatus* in conversion of acetate to hydrogen [37].

5.4. *Enhanced energy flow to nitrogenase*

The charge or redox status of the cells is very important for biohydrogen production since the nitrogenase needs electrons and ATPs to reduce protons to hydrogen. It is known that the hydrogen production under nitrogen-fixing conditions in the presence of molecular nitrogen is inefficient process due to the fact that 75% of the reducing power is consumed by nitrogen fixing and only the 25% of the reductant is used for H_2 evolving [84,85]. Therefore, hydrogen production under non-nitrogen fixing conditions using fixed nitrogen sources such as glutamate is a more efficient process. Furthermore, an enhancement in the electron and ATP flow to the nitrogenase would certainly lead to a burst of H_2. In the photosynthetic bacterium *R. sphaeroides* and *R. capsulatus*, a putative membrane-bound complex encoded by the *rnfABCDGEH* operon is thought to be dedicated to electron transport to nitrogenase [10]. It was shown that overexpression of *rnf* operon in *R. capsulatus* enhanced in vivo nitrogenase activity. In another study done by Öztürk et al. (2006), it was shown that a loss of function in the electron carriers in the membrane of *R. capsulatus* resulted in significant decrease in H_2 production (Table 2) [5]. These results suggest that the electron flow to nitrogenase is one of the bottlenecks of the H_2 production process and needs to be manipulated for the enhanced H_2 production.

5.5. *Reduced pigment content*

The efficiency of photobiological hydrogen production by PNS bacteria is highly dependent on the intensity of the light which functions to drive electrons from photosystem of bacteria to the electron carriers in the membrane. Thus, light intensity/availability is a very important parameter for the hydrogen production and the activity of nitrogenase enzyme. It was observed that light strongly stimulates activity and amount of nitrogenase in *R. capsulatus* and therefore the hydrogen production activity was higher [13,86]. The nitrogenase content under high light intensities was found to be up to 25% of all soluble proteins in this bacterium. The photophosphorylation capacity is also slightly greater in cells grown under high light intensity than in cells grown under low light intensities [87]. Therefore, it could be proposed that high ATP production rate under well-illuminated conditions results in higher hydrogen production activity. Although this type enhancement of H_2 production is not a result of genetic manipulation but it proves that H_2 production could possibly be increased by just adjusting the light conditions. In addition, there are genetic manipulations by which the enhancement of H_2 production was recorded through alterations in light harvesting complexes (LH) [88,89]. In a study done by Kondo et al. (2002), a mutant strain of *R. sphaeroides* with reduced LH1 complex produced 50% more hydrogen than its wild type parent [88]. This finding is of special importance since it shows the negative effect of shading due to the high pigment concentration on H_2 production process. Reducing pigment content of the bacteria by genetic manipulations increased the light penetration inside the bioreactor, thereby increasing the H_2 production.

5.6. *Development of ammonia insensitive PNS bacteria*

The first record of photobiological hydrogen production by photosynthetic bacteria dates back to 1949 by Gest and Kamen

[90,91]. And, even at that time, the suppressive effect of ammonia on hydrogen production was realized such that a burst of H_2 production by *R. rubrum* occurred when the bacteria were grown using glutamate instead of ammonia. However, today the mechanisms of ammonia repression of nitrogenase at both genetic and protein level are known more clearly [22,84,92–97]. Recently, as soon as the mechanisms of ammonia repression of the nitrogenase are being disclosed, the enzyme have been rendered to be insensitive to ammonia repression by several approaches so that H_2 production occurs even in the presence of suppressive concentrations of ammonia. In *R. rubrum* and *R. capsulatus*, the nitrogenase enzyme activity is reversibly inactivated in a way that an ADP-ribose group from NAD^+ is attached to an arginine residue in one subunit of the homodimeric NifH protein resulting in NifH inactivation (switch-off) in response to the addition of ammonia or darkness. This process is catalyzed by dinitrogenase reductase ADP-ribosyltransferase (DraT). However, this inactivation is reversible such that when the added ammonium is exhausted by cellular metabolism or the cells are moved to light, the ADP-ribose group is removed by dinitrogenase reductase activating glycohydrolase (DraG) leading to NifH activation (switch-on) [92–95]. The mutant strain of *R. rubrum* which lacks functional *draT* gene retained dinitrogenase activity upon ammonia addition or darkness [95]. In the case of *R. sphaeroides* neither *draTG* genes nor the ADP-ribosylation of dinitrogenase reductase were detected [98].

One of the interesting findings about the relationship of three fundamental biological processes; photosynthesis, biological nitrogen fixation, and carbon dioxide assimilation was well documented by Joshi and Tabita (1996) and Qian and Tabita (1996) [22,23]. Previously it was known that the RegA (PrrA)/RegB (PrrB) system controls the ability of *R. sphaeroides* and *R. capsulatus* to respond to different intensities of light for the anoxygenic photosynthesis gene expression [99–102], however, it was proved that this two-component regulatory system also influences the nitrogen fixation in addition to photosynthesis and carbon dioxide reduction [22,23]. It was shown that mutations in the *regB* (*PrrB*) gene of *R. sphaeroides* blocked transcription of the *cbb* regulon which contains genes that encode two forms of RubisCO and the enzymes in CBB cycle. And, inactivation of the *regB* (*PrrB*) gene of *R. sphaeroides* resulted in the production of active nitrogenase in the normally repressive concentrations of ammonia [23]. Similar result was obtained when the CBB route is blocked by mutation in the genes coding for form I and II RubisCO in *R. sphaeroides* and *R. rubrum* [22]. The explanation for why the nif system is on even in the presence of normally repressive levels of ammonia is that organisms seek alternative mechanisms to dispense the large amounts of reducing power generated via photosynthesis and the oxidation of organic carbon (such as malate) when the CBB route is blocked. In other words, CO_2 is no longer capable of functioning as the major electron sink when the CBB route is blocked, and the large amount of reducing power is forced to be dissipated through nitrogenase action [22,23]. In any case, the ammonia control on the nitrogenase enzyme has been abrogated.

Another mechanism of nitrogenase activity control in *R capsulatus* is mediated by *glnB–glnK* genes whose products are controlling the activity and synthesis of Mo-nitrogenase and iron-only nitrogenase in response to the ammonia in *R. capsulatus* [93]. It was shown that in *glnB–glnK* double mutant strain; ammonium regulation of Mo-nitrogenase was completely abrogated leading to an active Mo-nitrogenase in the repressive concentrations of ammonia [93]. Since *glnK* was thought to be co-transcribed with *amtB* which encodes a high-affinity (methyl-)ammonium transporter, the loss of ammonium control on nitrogenase might be due to the loss of amtB activity. The effect of *amtB* gene on the activity of Mo-nitrogenase was demonstrated by Yakunin and Hallenbeck (2002) such that *amtB* mutant strain of *R. capsulatus* lost the ability to regulate Mo-nitrogenase activity in response to ammonium [103].

In proteobacteria, the *nif* structural genes are positively regulated by nifA transcriptional activator which is a member of enhancer-binding proteins (EBPs) [104]. NifA interacts with the RNA polymerase sigma factor σ^{54} which is different from commonly observed sigma factors such as the σ^{70} family. The interaction between DNA bound σ^{54} RNA polymerase holoenzyme and nifA occurs through DNA loop formation which is sometimes facilitated by other DNA-binding proteins, such as integration host factor (IHF). Then, the correct interaction initiates the transcription of nitrogen related genes. For a detail information about the genetic regulation of nitrogen fixation process, please read the review written by Dixon and Kahn (2004) [104]. Therefore, this interesting transcriptional activator has a paramount effect on the nitrogenases. In a study done by Rey et al. (2007), constitutive hydrogen production in the presence of repressive concentrations of ammonia by *R. palustris* having mutations in the four different sites in the NifA transcriptional regulator was reported (Table 2) [84]. This mutation made the bacteria escape from the repressive effect of ammonia too.

6. Concluding remarks

Biohydrogen production is increasingly becoming challenging as the basic science disclose the unknown metabolic networks and develop tools for manipulating the microorganisms. It is obvious that the efficiency of biohydrogen production is low and the scaling up is not feasible, therefore, biohydrogen production, as being a biotechnological process, requires a sophisticated and multidirectional approach to be a feasible and an efficient bioprocess. Genetic improvements will certainly contribute to the efficiency of biohydrogen production process but it additionally requires considerations on the type of microorganism, renewable biomass source, optimization of bioprocess parameters and bioreactor design. However, it is inevitable that overall achievements in the basic and applied sciences will help to develop an applicable biohydrogen production process.

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