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# Photofermentative Hydrogen Production in Outdoor Conditions

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Additional information is available at the end of the chapter

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## 1. Introduction

Today, we are consuming the solar energy accumulated on earth in million years as fossil fuels at a rate which is much faster than it is stored by photosynthesis. Alternative energy sources such as solar, wind, wave, geothermal and nuclear are today's need of carbon neutral technologies either as replacement of some of the existing ones or as producing new sources such as; biofuel, biogas and biohydrogen, to increase the energy supplement of the world. Besides the source, nowadays engineers are very much concerned about how to utilize these energies in a more efficient way. There is a solution for the future, a new energy carrier system that is hydrogen. Hydrogen can be produced from primary energy sources; it can be stored and directly converted to electricity in fuel cells efficiently when needed.

Hydrogen energy system is bio-analog strategy for the sustainable future. Photosynthesis is the most efficient way to store solar energy. Plants, algae and photosynthetic microorganisms have developed their energy transduction centers and they know how to do this energy transformation and storage. Man exploits photobiological and photobiomimetic production of hydrogen. Biological hydrogen production processes, namely biophotolysis, dark fermentation and photofermentation, offer the prospect of producing hydrogen from renewable sources. *Rhodobacter* species are photosynthetic PNSB that can produce hydrogen from small-chain organic acids derived from biomass at the expense of light energy.

Since fuel consumption rate is very high, biohydrogen needs to be produced at a much faster rate with new strategies basing on energy bionics. These processes should provide a net energy gain, be economically competitive, and be producible in large quantities without reducing the food supplies. Further research is needed in all fields to be competitive with conventional technologies. Hydrogen- powered fuel cell electric vehicle option is a clue for 21<sup>st</sup> century's people how to change their consumption habits for a sustainable future.

In this chapter we give the fundamentals of photobiological hydrogen production by PNSB and review the research published on photofermentative hydrogen production at outdoor conditions. We discuss the most critical factors in PBR design and compare the technological development, availability and economics of other biohydrogen production techniques with photofermentation.

## 2. Photobiological hydrogen production

Photobiological hydrogen production is a microbial process that requires light as energy source, an electron donating substrate, and a biological catalyst that generate  $H_2$  by combining protons and electrons. Basically, a biocatalyst is used to convert light energy into  $H_2$ . The process can be categorized as oxygenic and non-oxygenic photobiological hydrogen production, depending on the formation of oxygen during the process (Table 1). Oxygenic photobiological hydrogen production is carried out by microalgae and cyanobacteria, which produce hydrogen during photoautotrophic growth using water as electron donor,  $CO_2$  as carbon source and light as energy source. In microalgae, biohydrogen production, also termed direct biophotolysis, is catalyzed by [FeFe]-hydrogenase, which accepts electrons from ferredoxin. Water is the proton and electron donor, which is split by PSII reaction center using light energy. Electrons travel through the Z-scheme (PSII-PQ-cyt  $b_6/f$ , PC and PSI, sequentially). Ferredoxin is the final electron acceptor and it delivers electrons to [FeFe]-hydrogenase. The [FeFe]-hydrogenase combines protons and electrons to form molecular hydrogen. The hydrogenase enzyme is extremely sensitive to the presence of  $O_2$ , which limits industrial application of biohydrogen production using microalgae [1]. In order to circumvent the  $O_2$  inhibition, Melis and co-workers [2] introduced 2-stage photobiological hydrogen production by *Chlamydomonas reinhardtii* by utilizing sulfur deprivation, which resulted in PSII inactivation, hence  $O_2$  evolution. By this way, photosynthetic oxygen evolution and carbon fixation were temporally separated from consumption of cellular metabolites and  $H_2$  evolution.

In cyanobacteria, biohydrogen production is catalyzed by nitrogenase enzyme. In filamentous cyanobacteria, the reaction proceeds in two spatially separated vegetative and heterocyst cells, hence, the process is called indirect biophotolysis. In vegetative (photosynthetic) growth mode,  $CO_2$  is fixed into carbohydrates through photosynthesis using water as electron donor and light as energy source. Heterocyst cells are specialized in nitrogen fixation and contain nitrogenase enzyme. In heterocysts, under anoxic and nitrogen limited conditions, stored carbohydrates from vegetative cells are oxidized to form ATP. Electrons generated during oxidation reactions are delivered to PSI reaction center where they are excited by light and travel through several electron transfer proteins. The final electron acceptor, heterocyst-type Ferredoxin, delivers the electrons to the nitrogenase enzyme that catalyzes  $H_2$  formation from protons and electrons using ATP. In indirect biophotolysis,  $O_2$  and  $H_2$  production reactions are spatially separated, therefore eliminating  $O_2$ -induced nitrogenase repression [1]. Cyanobacteria also harbor bidirectional and uptake hydrogenases which function to maintain redox balance within the cell.

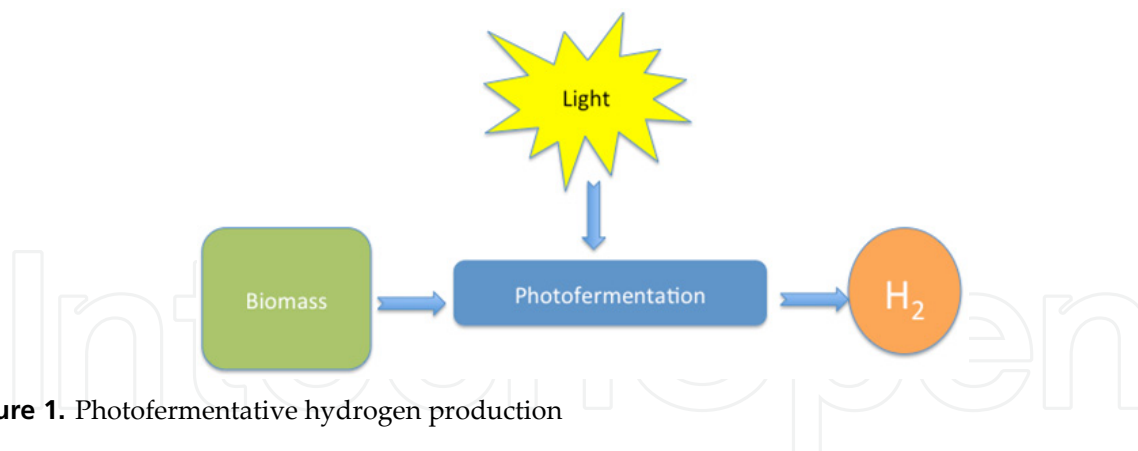
Non-oxygenic photobiological hydrogen production is carried out by PNSB, which produce hydrogen during photoheterotrophic growth on organic carbon sources utilizing energy from sunlight. Electrons generated during oxidation of substrates are converted to  $H_2$  by nitrogenase. During photofermentative hydrogen production,  $O_2$  is not produced and  $O_2$ -induced nitrogenase repression is not a concern. Photoheterotrophic  $H_2$  evolution by PNSB is well characterized. Similar to cyanobacteria, PNSB also have uptake and bidirectional hydrogenases, which regulate  $H_2$  cycling within the cell. The details of photofermentative hydrogen production by PNSB will be given in the following sections.

	Process	Organism	Enzyme	Reactions
Oxygenic	Direct Biophotolysis	Microalgae and Cyanobacteria	[FeFe] Hydrogenase	$2H_2O + \text{Light} \rightarrow 2H_2 + O_2$ <i>Hydrogenase reaction:</i> $2H^+ + 2e^- \rightarrow H_2$
	Indirect Biophotolysis	Filamentous Cyanobacteria	Nitrogenase	<i>In vegetative cells:</i> $6CO_2 + 6H_2O + \text{Light} \rightarrow C_6H_{12}O_6 + O_2$ <i>In heterocyst:</i> $C_6H_{12}O_6 + 6H_2O + \text{Light} \rightarrow 6CO_2 + 12H_2$ <i>Nitrogenase reaction:</i> $N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$
Non-oxygenic	Photofermentation	Purple non-sulfur bacteria	Nitrogenase	$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$ <i>In the absence of <math>N_2</math>:</i> $2H^+ + 2e^- + 4ATP \rightarrow H_2 + 4ADP + 4P_i$

**Table 1.** Photobiological hydrogen production routes.

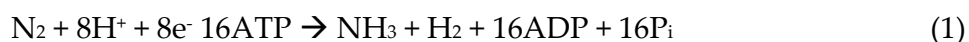
### 3. Photofermentative hydrogen production by purple non-sulfur bacteria (PNSB)

Photofermentative hydrogen production is a microbial process in which electrons and protons generated through oxidation of organic compounds are used to produce molecular hydrogen under anaerobic, nitrogen-limited conditions, utilizing light as energy source (Figure 1).

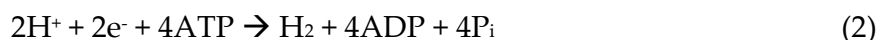


**Figure 1.** Photofermentative hydrogen production

The process is mainly mediated by nitrogenase enzyme, which catalyzes the reduction of  $N_2$  to  $NH_3$ . Hydrogen production is an inherent activity of the nitrogenase enzyme, which forms 1 mole of  $H_2$  per mole of  $N_2$  fixed.



However, under limited nitrogen source, the enzyme functions as hydrogenase and catalyzes the reduction of protons to form molecular hydrogen with the expense of 4 moles of ATP.



Hence, with the same energy requirement, 4 times more hydrogen can be produced under nitrogen-limiting conditions. There is also membrane-bound  $H_2$ -uptake [NiFe]-hydrogenase, which mainly catalyzes the oxidation of  $H_2$  to protons and electrons by the following reversible reaction:



A wide range of photosynthetic bacteria was reported to produce hydrogen. Among them, PNSB is the most widely studied and well characterized.

Purple non-sulfur bacteria (PNSB) are facultative anoxygenic phototrophs belonging to the class of *Alphaproteobacteria* and include several genera within order of *Rhodobacterales*, *Rhodospiralles* and *Rhizobiales* [3]. They are a diverse group of photosynthetic microorganisms that are capable of photobiological hydrogen production under anaerobic, nitrogen limiting conditions. Various species of PNSB were utilized in hydrogen production studies, *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, *Rhodoseudomonas palustris* and *Rhodospirillum rubrum* being the most famous strains. They prefer photoheterotrophic growth in the presence of an organic carbon source, preferentially, small organic acids. Photoheterotrophic growth is the only growth mode that results in hydrogen production, however, PNSB are capable of growth under photoautotrophic, respiratory, fermentative or chemotrophic conditions, depending on the presence of light, type of carbon source and availability of  $O_2$  (Table 2).

Growth Mode	C-source	Energy source	Notes
Photoheterotrophy	Organic carbon	Light	Only mode that results in H <sub>2</sub> production
Photoautotrophy	CO <sub>2</sub>	Light	CO <sub>2</sub> fixation occurs. H <sub>2</sub> is used as electron donor
Aerobic respiration	Organic carbon	Organic carbon	O <sub>2</sub> is the terminal electron acceptor
Anaerobic respiration/chemoheterotrophy	Organic carbon	Organic carbon	Requires a terminal electron acceptor other than O <sub>2</sub> (N <sub>2</sub> , H <sub>2</sub> S or H <sub>2</sub> )
Fermentation/anaerobic, dark	Organic carbon	Organic carbon	

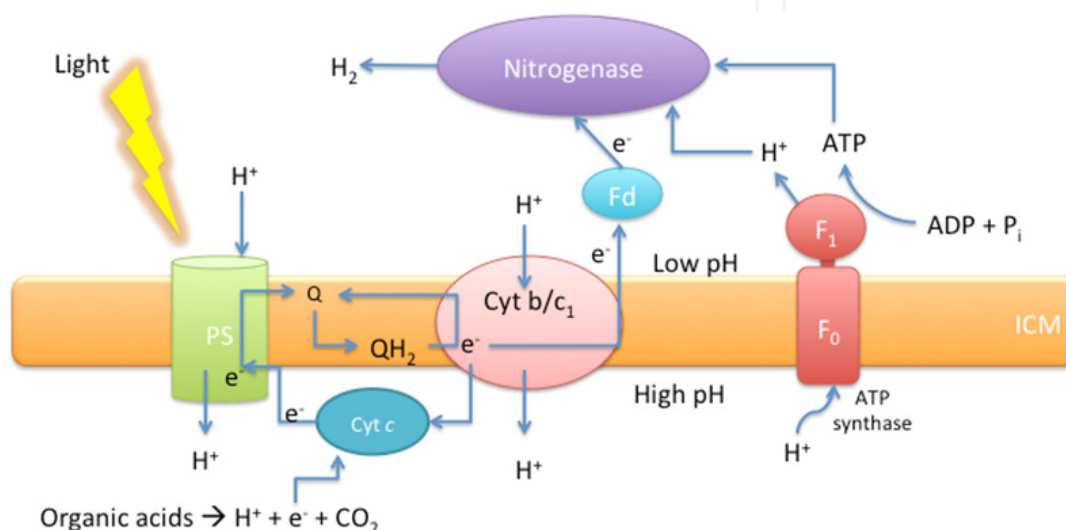
**Table 2.** Various growth modes of PNSB [4].

This versatility of growth modes attracted research interest for many years, and made PNSB a model organism to study metabolic regulations of carbon, nitrogen and energy metabolism. There are three important external factors that determine the metabolic route: the carbon source, light and O<sub>2</sub> availability. PNSB are capable of growth on a variety of organic carbon sources including sugars (glucose, sucrose), short chain organic acids (acetate, malate, succinate, fumarate, formate, butyrate, propionate, lactate), amino acids, alcohols and even polyphenols. They also grow on inorganic carbon (CO<sub>2</sub>) under photoautotrophy and chemoautotrophy. Under photoheterotrophic hydrogen production conditions, these bacteria preferentially use short chain organic acids as electron donors to obtain ATP for their metabolic processes. Short chain organic acids are assimilated through tricarboxylic acid cycle, which yields CO<sub>2</sub>, protons and electrons, which are shuttled through electron transport chain that uses NAD/NADH and ferredoxin.

Photosynthetic apparatus in PNSB is located in the intracytoplasmic membranes, the invaginations of cytoplasmic membrane forming a parallel lamella underlying the cytoplasmic membrane. It is composed of a photosystem, a series of electron transport proteins (cytoplasmic cytochrome *c*, lipid soluble quinones (Q/QH), cytochrome *b*/C<sub>1</sub> complex, and) and a transmembrane ATP synthase protein. The photosystem contains light harvesting complex 1 (LH1) and 2 (LH2) and a reaction center [5]. The LH complexes trap light in the visible (450-590 nm) and near infrared (800-875 nm) wavelength and transfer the excitation energy to the reaction center, and starts cyclic electron transfer. LH1, LH2 and reaction center are protein-pigment complexes that contain different types of carotenoids and bacteriochlorophyll *a*. Biosynthesis of photosynthetic apparatus is primarily controlled by the presence of O<sub>2</sub> and light [6,7,8]. During aerobic growth, the synthesis of bacteriochlorophyll is repressed. Once the O<sub>2</sub> tension is removed, the synthesis resumes. Light intensity and quality also controls the synthesis of photosynthetic apparatus. Under low light intensity, photosystem biosynthesis increases to gather more light energy, and at high light intensity, less photosystem is biosynthesized.



The photosystem of PNSB is not powerful enough to split water; hence, no  $O_2$  evolves, which makes it very suitable for biohydrogen production. Electrons that are liberated through oxidation of organic carbon are funneled through a series of electron carriers, during which protons are pumped through the membrane. This leads to a development of a proton gradient across the membrane, which drives ATP production by ATP synthase. The electrons are either used for replenishment of quinone pool or donated to Ferredoxin, which delivers electrons to nitrogenase enzyme to reduce molecular nitrogen to ammonia. When molecular nitrogen is not available, nitrogenase functions as hydrogenase and catalyzes the proton reduction with the electrons derived from ferredoxin (Figure 2). By this way, electrons from organic compounds are stored in the form of  $H_2$  by using light energy.



**Figure 2.** Photofermentative hydrogen production in PNSB. Oxidation of organic acids generates electrons, which are delivered to cytochrome *c* and travels through number electron transport proteins and delivered to ferredoxin. During this process, protons are pumped through the membranes forming a proton gradient. This proton motive force derives ATP production by ATP synthase. Ferredoxin delivers electrons to nitrogenase, which catalyzes the reduction of protons to molecular hydrogen using ATP.

The synthesized ATP is primarily used for biomass production. In order to produce  $H_2$ , ATP flux to the cell should surpass the amount of ATP necessary for growth. Bacteria produce hydrogen when there is an excess of reducing powers to maintain cellular redox balance. There are mainly three metabolic pathways that compete for electrons:  $CO_2$  fixation,  $N_2$  fixation/ $H_2$  production and polyhydroxybutyrate (PHB) biosynthesis. PNSB use  $CO_2$  as electron sink under photoheterotrophic conditions to get rid of excess reducing equivalents and maintain redox homeostasis. It uses Calvin-Benson-Bassham (CBB) pathway to fix  $CO_2$  at the expense of ATP and NADH. The primary function of CBB pathway is to provide carbon for the cell under photoautotrophic growth on  $CO_2$ . However, under photoheterotrophic growth on organic carbon, it mainly functions for redox balancing [3,9,10]. The regulatory enzyme of the  $CO_2$  fixation is ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) which catalyzes the conversion of RuBP (ribulose-1,5-bisphosphate) into glyceraldehyde-3-phosphate. Genes involved in  $CO_2$  fixation is located in *cbb* operon that is transcriptionally regulated by CbbR [11].

Another electron sink is the molecular nitrogen ( $N_2$ ), which is fixed to  $NH_3$  by nitrogenase enzyme at the expense of 16 moles of ATP. The primary function of nitrogenase is to fix  $N_2$  to ammonia when the cells are grown on ammonia-free environment. However, nitrogenase also functions in redox balancing. Hydrogen production is an inherent activity of nitrogenase enzyme, which produces 1 mole of  $H_2$  for 1 mole of  $N_2$  fixed. When  $N_2$  is not present, the enzyme functions as hydrogenase and catalyzes the proton reduction to form molecular hydrogen ( $H_2$ ), with 4 times higher efficiency (4 ATP is utilized per mole of  $H_2$  produced in the absence of  $N_2$ ). This energy intensive process is under tight metabolic control that is regulated mainly by cellular nitrogen status. As discussed below, nitrogenase activity is tightly regulated by several environmental factors, including ammonia,  $O_2$ , and light. For this reason, hydrogen production studies are carried out under anaerobic, nitrogen-limited conditions in the presence of light.

Polyhydroxyalkanoates (PHAs) are the polymers of hydroxyalkanoates, which are accumulated as an energy storage material usually under the condition of limiting nutritional elements such as N, P, S, O, or Mg in the presence of excess carbon source [12]. Polyhydroxybutyrate (PHB) is the best-known representative. PHB synthesis and expenditure are closely connected with the energy requirements of the cell. Batch cultures of *R. palustris* growing photoheterotrophically on acetate with varying nitrogen sources and regimens of nitrogen supplementation, demonstrated that some competition for reducing equivalents exists between nitrogenase activity and PHB biosynthetic pathway [13]. Acetyl-CoA is the substrate for PHB biosynthesis in PNSB, hence, in cultures grown on acetate higher PHB accumulation was reported [14]. In *R. rubrum*, it is reported that S-deprivation caused inhibition of nitrogenase activity, including  $N_2$  fixation and  $H_2$  production, and a concomitant enhancement in PHB accumulation [15].

### 3.1. Enzymes involved

**Nitrogenase:** Nitrogenase is a metalloprotein complex that catalyzes the reaction of biological nitrogen fixation. At least three genetically distinct nitrogenase systems have been confirmed in PNSB, namely Nif, Vnf, and Anf, in which the active-site central metals are Mo, V, and Fe, respectively [16]. In general, the nitrogenase enzyme is composed of two oxygen labile and separable metalloproteins, dinitrogenase (component I; MoFe protein, VFe protein, FeFe protein) and dinitrogenase reductase (component II; Fe protein). Component I contain the active site for  $N_2$  reduction, with a molecular weight of approximately 240 kDa and is composed of two heterodimers. Component II is a 60–70 kDa homodimer coupling ATP hydrolysis to inter-protein electron transfer. Mo-nitrogenase, coded by *nifHDK* genes, is the most widely distributed nitrogenase in PNSB, but many PNSB also contain alternative forms of nitrogenases. In *R. sphaeroides*, only Mo-nitrogenase is found, but in *R. capsulatus* and *R. rubrum* there is also Fe-only nitrogenase [17; 18]. *R. palustris* is known to contain all three forms of nitrogenases [19]. Alternative nitrogenases have been proposed to serve as a route for nitrogen fixation in situations where molybdenum is limited in the environment.



Due to highly endothermic nature of nitrogen fixation, bacteria developed a tight control of nitrogenase at both transcriptional and posttranslational level [20]. Availability of ammonium and cellular nitrogen status is the primary regulator of the nitrogenase synthesis and activity. Presence of high concentrations of ammonia inhibits nitrogenase activity and represses the expression of nitrogenase structural at transcriptional level in *R. sphaeroides* [21]. In addition, N<sub>2</sub> fixation is controlled by environmental factors, molybdenum, light, and oxygen.

Three levels of regulation in response to ammonium availability are proposed in *R. capsulatus* (i) transcriptional activation of the regulatory genes *nifA1*, *nifA2* and *anfA*, (ii) posttranslational regulation of NifA and AnfA activity, and (iii) post-translational control of nitrogenase activity by reversible modification of NifH and AnfA. A diversity of regulatory proteins are involved in control of nitrogen fixation. Among these are two-component regulatory systems (NtrB/NtrC, RegB/RegA), two signal transduction proteins (GlnB, GlnK), three specific transcriptional activator proteins (NifA1, NifA2, AnfA), two molybdate-dependent repressor proteins (MopA, MopB), an ADP-ribose transferase/glycohydrolase system (DraT, DraG), two (methyl)-ammonium transporter proteins (AmtB, AmtY), and a histone-like protein (HvrA) [20]. Nitrogen-fixing bacteria have been shown to regulate nitrogenase in the short term by post-translational covalent modification via reversible ADP-ribosylation of the Fe protein in response to different environmental stimuli: ammonium addition, darkness, and the absence of oxygen. This process is catalyzed by two non-*nif*-specific enzymes: dinitrogenase reductase ADP-ribosyltransferase (DRAT) and dinitrogenase reductase-activating glycohydrolase (DRAG). Another post-translational regulation that does not involve ADP-ribosylation was also proposed in *R. capsulatus* [22,23]. Due to the modulation of nitrogenase activity according to cellular nitrogen status, hydrogen production studies on PNSB are carried out on media with limited nitrogen source. Regulation of nitrogenase activity by posttranslational ADP-ribosylation has been shown to occur in response to light and O<sub>2</sub> status, as well. Rapid inhibition of nitrogenase activity by O<sub>2</sub> through ADP-ribosylation was reported in *R. capsulatus* [24].

Hydrogen production is an inherent activity of nitrogenase enzyme, producing one mole of H<sub>2</sub> per mole of N<sub>2</sub> fixed, at the expense of 16 moles of ATP. In the absence of nitrogenase, the enzyme acts as ATP-dependent hydrogenase and catalyzes the proton reduction for molecular hydrogen at the expense of 4 ATP. Nitrogenase mediated hydrogen production in PNSB also plays a role in maintaining cellular redox status.

**Hydrogenase:** Hydrogenases, the key enzymes of hydrogen metabolism, are metalloenzymes that catalyze either the oxidation of H<sub>2</sub> to form protons and reducing equivalents or, the reduction of protons to form molecular hydrogen. There are three distinct types of hydrogenases classified according the type of metal cofactor present in the active site: [FeFe]-hydrogenases, [NiFe] hydrogenases, and [Fe]-hydrogenase, the first two are divided into a variety of sub-types depending on the structures and functions in the cell [25]. [NiFe]-hydrogenases tend to be involved in H<sub>2</sub> consumption, while [FeFe]-hydrogenases are usually involved in H<sub>2</sub> production [1].

[FeFe]-hydrogenases are generally monomeric and consist of a catalytic subunit of ca. 45-48 kDa, which bidirectionally catalyze  $H_2$  production [25]. The direction of the reaction is determined by the redox status of the cell. They are found in green algae and anaerobic prokaryotes, and characterized by high catalytic activity (turnover rates: 6000-9000  $s^{-1}$ ) [1]. However, they are particularly sensitive to  $O_2$ , which causes irreversible inactivation of the enzyme. Hence, anaerobiosis is a prerequisite for algal hydrogen production. PNSB bacteria do not contain [FeFe]-hydrogenase except *R. palustris*, which was reported to possess a [FeFe]-hydrogenase [26].

[NiFe]-hydrogenases are heterodimeric enzymes consisting of a large subunit ( $\alpha$ -subunit) of ca. 60 kDa hosting the bimetallic active site and the small subunit ( $\beta$ -subunit) of ca. 30 kDa hosting the Fe-S clusters. [NiFe]-hydrogenases are present in cyanobacteria and PNSB. Membrane-bound uptake hydrogenases (i.e., HupSL and hynSL), hydrogen sensors (HupUV), NADP-reducing (HydDA), bidirectional NADP/NAD-reducing (hoxYH) and energy-converting membrane-associated  $H_2$ -evolving hydrogenases are the subgroups of [NiFe]-hydrogenases. Cyanobacterial  $H_2$ -uptake hydrogenases are cytoplasmic that are induced under  $N_2$ -fixing conditions. Cyanobacteria also contain bidirectional NAD(P)-linked [NiFe]-hydrogenase, which is responsible for catalyzing  $H_2$  photoproduction in the absence of a functional nitrogenase. The bidirectional enzyme probably plays a role in fermentation and/or acts as an electron valve during photosynthesis [1,25,27].

Most PNSB harbor membrane-bound [NiFe]-hydrogenase, also called  $H_2$  uptake hydrogenase (Hup) that catalyzes the oxidation of  $H_2$  to protons and electrons. They are connected to the quinone pool of the respiratory chain in the membrane by a third subunit, which anchors the hydrogenase dimer to the membrane. Unlike those in cyanobacteria,  $H_2$  uptake hydrogenases in PNSB are characterized by the presence of a long signal peptide at the N terminus of their small subunit. It serves as signal recognition to target the fully folded heterodimer to the membrane and the periplasm. In some PNSB (*R. capsulatus* and *R. palustris*) a cytoplasmic [NiFe]-hydrogenase is also present, which functions as  $H_2$  sensors of the cell and trigger a cascade of cellular reactions controlling the synthesis of hydrogenases. In *R. rubrum*, there is also a CO-induced [NiFe]-hydrogenase (CooLH), which, together with CO-dehydrogenase, oxidizes CO to  $CO_2$  with concomitant production of  $H_2$ . This allows *R. rubrum* to grow in the dark with CO as the sole energy source.

## 4. Photofermentation in outdoor conditions

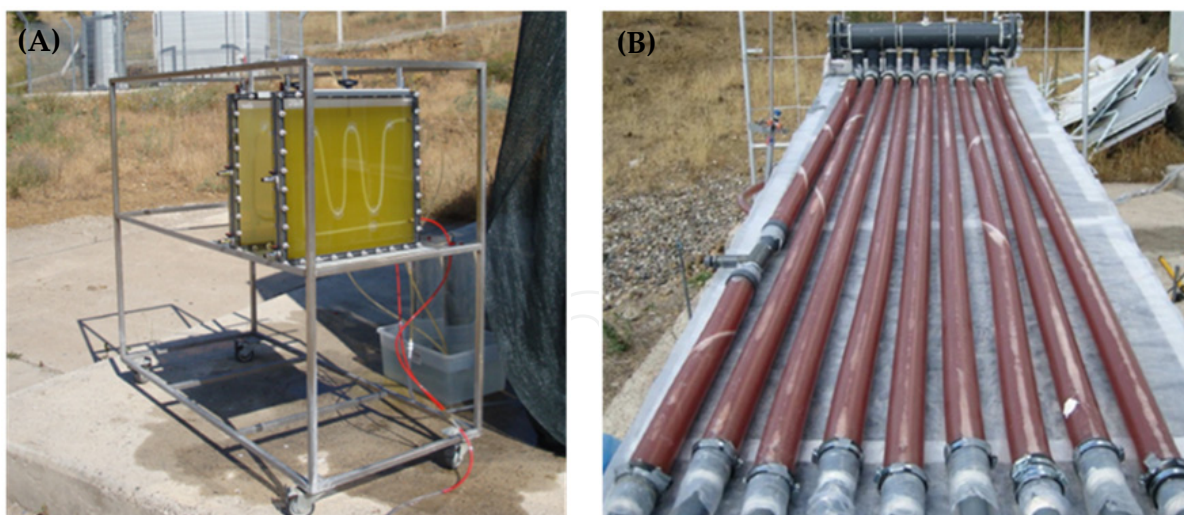
### 4.1. Photobioreactors

Photobioreactors (PBRs) are systems designed to grow photosynthetic microorganisms under a given environmental condition [28]. They can be classified as open (raceway ponds, lagoons and lakes) or closed (flat plate, tubular) systems. Open systems are mostly suited to biomass production since they cannot provide the anaerobic conditions required for hydrogen production. Also, control of parameters like temperature, nutrients and pH is poor in such systems. On the other hand, closed systems allow better control of these parameters and result in higher biomass production and biohydrogen production [29].

Different types of PBRs are used in photofermentative hydrogen production studies. They are generally classified according to their: (i) Design - flat or tubular, horizontal, inclined, vertical or spiral and manifold or serpentine [30] (ii) Mode of operation batch, fed-batch and continuous [29]. In order to achieve sustainable photofermentative hydrogen production in outdoor conditions, the development of an optimized photobioreactor system that has the following properties is targeted: (i) a simply designed enclosed system that is impermeable to hydrogen (ii) a transparent system that allows maximum light penetration, preferably at high visible light or near red-infrared transmissions (iii) a system with high surface-to-volume ratio for better/wide distribution of light (iv) a system made from an unreactive material that is durable, easy to clean and sterilize [29,31-33]. Flat plate and tubular types of PBRs are commonly used in photofermentative hydrogen production (Figure 3). This is probably due to their high efficiencies brought about by their high illumination areas.

Flat-plate (panel) PBRs consist of frames placed in between two transparent rectangular plates (PMMA or glass plates). They generally have a depth of 1-5 cm and vary in height and width (smaller than 1 m in practice) [31]. These conventional panel PBRs are considered as the first generation plate-type bioreactors. The second generation is a flat panel airlift PBR made up of two deep-drawn plates glued together while the third generation comprises deep-drawn plates fused together under pressure and heat [29]. Panel PBRs have a short light path and facilitate the measurement of irradiance at the culture surface [28,3,34]. They can be placed vertically or tilted at optimal angles for maximum exposure to direct sunlight [35-37] and can be arranged in stacks close to each other, therefore providing a large illuminated area in a small ground area [38]. However, a drawback of this system is the lack of mixing. Mixing by aeration [31] or agitation via rocking motion [39] is suggested, but an impediment to these techniques is their high power consumption for pumping gas and shaking the photobioreactor. Also, mixing via aeration would lead to dilution of the gas produced and incur extra costs for gas separation. Recirculation of the evolved gas has been proposed [34], however, the PNSB have hydrogenase enzyme that can breakdown the produced hydrogen to protons and electrons, thus reducing the amount of gas produced. Moreover, reduction of hydrogen partial pressure by decreasing total gas pressure in the PBR headspace was shown to improve hydrogen production [40].

Tubular PBRs are made of long transparent tubes through which liquid culture is circulated using mechanical or gas-lift pumps. The tubes have diameters ranging between 3 and 6 cm and length between 10 and 100 m [31]. The PBRs fall under different categories: simple airlift or agitated bubble column (vertical type) [41-44], horizontal or nearly horizontal tubular PBRs [28,38,45] and helical type PBRs [46-48]. Tubular PBRs can be scaled-up by connecting a number of tubes to manifolds, but the length of the tubes is limited by the accumulation of gas [31]. A disadvantage of these PBRs is that they require large ground area. In comparing the hydrogen production performance of the panel and tubular PBRs, Gebicki et al. [39] reported that the ratio of the illuminated reactor surface to the installed ground area was 8:1 in the panel PBR, while that in the tubular PBR was 1:1.



**Figure 3.** (A) Flat plate and (B) tubular photobioreactors.

## 4.2. Parameters affecting bacterial growth and hydrogen production in outdoor conditions

The ultimate goal of photobiological hydrogen production is to carry out the process in large scale PBRs operated at outdoor conditions, under natural sunlight. Solar light energy is warranted because it is a free resource that is abundant in nature. The earth receives about  $5.7 \times 10^{24}$  J of solar light energy per annum [49]. Its usage for photofermentative hydrogen production not only saves on operating costs but also supports the concept of sustainable hydrogen production from renewable resources. It promotes waste reduction and recycling [50].

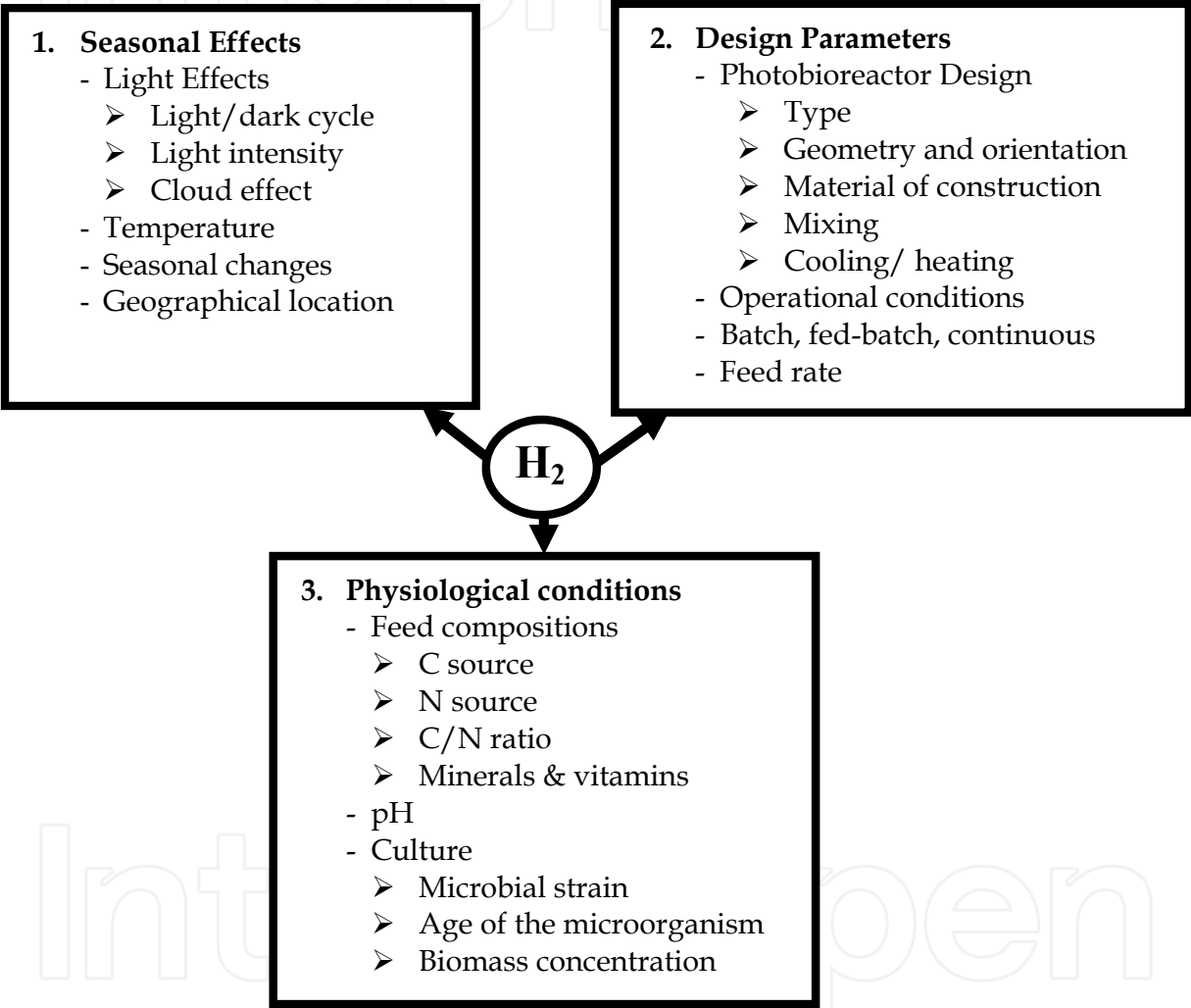
Photofermentative hydrogen production in outdoors is affected by several conditions. The major parameters being physical variations like solar light energy and temperature, which are uncontrolled. These parameters regulate photosynthetic bacterial activity, therefore their daily (day/night cycle), seasonal and geographical variation greatly influence the amount of hydrogen produced [38,45,51-55]. In addition, other parameters such as PBR type, mode of operation, nutrients, carbon to nitrogen ratio, type and age of the microorganism are critical. Shown in Figure 4 are the general parameters influencing photofermentative hydrogen production.

### 4.2.1. *Effects of the variation in solar light intensity and temperature on biomass and hydrogen production*

The changing intensities of solar light energy and temperatures experienced in outdoor conditions greatly influence PNSB growth and hydrogen production. These enzymatic processes rely on chemical bond energy (ATP) [25] generated by the conversion of absorbed light energy to ATP as discussed in Section 3. This energy is utilized in different basic cellular metabolic activities such as biomass formation, biomass maintenance, hydrogen production and the excess is dissipated as heat [4,56]. Indoor studies have demonstrated



that biomass increased with increasing light intensity [57] and temperature [58,59]. He et al. [58] investigated the growth and hydrogen production of two mutants of *Rhodobacter capsulatus* (JP91 and IR3) at different temperatures (26, 30 and 34 °C). They reported good cell growth and high substrate conversion efficiencies of 52.7% and 68.2% at 30°C for JP91 and IR3 strains, respectively. Likewise, in batch experiments using different light intensities (1.5-5 klux) and temperatures (20°C, 30°C and 38°C), Sevinç et al. [59] reported optimum light intensity for growth and hydrogen production to be 5 klux. The cell growth of the PNSB was found to fit the logistic model [4,58-60].



**Figure 4.** Parameters affecting photofermentative hydrogen production.

In outdoor operations, cell growth and hydrogen production were indicated to be dependent on the solar light intensity received [45,49,51-54,61]. Temperature and the intensity of solar radiation vary daily, seasonally and geographically. Northern Europe experiences lower temperatures and light intensities compared to Southern Europe; solar light intensities of up to 850-950 W/m<sup>2</sup> are reported to be common for most parts of Europe (40°-55°N) during summer [62]. The Mediterranean region, for example Turkey which lies between 36°-42°N latitudes receives abundant solar radiation (circa 3.6 kWh/m<sup>2</sup> day) [63].

Daily and seasonal variations in light intensity affect growth of PNSB. Eroglu et al. [51] observed that *R. sphaeroides* cell concentrations increased during the day but either remained the same or decreased at night. While investigating continuous hydrogen production using a fed-batch 90 L pilot tubular photobioreactor in outdoor conditions, Boran et al. [64] found that the specific growth rate of *R. capsulatus* increased exponentially with the total light intensity. Moreover, the growth rate of *R. capsulatus* YO3 (Hup<sup>-</sup>) was found to be lower ( $0.0042\text{ h}^{-1}$ ) during winter (low light intensity and temperatures) compared to that during summer (high light intensity and high temperature) ( $0.035\text{ h}^{-1}$ ) [53].

Varying light intensities also influences the amount of hydrogen produced by the PNSB. Wakayama et al. [65] observed that the levels of hydrogen produced by batch cultures of *R. sphaeroides* depended on the irradiation intensity of sunlight. During the experiments, the light intensity and total irradiation (ranging between 6 to 7 kWh/m<sup>2</sup>) fluctuated on a regular basis (up to 60%). Maximum hydrogen production rate of 4.0 L/m<sup>2</sup>/h and light conversion efficiency of 2.2% was obtained. In another continuous hydrogen production study using an 8 L flat plate PBR, Androga et al. [53] observed that the daily amount of hydrogen produced decreased with the decreasing daily total global solar radiation (from 4000 Wh/m<sup>2</sup> to 2350 Wh/m<sup>2</sup>). Avcioglu et al. [54] reported similar results using continuous cultures of *R. capsulatus*, fed with molasses dark fermenter effluent. Hydrogen yield factor was reported to linearly increase with increasing solar radiation [53,66].

#### 4.2.2. Effects of light/dark cycle

Diurnal light/dark cycle adversely affects photofermentative hydrogen production [52,62,67]. During winter, shorter daylight periods (circa. 9 h) and shorter night periods are experienced while during summer, longer daylight periods (circa. 14 h) and shorter night periods are experienced [53]. Excessive light energy leads to photo-inhibition, which in turn reduces hydrogen production efficiency [62,67,68]. Sunlight intensity of over 1.0 kW/m<sup>2</sup> was found to be deterrent to hydrogen production [69]. Studies on light/dark cycle have demonstrated that little or no hydrogen was produced during the dark periods but bacteria survived and hydrogen production recovered once illumination resumed [4,40].

Wakayama et al. [67] observed that short intermittent light/dark cycles increased hydrogen production efficiency while longer intermittent periods reduced it. In experiments carried out under excessive light energy (1.2 kW/m<sup>2</sup>), they found that a 30-min light/dark cycle improved efficiency to 150%, while a 12-h cycle reduced it to 73%, in comparison to the reference of continuous illumination. A 12-h light/12-h dark diurnal cycle yielded the same amount of biomass and volume of hydrogen as continuously illuminated bioreactors in batch studies using olive mill waste water, however, it resulted in a longer lag in biomass and hydrogen accumulation [70]. Under continuous illumination, maximum hydrogen production rate and substrate conversion efficiency of 92.41 ml H<sub>2</sub>/L/h and 90.54% were obtained respectively. These values decreased to 89.96 ml H<sub>2</sub>/L/h and 85.35% under 12-h light/12-h dark cycle and 86.91 ml H<sub>2</sub>/L/h and 80.97% under 12-h dark/12-h light cycle, respectively [40]. Similar results were reported by Uyar et al. [62], who found that the



average hydrogen production rate and the total hydrogen produced by *R. sphaeroides* cells exposed to light/dark cycles were lower compared to the continuously illuminated cultures. This could be attributed to lack of light energy to produce ATP (that is needed for hydrogen production) during the dark period [4]. Also, the metabolism of the bacterial cells may change to adapt to non-light conditions (fermentation) as observed by Eroglu et al. [51]. They reported that under limited sunlight intensity (<10 klux), no hydrogen production occurred, instead *R. sphaeroides* cells fed with malate performed fermentation, producing formate as the end product.

Fluctuating day and night temperatures have also been reported to significantly affect hydrogen production. In investigating the effect of temperature cycles and temperature plus light/dark cycle conditions on hydrogen production using batch cultures of *R. capsulatus* YO3 (Hup<sup>-</sup>) grown outdoors, Özgür et al. [52] observed significantly lower substrate conversion efficiencies, yields and hydrogen productivities. The maximum hydrogen productivity and yield was obtained at reactor temperatures of 33°C. Light/dark cycle was reported to cause a further 50% decrease in hydrogen productivity.

### 4.3. Process technology for photobioreactors operation in outdoor conditions

#### 4.3.1. Mode of operation: Batch, continuous and fed-batch systems

Photobioreactors used in biological hydrogen production can be classified depending on their mode of operation as batch, continuous and fed-batch. Batch PBRs have no flow of material in or out of the bioreactor and the reactions are time dependent. Continuous PBRs have both inflow and outflows (at the same time during operation) and operate at steady state (time independent). Fed-batch PBRs have either an inflow or outflow of material and the reactions are time dependent [33,71]. The mode of operation influences the growth of the microorganisms and hydrogen production rate and yield.

Batch PBRs are the most widely used bioreactors in photofermentative hydrogen production studies given in literature. The bacterial cells are left to grow, consuming the initially fed media, generating and accumulating products in a given time period. These systems are easy to operate, flexible and can be adapted to investigate various parameters. Most studies using batch reactors are carried out in small scale in the laboratory. However, they are usually liable to substrate and product inhibitions, , therefore resulting in low hydrogen production rates and yields [71]. There are a few outdoor batch studies reported in literature. Eroglu et al. [51] used a 6.5 L (working volume) temperature controlled flat plate solar photobioreactor to cultivate *R. sphaeroides* O.U 001 cells in batch mode using malate, lactate, acetate and olive mill waste water. They obtained the highest hydrogen production rate of 10 ml H<sub>2</sub>/L/h using malate. Özgür et al. [53] investigated the effects of temperature fluctuations and day/night cycles on hydrogen production using *R. capsulatus* (a wild type and YO3 (Hup<sup>-</sup>) strain) grown in 550 ml glass bottle PBRs operated in batch mode under outdoor conditions. They found that temperature oscillations and day/night cycles greatly reduced hydrogen production in both strains and the YO3 (Hup<sup>-</sup>) strain performed better than the wild type.

PNSB have been shown to be able to produce hydrogen in the absence of growth. This avails the possibility of developing continuous photobioreactor systems that can produce hydrogen in long-term. Continuous systems operating under steady state conditions can generate products at constant rate, yield and quality, therefore are more advantageous than batch systems [71]. They may require less maintenance but long-term operation may cause contamination in bioreactors [33,72] and maintaining a stable cell concentration is challenging as the bacteria's cell growth and hydrogen production capability is highly susceptible to environmental (especially in outdoor conditions) and medium composition changes [32,53,73]. The hydraulic retention time (HRT) which is the average amount of time that a soluble compound spends in the reactor, is an important factor influencing the biomass, hydrogen production rate, hydrogen yield, and light conversion efficiency. Continuous PBRs can be operated as suspended cell processes or immobilized cell reactors. Suspended systems are prone to washouts and product inhibition, which could be overcome by cell recycle. Immobilized systems offer the advantages of cell longevity and have been shown to produce hydrogen at higher rates and yields [74,75]. Studies using different HRT values indicate that longer HRT is more suitable for photofermentation as the PNSB utilized the fed organic acids slowly [34,76-78]. It is postulated, especially for continuous systems, that HRT should be long enough to curtail cell growth so as to direct metabolic activity towards hydrogen production [33]. Chen et al. [99] carried out continuous fermentation at 96 h HRT employing *Rhodopseudomonas palustris* WP3-5, fed with dark fermenter effluent. The continuous culture ran stably for 10 days and produced an average yield of 10.21 mol H<sub>2</sub>/mol sucrose. There are limited numbers of outdoor photofermentative hydrogen production studies using continuous systems reported in literature [80,81]. Most of the ones described were carried out in indoor conditions [34,46,56,82,83].

Fed-batch systems are one of the most promising processes for cell growth and metabolite production. They offer the prospects of having high cell densities and the feed flow rates and composition can be adjusted to control the reaction rates [84]. The substrates are added at controlled levels that adequately support cell growth, therefore alleviating substrate or product inhibition [71]. Repeated fed-batch operations, whereby products and parts of the settled bacteria are removed and replaced with fresh media (semi-continuous operation) to adjust the cell age and concentration of microorganisms, have been shown to be viable for hydrogen production [85]. Comparisons based on the effects of operation modes on biomass, substrate and product concentrations reveal fed-batch operation to be more promising for attaining high hydrogen rates and yields [71,79,85]. Controlled feeding and sequential product removal in fed-batch systems facilitate continuous photofermentative hydrogen production and allow feed media optimization. There are several outdoor fed-batch studies reported in literature [38,45,53-55,64,66,86].

#### 4.3.2. Photobioreactor positioning

The performance of PBRs is highly dependent on the amount of sunlight received, therefore the location and orientation of the PBRs in outdoors is critical. An East-West positioning of

PBRs has been indicated to be the most suitable for receiving maximum sunlight energy and utilization [35,38,49,51,87]. PBRs facing East-West position were shown to receive higher amount of irradiance than South-North facing PBRs [87] and better utilization of long wavelength (red and infrared) that are prevalent in the mornings and evenings [72]. Orientation of the PBRs with inclinations of about 90° or less were demonstrated to be more suitable for cell growth and hydrogen production [35,38,49] than their counterparts without inclination. With inclination of flat plate PBRs towards the sun, major sunlight was received on the inclined surface of the PBR and the backside surface of the PBR was illuminated by diffuse and reflected light that may be good for photosynthesis. Vertical flat PBRs were suggested to be placed in a 30° and 60° inclinations for summer and winter operation [35]. In investigating methods of illumination to simulate the daily sunlight irradiation pattern, a Roux flask PBR with an irradiation area of 159 cm<sup>2</sup>, working volume of 700 cm<sup>3</sup> and a light path of 4.5 cm was set at an angle of 30° from the horizontal. A solar tracking device was used to reposition the PBR to receive maximum sunlight every 30 minutes and maximum hydrogen productivity and maximum light conversion efficiency of 2.8 L/m<sup>2</sup>/h and 1.5% were obtained, respectively. Hydrogen production experiments using batch cultures of *R.sphaeroides* fed with different carbon sources (malate, lactate, and acetate and olive mill waste water) were carried out in flat plate PBR (8 L) inclined at 30°. Inclinations of 92°–93° were observed to provide the highest internal circulation and gas separation with a vertically plate photobioreactor [38]. In tubular PBRs, slight inclination (circa 10°) and pumping were applied to assist in gas separation [28,38,45]. Stacking of the flat plate PBRs next to each other provides a large illuminated area under small ground area. The packed arrangement caused a lamination effect where solar irradiance at the surface of the PBR was diluted therefore greatly improving the efficiency of conversion of solar radiance to biomass [88,89]. Temperature distribution experiments demonstrated that a 15 cm gap between the flat panel PBRs was optimal for hydrogen production. With the 15 cm spacing, the maximum temperature of the culture remained close to the physiological limit (31°C) at the occurrence of the highest daytime temperature [38].

#### 4.3.3. Light distribution

Activity of the photosynthetic bacteria is dependent on the light distribution within the photobioreactor. Parameters such as the light path length [90,91], biomass concentration [92] and type and composition of the feed media [54,64] affect light distribution in the PBRs. Light intensity has been described to decay exponentially with the culture depth, following the Lambert-Beer law [38,93,94]. Nakada et al. [90] investigated the light penetration into a photobioreactor and its effect on hydrogen production using an A-type four-compartment bioreactor. They observed that 69% of the incident light energy was absorbed in the first compartment (0-5 mm) and 21% was absorbed in the second compartment (5-10 mm). Similarly, cells in the deeper parts of the bioreactor were demonstrated to be poorly illuminated as much of the incident light energy was absorbed as 69%, 21%, 7%, 2% in the first, second, third and fourth compartments of a 20 mm bioreactor [49]. The reduction of a flat panel bioreactor depth from 4 cm to 2 cm led to an increase in the overall light

conversion efficiency from 0.13% to 0.53% [95]. Evaluations on the effect of bioreactor light path using a flat panel photobioreactor revealed that the highest hydrogen productivity was obtained at 20 mm depth with a mean biomass content of 0.7 g/L and illumination provided from both sides of the photobioreactor [38]. In comparing the percent penetration of light intensity in a photobioreactor, Boran et al. [96] observed that for each 1 cm of depth there was a decrease of 89 % for artificial medium, 70 % for thick juice DFE and 51 % for molasses dark fermenter effluent. Having a large tube diameter (6 cm) was concluded to have led to the decrease in light penetration in the tubular photobioreactor, thus decreasing hydrogen productivity. Decreasing the tube diameter to below 6 cm and increasing the wall thickness to prevent hydrogen loss was suggested to improve photofermentative hydrogen production in the pilot scale 90 L tubular photobioreactor [64].

Utilization of light energy by the bacterial cells is evaluated by the light conversion efficiency, which is the ratio of the total energy value of the hydrogen produced to the total energy input to the photobioreactor by light radiation as shown in Equation 4 [62].

$$\begin{aligned}\eta (\%) &= (\text{Amount of H}_2 \text{ produced} \times \text{H}_2 \text{ energy content}) / \text{Light energy input} \times 100 \\ &= (33.61 \times Q_{\text{H}_2} \times V_{\text{H}_2}) / (I \times A \times t) \times 100\end{aligned}\quad (4)$$

where 33.61 is the energy density of hydrogen gas in W.h/g,  $Q_{\text{H}_2}$  is the density of the produced hydrogen gas in g/L,  $V_{\text{H}_2}$  is the volume of hydrogen gas produced in L,  $I$  is the light intensity in W/m<sup>2</sup>,  $A$  is the irradiated area in m<sup>2</sup>, and  $t$  is the duration of hydrogen production in hours.

Low light conversion efficiencies of 0.5 to 6% for solar and tungsten lamps, are considered to be the bottleneck in the scale-up of photofermentative hydrogen production systems. Generally, higher light conversion efficiencies were obtained at low light intensities [56]. Light conversion efficiency reduced from 1.11% to 0.25% as the light intensity increased from 88 to 405 W/m<sup>2</sup> [62]. In outdoor experiments using cultures of *R.sphaeroides* 8703, the solar light conversion efficiency was observed to decrease from 7% at low sunlight intensities (100 W/m<sup>2</sup>) to 2% at high light intensities (1000 W/m<sup>2</sup>). Average solar light conversion efficiencies of about 1% were obtained in photofermentative experiments carried out under natural sunlight [45,49].

Self-shading effect brought about by high biomass concentration is another important parameter affecting light distribution within the photobioreactor. Bacterial cells close to the illuminated surface prevent light from penetrating the PBR, therefore blocking the inner cells from receiving enough light energy. This reduces the PBR performance as it hinders cell growth and hydrogen production [92]. High sunlight intensities necessitated quick bacterial growth and higher biomass concentrations compared to low light intensities, which exhibited slow growth rate and lower biomass concentrations [53,54].

The use of supplementary light sources like LED or tungsten lamps at night to necessitate bacterial growth [45], use of optic fibers and solar tracking device to provide internal illumination at night and on cloudy days [49,79,97-99] and the use of covering material or



optical fibers like Rhodamin B and CuSO<sub>4</sub> solution to reduce light intensity or block specific ranges of light wavelength [62,100,101] have been applied to improve light distribution in PBRs operated in outdoor conditions. Uyar et al. [62] suggested the use of artificial illumination in outdoor PBRs to necessitate growth during the night. Boran et al. [96] provided illumination using two 500 W halogen lamps during night to enable growth of bacterial cells and decrease lag time. Chen et al. [99] developed a solar-energy-excited optical fiber (SEEOF) PBR and observed improved hydrogen production using *Rhodospseudomonas palustris* WP3-5 cultures fed with acetate as sole carbon source. They showed that the provision of radiation using a combination of an optical fiber (excited by solar energy) and tungsten lamp improved hydrogen production and yield by 138% and 136%, respectively. Also combination of the optical fiber system with tungsten lamp and a light dependent resistor that monitored sunlight online and controlled irradiation intensity on the PBR, resulted in 27% increase in hydrogen production and yield. An experiment carried out using *R. sphaeroides* DSM 9483 cultures fed with lactate and grown in a column shaped 1.4 L bioreactor that was operated in outdoor conditions in the Sahara desert demonstrated that the use of fluorescent (laser) dye filters enhanced hydrogen production. Hydrogen production rate of 85 mL H<sub>2</sub>/L/day was achieved. It was supposed that the laser dyes prevented the PNSB from being damaged by excessive sunlight and may have transformed the absorbed wavelengths into longer ones, which were more effective for photosynthesis [100]. In investigating the effect of wavelength on hydrogen production by *R. sphaeroides* O.U001, Rhodamin B and CuSO<sub>4</sub> solutions were used as optical filters. It was found that the blockage of infrared light negatively affected bacterial growth and reduced photofermentative hydrogen production by around 40% [62]. PNSB absorb light at near infrared (750-950 nm) for photofermentative hydrogen production [49]. Shading light bands were successfully employed to spatially disperse excessive light intensity and improve light conversion efficiencies of photosynthetic bacteria [101]. Experiments that were carried out in indoor and outdoor conditions using *R. sphaeroides* RV strain resulted in an increase of 1.4 times (2.1%) and 1.3 times (1.4%) of light conversion efficiencies in indoor and outdoor conditions, respectively.

#### 4.3.4. Temperature control

Most PNSB produce hydrogen optimally between 30 and 35°C [58,59,102]. They cannot grow or produce hydrogen above 38°C [59], except for a few such as *R. centenum*, which was able to grow optimally at 40-42°C but could not utilize substrate at high concentration, therefore limiting its usage in hydrogen production studies [103].

Fluctuating day and night temperatures significantly affect bacterial growth and hydrogen production in outdoors. During summer, the daytime outdoor temperatures fluctuate between 20 and 40°C, but during winter, it remains below 10°C [52,53]. During winter, electric heaters were used at night to maintain temperatures above freezing point. Slow bacterial growth rate attributed to low temperatures and low light intensities were reported in the experiments carried out a glasshouse during winter [45,53,54].

During the summer, due to the high temperatures experienced in outdoors (circa. 40°C), cooling of the PBRs is necessary to maintain temperatures at optimal PNSB growth and

hydrogen production conditions. The major strategies that have been devised to control temperatures within the PBRs include: (i) external cooling by water spraying and shading [52] and submersion of the photobioreactor in a water bath/basin [86,104,105] (ii) internal cooling using internal coils [51-55,64,66].

Partial shading (60%) and cooling by water spraying were employed in hydrogen production studies carried out using batch cultures of *R. capsulatus* YO3. The cells were grown in a glass bottle photobioreactor operated in outdoor conditions. The PBR temperature was maintained at 33°C and a maximum hydrogen productivity and hydrogen yield of 0.63 mgH<sub>2</sub>/L/h and 0.045 gH<sub>2</sub>/g<sub>substrate</sub> were obtained, respectively [52]. The temperature of a 0.8 L floating-type bioreactor was successfully controlled by submersion in seawater. When the atmospheric temperature rose to 36°C, the temperature of the seawater was 25°C and that of the reactor remained at 28°C [78]. Carlozzi and Sacchi [104] operated a temperature controlled underwater tubular PBR for 6 months in outdoor conditions to produce *Rhodospseudomonas palustris* strain 42OL biomass. They obtained an average productivity of 0.7 gram biomass dry weight per gram acetic acid. Adessi et al. [86] investigated hydrogen production using a 50 L tubular PBR submerged in a thermostated stainless steel water basin containing demineralized water set at 28±0.5°C. Maximum hydrogen production rate of 27.2 mL H<sub>2</sub>/L/h and substrate conversion efficiency of 49.7% were attained in the outdoor operated PBR. A glass tube internal coil measuring 1.70 m in length and 0.01m in internal diameter was used to cool a 6.5 L (working volume) flat panel PBR [51]. Flexible polyvinylchloride (PVC) cooling coils were integrated into flat panel PBRs and chilled water (5°C) was passed through to maintain the culture temperatures below 35°C [53,54]. PVC cooling coils were also utilized in a pilot-scale 90 L tubular PBR [64,66].

#### **4.4. Comparison of photofermentative hydrogen productivities and yields obtained in outdoor conditions**

The performances of outdoor operated PBRs are compared in Table 3. The parameters evaluated are hydrogen productivities, hydrogen yields (substrate conversion efficiencies) and light conversion efficiencies. Over the years, as advances in hydrogen production studies are made, more pilot studies are being carried out using PNSB and microalgae [38,45,64,66,86,105]. One of the highest hydrogen production rates was reported as 1.21 mol/m<sup>3</sup>/h by Adessi et al. [86] using a water basin cooled 50 L tubular PBR. The use of dark fermenter effluents of agricultural wastes such as molasses and sugar beet juice as feed media has also been shown to be viable in photofermentative hydrogen production [52,54,55,106]. In some cases it even led to better hydrogen production than the artificial feed media. This could be attributed to its multi-component nature in which the presence of extra nutrients enhanced hydrogen production and yield [54,55]. Different hydrogen productivities and hydrogen yields are reported (Table 3). Light conversion efficiencies not exceeding 1% are generally observed in outdoor studies. The differences in performances of the PBRs could be ascribed to the differences in: geometry and volume of the PBRs, type of microorganisms, mode of operation, nature of feed and composition, season and geographical location of the PBR operation.



Reactor type and volume	Mode of operation	Microorganism	C and N sources	H <sub>2</sub> Productivity	H <sub>2</sub> Yield	Substrate Conversion Efficiency (%)	Light Conversion Efficiency (%)	Ref.
Flat plate (33L)	Batch	<i>Rhodopseudomonas sphaeroides</i> B5/A	53mM lactate, 5 mM glutamate	0.13 mol H <sub>2</sub> /m <sup>3</sup> /h	-	69.1	-	[107]
	Batch	<i>Rhodopseudomonas sphaeroides</i> B5/B		0.18 mol H <sub>2</sub> /m <sup>3</sup> /h		78.1		
Flat plate (6L)	Semi-continuous	<i>Rhodopseudomonas sphaeroides</i> B6	60 mM lactate, 6 mM glutamate or and 150 mM lactate, 30 mM glutamate	0.84 mol H <sub>2</sub> /m <sup>3</sup> /h	-	62.5	-	[108]
Flat plate (4.4 L)	-	<i>Rhodobacter sphaeroides</i> RV	Lactate	0.56 mol H <sub>2</sub> /m <sup>3</sup> /h				[109]
Column (1.4L)	Batch	<i>Rhodobacter sphaeroides</i> DSM 9483	Lactate	0.16 mol H <sub>2</sub> /m <sup>3</sup> /h	-	-	-	[100]
Roux flask (0.7 L)	Batch	<i>Rhodobacter sphaeroides</i> RV	50 mM sodium lactate, 10mM sodium glutamate	0.13 mol H <sub>2</sub> /m <sup>2</sup> /h	-	-	1.1	[49]
Helical tubular (4.35 L)	Batch	<i>Anabaena variabilis</i> PK84	2% Carbondioxide, air	0.82 mol H <sub>2</sub> /m <sup>3</sup> /h	-		0.14	[80]
Helical tubular (4.35 L) <sup>a</sup>	Chemostat			0.36 mol H <sub>2</sub> /m <sup>3</sup> /h	-		-	
Tubular (4.35 L)	Batch	<i>Anabaena variabilis</i> PK84	2% Carbondioxide, 98% air	0.31 mol H <sub>2</sub> /m <sup>3</sup> /h	-	-	0.38	[81]
	Continuous			0.50 mol H <sub>2</sub> /m <sup>3</sup> /h			0.57	
Flat plate (6.5 L)	Batch	<i>Rhodobacter sphaeroides</i> O.U.001(DSM 5864)	15 mM acetate, 2 mM glutamate	0.45 mol H <sub>2</sub> /m <sup>3</sup> /h	4.6 mol H <sub>2</sub> /mol <sub>substrate</sub>	-	-	[51]
			30 mM malate, 2 mM glutamate	0.01 mol H <sub>2</sub> /m <sup>3</sup> /h	0.6mol H <sub>2</sub> /mol <sub>substrate</sub>	-	-	
			30 mM acetate, 2 mM glutamate	0.36 mol H <sub>2</sub> /m <sup>3</sup> /h	1.2mol H <sub>2</sub> /mol <sub>substrate</sub>	-	-	
			20 mM lactate, 2 mM glutamate	0.09 mol H <sub>2</sub> /m <sup>3</sup> /h	0.8 mol H <sub>2</sub> /mol <sub>substrate</sub>	-	-	
			Olive mill waste water	0.13 mol H <sub>2</sub> /m <sup>3</sup> /h	-	-	-	
Glass bottle (0.55 L)	Batch	<i>Rhodobacter capsulatus</i> DSM 1710	30mM of acetate, 7.5mM of lactate, 2 mM glutamate	0.14 mol H <sub>2</sub> /m <sup>3</sup> /h	0.027 gH <sub>2</sub> /g <sub>substrate</sub>	19	0.79	[52]
		<i>Rhodobacter capsulatus</i> YO3 (Hup-)		0.32 mol H <sub>2</sub> /m <sup>3</sup> /h	0.045 gH <sub>2</sub> /g <sub>substrate</sub>	33	2.41	

Tubular nearly horizontal <sup>a,bi</sup> (80 L)	Fed-batch	<i>Rhodobacter capsulatus</i> DSM 1710	40 mM acetate, 2 mM glutamate	0.74 mol H <sub>2</sub> /m <sup>3</sup> /h	0.60 mol H <sub>2</sub> /mol acetate	16	1	[45]
Flat plate (4×25 L)	Fed-batch	<i>Rhodobacter capsulatus</i> DSM 155	Acetate, lactate, glutamate	0.94 mol H <sub>2</sub> /m <sup>3</sup> /h	-	-	-	[110]
Tubular nearly horizontal <sup>a</sup> (65 L)	Fed-batch	<i>Rhodobacter capsulatus</i> DSM 155	Acetate, lactate, glutamate	0.74 mol H <sub>2</sub> /m <sup>3</sup> /h	-	-	-	
Flat plate <sup>b</sup> (8L)	Fed-batch	<i>Rhodobacter capsulatus</i> YO3 (Hup-)	40 mM acetate, (2-10) mM glutamate	0.30 mol H <sub>2</sub> /m <sup>3</sup> /h	-	44	-	[53]
Flat plate <sup>c</sup> (4L)	Fed-batch	<i>Rhodobacter capsulatus</i> YO3 (Hup-)	40 mM acetate, 4 mM glutamate	0.51 mol H <sub>2</sub> /m <sup>3</sup> /h	-	53	-	[53]
Flat plate (4 L)	Fed-batch	<i>Rhodobacter capsulatus</i> DSM 1710	molasses dark fermenter effluent	0.50 mol H <sub>2</sub> /m <sup>3</sup> /h	-	50		[54]
	Fed-batch	<i>Rhodobacter capsulatus</i> YO3 (Hup-)		0.67 mol H <sub>2</sub> /m <sup>3</sup> /h	-	78		
Tubular nearly horizontal (50 L)	-	<i>Rhodopseudomonas palustris</i> strain 42OL	malate, glutamate	1.21 mol H <sub>2</sub> /m <sup>3</sup> /h	-	49.7	0.92	[86]
Tubular nearly horizontal (90 L)	Fed-batch	<i>Rhodobacter capsulatus</i> YO3 (Hup-)	20 mM acetate, 2 mM glutamate	0.40 mol H <sub>2</sub> /m <sup>3</sup> /h	0.35 mol H <sub>2</sub> /mol acetate	12	0.2	[64]
Tubular nearly horizontal (90 L)	Fed-batch	<i>Rhodobacter capsulatus</i> DSM 1710	Thick juice dark fermenter effluent	0.27 mol H <sub>2</sub> /m <sup>3</sup> /h	0.40 mol H <sub>2</sub> /mol acetate fed	-	-	[66]
Flat plate (4 L)	Fed-batch	<i>Rhodobacter capsulatus</i> YO3 (Hup-)	Thick juice dark fermenter effluent	1.12 mol H <sub>2</sub> /m <sup>3</sup> /h	-	77	-	[55]
Tubular nearly horizontal (50 L)	-	<i>Chlamydomonas reinhardtii</i>	Tris-Acetate-Phosphat medium (TAP)	0.03 mol H <sub>2</sub> /m <sup>3</sup> /h	-	-	-	[105]

<sup>a</sup> Continuous circulation, <sup>b</sup>operation during winter, <sup>c</sup>operation during summer, <sup>i</sup>illumination provided at night using artificial light.

**Table 3.** Comparison of photofermentative hydrogen production performance in photobioreactors operated at outdoor conditions.

#### 4.5. Scale up

The eventual goal of photofermentative hydrogen production research is to produce hydrogen in large scale in outdoor conditions. For this to be realized, scale up of the

hydrogen production systems, which have so far been used in small scale studies in the laboratory, need to be done. There are a few large-scale systems (pilot scale) studies reported in literature as shown in Table 3.

Scale up of the PBR systems faces several challenges. The first is related with the geometry of PBR. With scale up, the depth of the PBRs increases, this in turn increases the distance that light and evolved gas travels. Hence the possibility of hydrogen being used up by the microorganisms or diffusing through the PBR surface rises. Increasing the heights of the PBR may lead to build up of pressure at the bottom of the PBR, which may be detrimental to the bacterial cell growth. Scale up of flat plate PBR to 1 m in both height and width was reported suitable to reduce light deflection by the plates and allows gas-tightness of the enclosed volume without excessive pressure build [110,111]. Flat plate PBRs can be scaled up by cascading in stacks [38,53,54,88,89] while the size of tubular PBRs can be increased by connecting more tubes on the manifolds [28,45,64,66,86,105].

Another problem of scale up is that self-shading of cells becomes more prominent. The effect increases with increasing reactor size and cell concentration, negatively affecting cell growth and hydrogen production [92]. Due to the lack of sufficient light the bacterial growth rate is hampered and hydrogen production reduces as the organisms switch to alternative modes of growth [4,51]. The problem of self-shading can be alleviated by use of PBRs with larger illumination areas, use better light distribution methods such as integration of optic fibers within the PBR or genetically tailoring the photosynthetic apparatus of the photosynthetic bacteria [98,112]. These solutions may lead to the requirement of larger ground area, increasing the system costs and bring about ethical issues of using genetically modified microorganisms at industrial scale.

Light distribution in the scale up system could also be improved by providing artificial illumination at night or during cloudy days [62], however, extra expenses incurred will have to be considered.

Another difficulty of scale up is mixing. Mechanical mixing in large scale-systems is difficult because of the large surface to volume ratio in the reactors. Sparging of inert gas [31] or recycling of the produced gas through the culture [34] is preferred; however, the former leads to dilution of the total gas while the latter incurs extra operating costs due to pumping.

Sterilization of the PBRs and feed media is another concern of scale up. For large-scale application, it is suggested to operate the systems in non-sterile conditions. Constant hydrogen production was obtained for a period of almost two months using a semi-continuously operated PBR that was fed with non-sterile media [72]. Nonetheless, it is possible to operate large scale PBRs using sterilized PBR and media under optimal conditions, therefore reducing contamination risks [111]. However, further studies on sterilizing to avoid contamination in the scaled up PBR systems remain to be done.

## 5. Improvement of photofermentative hydrogen production

### 5.1. Photobioreactor design

#### 5.1.1. Material of construction

PBRs can be constructed from a wide variety of materials such as; glass, low-density polyethylene film (LDPE), rigid acrylic or polymethyl methyl acrylate (PMMA), polycarbonate and transparent polyvinylchloride (PVC). Glass is considered a very good construction material because it is transparent, has low hydrogen permeability and a long lifespan (circa. 20 years). However, it is brittle, rigid, heavy, not easily workable and expensive, therefore not suitable for large scale systems [113]. PBRs constructed from glass have been reported in several studies [41,42].

LDPE is a flexible thin material that is mostly used in greenhouse covering. It has been applied in constructing tubular photobioreactors [38,45,64,66]. Its desirable properties include transmission of high visible and near infrared that is required by the PNSB for growth and hydrogen production, low UV transmission and low cost [114]. A major disadvantage is its thin wall thickness, which may lead to high hydrogen permeability [64]. Also, it has a short lifespan of about 3 years (maximum) [113].

PMMA is another suitable material for PBR construction. It is a highly transparent thermoplastic that is lighter, softer and easier to work with compared to glass. It transmits 92% of visible light (3 mm thickness) and filters ultraviolet (UV) light wavelengths below 300 nm while allowing infrared light of up to 2800 nm wavelength to pass. Also, it is weather resistant and can withstand outdoor conditions better compared to other plastics such as polycarbonate. However, it is brittle, inflexible and has higher hydrogen permeability compared to glass. A wall thickness of 4 mm minimum is needed to avoid leakage and cracking due to mechanical stress. It is stated to have a minimum lifespan of 10 years in outdoor operations [113]. PMMA has been used to construct flat plate [38,53-55] and tubular [36,115] PBRs.

Other materials such as polyethylene (PE), polypropylene (PP), polycarbonate (PC), polyvinyl chloride (PVC) have been used, especially in constructing tubular PBRs. However a major drawback in using them in outdoor conditions is that they can lose their transparency due to exposure to sunlight, thus are less durable [30].

Criteria in selecting the material of construction of PBRs for outdoor operations are such that they must: be transparent, be chemically unreactive and metal free, nontoxic, have low hydrogen and oxygen permeability, have high mechanical strength, have high durability and resistivity to sterilizing chemicals (i.e. hydrogen peroxide), be easy to clean and be available at low cost [29]. These factors affect the choice of material of construction and the overall system construction and sustainability costs.

#### 5.1.2. Geometry, mixing and mode of operation

The geometry of the PBR impacts the illuminated surface area. Flat plate and tubular PBRs have been widely used in photofermentative hydrogen production studies because of their

high illuminated surface area [33]. However, new PBR concepts with larger surface areas can be developed to improve hydrogen production. Some of the PBRs designed to enhance photofermentative hydrogen production studies include: helical tubular PBR [100], hollow channel plate PBR [72], a PBR with an integrated active gas separating membrane system [116], a multi-layered PBR [117] and a PBR with integrated solar excited optic fibers [79]. Moreover, in the design of the PBR geometry, PBR with a short light path is targeted to prevent exponential decay of light passing through the culture. A light path length of about 20 mm or less has been shown to be sufficient for the design of flat plate PBRs [38,49].

Mixing is another crucial parameter that needs to be considered in improving photofermentative hydrogen production. Good mixing facilitates the separation of evolved gas and assists in the homogenous distribution of cells, substrates and light within the PBR [118]. Stirring of the culture media using a magnetic stirrer was found to enhance hydrogen production and the amount of total gas produced. The experiment was carried out in a 400 ml water jacketed-glass column PBR using a combined system of *H. salinarum* packed cells with *R. sphaeroides* O.U.001 cells [42]. Shaking during the stationary phase of cell growth was shown to enhance hydrogen production more rather than mixing during growth (exponential) phase [40]. However, vigorous mixing at high circulation rates may lead to cell damage and incur higher running costs. The use of mechanical agitators was depicted to be not suitable for mixing cultures in flat plate PBRs as their light path is short (narrow width). Sparging of inert gas [31], re-circulation of the evolved gas [34], and agitation through rocking motion [39] have been suggested. For tubular PBRs, mixing through continuous circulation of cell culture using a pump was stated to improve mass transfer between cells and necessitate easier hydrogen gas separation compared to intermittent circulation [64].

The choice of the mode of operation to be used in running outdoor PBRs for biohydrogen production is dependent on the production capacity, which in turn affects the capital investment and operating costs [33]. For outdoor operations, development of large scale continuous hydrogen production systems is targeted. Continuous and fed-batch systems are suitable for these processes as they enable long-term operations through regular feeding of the bacteria at certain dilution rates. However, fed-batch operation was described to be the most favorable mode of operation. It operates under high cell densities and allows the control of the reaction rates by adjustment of feed flow rates and compositions, thus preventing substrate and product inhibitions [71,84].

## 5.2. Immobilization studies

Hydrogen production can be improved by immobilizing cells within the PBR. Immobilized cultures have been shown to be more attractive for hydrogen production studies compared to suspended systems. The systems are easier to operate, the bioreactor effluent is cell-free and higher cell concentrations can be used, therefore enabling hydrogen production at higher rate and yields. Drawbacks of this system include substrates and products ( $H_2$ ) diffusion limitation brought about by the high cell concentrations and difficulty in controlling parameters such as pH and hydrogen content. Continuous operation may be



applied to curb this problem. Heterogeneity in parts of the PBR may exist, but this can also be overcome by growing the cells in matrix spaces [119].

Several techniques have been applied to immobilize the growing bacterial cells on the matrix spaces. They include adsorption, covalent bonding, cross-linking, entrapment and encapsulation [120]. Likewise, different materials have been used to immobilize the photosynthetic cells. Agar, alginate, carrageenan, cellulose and its derivatives, collagen, gelatin, epoxy resin, photo cross-linkable resins, polyacrylamide, polyester, polystyrene, polyurethane and porous glass are the most commonly used materials [74,82,120-122]. Gel entrapment was stated to be the best means of immobilizing bacterial cells [121,123].

Improvement of hydrogen production by immobilized systems has been reported in literature. Around 2-10 fold increase of hydrogen production rate was reported by immobilization [124,125]. A 4-fold increase in hydrogen production was observed in immobilized cells compared to suspended cells [126]. Entrapment of whole cells of *R. sphaeroides* inside reverse micelles led to 25-35 fold increase in hydrogen production [127]. *Rhodobacter sphaeroides* GL-1 was immobilized in polyurethane foam in continuously operated PBR. Hydrogen productivity and substrate conversion efficiency of 0.21 ml/h/ml foam-matrix and 86% were obtained, respectively [128]. Hydrogen production by immobilized *R. capsulatus* DSM1710 and *R. capsulatus* YO3 (Hup-) were investigated in agar immobilized systems. The optimization studies showed that artificial feed media containing 60 mM acetate (carbon source) and 4 mM glutamate (nitrogen source) produced the highest hydrogen yield of around 90-95%. Long-term hydrogen production of 67-82 days and 69-72 were also achieved in the *R. capsulatus* DSM 1710 and *R. capsulatus* YO3 (Hup-), respectively [120].

Moreover, immobilization may provide protection of bacteria cells against inhibitory effects of compounds like ammonium. In examining the suitability of using tofu wastewater for hydrogen production, it was found that agar protected the immobilized *R. sphaeroides* cells from the effect of ammonium [129]. Similar results were obtained in experiments carried out using *R. capsulatus* immobilized in agar gels and fed with artificial media containing different concentrations of ammonium chloride (2.5, 5 and 7.5 mM) [120].

### 5.3. Feed media: C/N and minor nutrients

PNSB are able to utilize a wide range of organic compounds as substrates for growth and hydrogen production [130]. They can use different C sources, preferably, volatile fatty acids (VFA) such as acetic, butyric, lactic and malic acid for hydrogen production and nitrogen sources such as glutamate and ammonium for growth [131]. Moreover, they can also use sugar containing wastes derived from various industries such as the tofu industry wastewater [129], olive mill wastewater [132], sugar refinery wastewater [133], dairy wastewater [134], palm oil mill wastewater [135] and ground wheat starch [136]. Effluents from dark fermentation have also been applied in several studies [54,55,66,106,137-139]. Studies using mixtures of VFAs demonstrated that the PNSB consume them at different sequences. *Rhodobacter sphaeroides* O.U.001 was found to initially deplete acetate, then



propionate and butyrate [138]. *R. capsulatus* was found to initially consume lactic acid then acetic acid in a study using a mixture of the organic acids [59]. Ammonium ions have been reported to inhibit hydrogen production [21,140,141] and glutamate has been widely used for growth and hydrogen production by the PNSB [4,73,131].

The C/N ratio in the feed media is another crucial parameter affecting the growth, hydrogen productivity and yield. A low C/N ratio was reported to enhance microbial growth but decrease hydrogen production [41,46,73,79]. The rise in biomass concentration reduces light penetration into the PBR, thus decreasing hydrogen productivity. A high C/N ratio also increases biomass concentration and reduces hydrogen production [73,79,142]. Stable biomass concentration of 0.40 g dry cell weight per liter culture (gDCW/L) and maximum hydrogen productivity of 0.66 mmol hydrogen per liter culture per hour (mmol/L<sub>c</sub>/h) were obtained with media containing 40 mM acetate and 4 mM glutamate (C/N = 25) for a period of over 20 days using fed-batch cultures of *R. capsulatus* YO3 (Hup-) [73]. The addition of minor nutrients, such as Fe and Mo which are co-factors to the nitrogenase enzyme, have been reported to enhance hydrogen production [137,138].

#### 5.4. pH

The pH of the culture regulates growth and hydrogen production by the photosynthetic bacteria. PNSB such as *R. capsulatus* are reported to optimally grow between pH 6-9 [102]. Good cell growth and biomass yield is obtained between pH 6.0-7.0, but it decreases with further increase in pH. In investigating the effects of initial pH on photofermentation using cultures of *R. sphaeroides* O.U. 001, Nath and Das [143] obtained high biomass yield and maximum cumulative hydrogen production at pH 7.0. Biomass decreased as pH increased from 7.0 to 8.0. A drop in pH caused by the accumulation of VFAs during photofermentation of glucose by *R. capsulatus* JP91 was observed to reduce hydrogen and cell growth [144]. During exponential growth phase, high hydrogen production rates and yields were observed between the pH range 6.8-7.5 [73] and no hydrogen production was reported above pH 9.0 [141]. It is postulated that high pH values could have prevented the PNSB cells from maintaining their membrane potential, therefore affecting cellular metabolism and eventually hindering cell growth and hydrogen production [139].

#### 5.5. Genetic modifications

Genetic engineering is a promising tool to increase the yield and productivity of photofermentative hydrogen production. Considering the H<sub>2</sub> metabolism of PNSB, genetic modifications can be done to (i) inhibit H<sub>2</sub> utilization, (ii) optimize the flow of reducing equivalents to nitrogenase by inhibition of PHB and CO<sub>2</sub> fixation, (iii) eliminate/decrease the effect environmental factors (NH<sub>3</sub>, O<sub>2</sub>, light, temperature), (iv) reduce the size of antenna pigments to increase light utilization efficiency.

Deletion of membrane-bound H<sub>2</sub>-uptake hydrogenase (Hup-) gene of PNSB has been the major strategy to increase hydrogen production. In *Rhodobacter sphaeroides* KD131 deletion of Hup and PHB synthase genes resulted in an increase in H<sub>2</sub> production from 1.32

to 3.34 ml H<sub>2</sub>/mg-dry cell weight, compared to the wild type strain [145]. Hup deletion in *R. rubrum* resulted in higher hydrogen production, however the rate of hydrogen production was not affected indicating that the capacity to recycle H<sub>2</sub> was not completely lost [146]. In *R. sphaeroides* O.U.001, HupSL deletion resulted in an increase in hydrogen production from 1.97 L H<sub>2</sub>/L<sub>c</sub> to 2.42 L H<sub>2</sub>/L<sub>c</sub> on malate containing culture, while on acetate both wild type and mutant strain produced hydrogen poorly [147]. In *R. capsulatus* MT1131, Hup deletion resulted in around 30% increase in H<sub>2</sub> production [148] in artificially illuminated batch cultures in indoor conditions, on malate as carbon source. The Hup- *R. capsulatus* MT1131 also showed enhanced photobiological H<sub>2</sub> production rates and yields in continuously operated outdoor photobioreactors on acetate as carbon source [53,64,66]. The strain also resulted in very promising H<sub>2</sub> production activities in studies carried out on real dark fermentation effluents in indoor batch studies [137,139] and in outdoor continuous PBR operations [54,55].

To optimize the flow of reducing equivalents to nitrogenase, genetic modifications were carried out targeting CO<sub>2</sub> fixation and PHB synthesis pathways, which compete for reducing equivalent. Spontaneous variants of *R. capsulatus* strains deficient in the CBB pathway have been shown to express nitrogenase structural genes to dissipate excess reducing equivalents, even in the presence of high concentrations of ammonia that is sufficient to repress nitrogenase expression in wild type [149]. In wild type and Hup- strains of *R. capsulatus* MT1131, inactivation of CO<sub>2</sub> fixation pathway resulted in improvements in the yield and productivity of hydrogen production [150]. In *R. sphaeroides* KD131, inactivation of PHB synthase resulted in 2 fold increase in hydrogen production on acetate and butyrate, in spite of depressed cellular growth and lower substrate utilization.

Removal of NH<sub>3</sub> inhibition on nitrogenase activity is important especially for integrated dark and photofermentation studies, in which the dark fermenter effluent is usually rich in NH<sub>3</sub>. To develop mutants with ammonia insensitive-nitrogenase activity, Pekgöz et al [140], deleted genes expressing two regulatory proteins of ammonia-dependent nitrogenase regulation, GlnB and GlnK in *R. capsulatus* DSM1710. However, glnB mutants showed lower hydrogen production, while glnK mutants were unviable. This observation suggests that GlnB/GlnK two component regulatory system most probably have role in other metabolic pathways, as well.

Increasing the light utilization efficiency of PNSB by reducing the size and quantity of photosynthetic pigments were addressed in *R. sphaeroides* RV. The mutant, which had lower LH2 content produced 50% more hydrogen compared to the wild type in plate-type photobioreactor [112].

Another strategy could be the development of recombinant PNSB, which express hydrogen-evolving hydrogenase. Kim et al. [151] developed a recombinant *R. sphaeroides* KCTC 12085 strain that harbor, with all the accessory genes necessary, Formate hydrogen lyase and Fe-only hydrogenase from *R. rubrum*, to enable dark fermentative hydrogen production from *R. sphaeroides*. The strain produced hydrogen during dark fermentative growth, and photofermentative hydrogen production increased by 2 fold.

## 6. Techno-economics of photofermentative hydrogen production

Biological hydrogen production through photofermentation is a sustainable way of producing hydrogen since it utilizes renewable resources (sunlight, water and biomass) and occurs under ambient conditions. Recently, a 6<sup>th</sup> framework EU integrated project, Hyvolution, entitled “ Non-thermal production of pure hydrogen from biomass” was completed where the aim was to produce hydrogen from biomass with integration of dark and photofermentation. Effluents obtained from dark fermentation were used to produce H<sub>2</sub> by photofermentation [152]. These effluents contained both acetic acid and lactic acid as carbon sources that could be utilized in a consecutive photofermentation step. *Rhodobacter capsulatus* was the selected PNSB in the photofermentation stage since it produced hydrogen most effectively by breaking down organic acids such as acetic acid and lactic acid under anaerobic conditions and illumination [139].

The HYVOLUTION plant was assumed to be in operation 8000 hours per year producing 60 kg H<sub>2</sub>/h (2MW thermal power). The PBR was assumedly in full operation during 10 hours per day, resulting in roughly 3330 hours of operation annually. The economic analysis included the capital cost for all four process steps, i.e. pretreatment, thermophilic fermentation, photofermentation and gas up-grading [153]. Four feedstocks (thick juice, molasses, potato steam peels and barley straw) were considered in the HYVOLUTION Plant. Aspen Plus was used to calculate mass and energy balances taking into account the integration of the processes. A net energy production, in form of hydrogen, showed that the production of hydrogen as an energy carrier was technically feasible with all the considered feedstocks. Thick juice had the lowest energy demand, but the other options required 20% more heat demand. This demonstrated that second generation biomass could compete with food biomass for the hydrogen production. Further investigations towards scale up and improved mass and energy balances (heat integration studies) for the various feedstocks would enable the betterment and selection of routes for the HYVOLUTION process [154].

From a costing perspective, the photofermenter was found to be bottleneck in the HYVOLUTION project. The final cost of photofermentative hydrogen production was estimated to be around 55-60 €/kg using a tubular photobioreactor and 385-390 €/kg in the case of a panel photobioreactor. These high production costs are mainly caused by the materials of construction of the PBRs; plastic and PMAA, at the current state-of-the-art. The total capital cost of the photofermenter was large, around €90 million and €320 million for the tubular and flat panel reactor, respectively. The cost of land was not considered; the ground area demand of the tubular and flat panel reactor was about 2.0 and 1.3 million square meters, respectively. Although photofermentation was the most expensive part of the HYVOLUTION process, other process steps have to be improved. To meet the proposed hydrogen cost of €10 /GJ, which corresponds to €1.21 /kg H<sub>2</sub> (based on the lower heating value, LHV) a maximum allowed capital investment of €5.3 million is necessary, neglecting all other costs (feedstock, labor, etc.). Presently the capital cost, excluding the photofermenter, is €24.6 million for the thick juice case and the capital costs of the tubular and flat panel photofermenter are €91 million and 332 million respectively. Clearly, vast

improvements are required starting with the utilization of co-products. Forecasts for the improvement of the performance of the photo-fermentation promise a reduction of the cost to 20 €/kg hydrogen.

HYVOLUTION has also been compared, in terms of €/GJ, with the costs for a bioethanol production plant equipped with a biogas installation for the utilization of the residues and pentose sugars. With barley straw as feedstock, energy from state-of-the-art HYVOLUTION is 452 €/GJ and 18 €/GJ for ethanol with biogas. The estimation for HYVOLUTION after 6 years and at small scale is a decrease to 153 €/GJ. Even though these costs are still significantly higher than for ethanol, hydrogen production may be supplementary to bioethanol production when feedstocks with high moisture content are considered. In this case, downstream processing of ethanol will not be economical due to prohibitive costs for distillation leaving room for hydrogen to add to the future biofuel mixture. However, the studies carried out in a two-step process, dark fermentation followed by photofermentation (FP6 HYVOLUTION Project [152]), revealed that photofermentative hydrogen production cannot be competitive with current productivities and yields. The current estimated costs for hydrogen from HYVOLUTION are higher than anticipated at the start of the project, mainly but not solely, to the costs of the PBR.

Currently, the long-term scenario (2030) predicts a cost of 6 €/kg H<sub>2</sub> provided that an overall yield of 85% and productivity of 53 and 3.3 mmol H<sub>2</sub>/L/h for thermophilic and photofermentation, respectively [106,152]. In order to obtain long-term operation, the reliability and the durability of the tubes should be increased by increasing the wall thickness of the tubes [64]. The tube diameter and wall thickness should be optimized for better light exposure of the cells. Decreasing the tube diameter can increase hydrogen productivity. However, feasibility of this approach should be investigated in terms of circulation energy [113] and land area requirements. Circulation should be continuous in order to increase the mass transfer between the cell (solid), liquid and the gas phases. This will also reduce the gas diffusion from the LDPE tubes.

## **7. Future prospects: Integration with other hydrogen production methods or alternative energy sources**

To estimate the productivity target for photofermentation, one may consider data from photovoltaic and biogas as benchmark technologies. The output of the primary product (electricity, biomass) is in both cases directly linked to the available ground area. In using photovoltaic as a benchmark, one has to consider that electricity is obtained as the primary product. According to various calculations in the literature, the obtained electrical power per m<sup>2</sup> depends strongly on the location. The yearly global irradiation on optimally oriented PV-modules is ranging from ca. 1000 kWh/m<sup>2</sup>.a in e.g. Germany and more than 2000 kWh/m<sup>2</sup>.a in certain areas of Greece, Turkey, Italy and Spain. Calculations for Germany (which can be extrapolated to other regions like The Netherlands) results in an energy harvest of between 75 and 125 kWh/m<sup>2</sup>.a (assuming a system efficiency of 75%) which corresponds to ca. 9-16 W/m<sup>2</sup> average (over the year) for Germany. For Nordrhein-Westfalen



an energy harvest of ca. 80 kWh/m<sup>2</sup>.a can be found in the literature and is used for any further calculations [155]. The calculation for a more southern country in Europe (ca. 150 kWh/m<sup>2</sup>.a) results in a produced electrical power of 18 W/m<sup>2</sup>. Converting finally the PV - electricity to hydrogen by electrolysis, one may obtain for the PV-system ca. 6.0 W H<sub>2</sub>/m<sup>2</sup> (Nordrhein-Westfalen) and 11.3 W H<sub>2</sub>/m<sup>2</sup> (Greece) assuming an efficiency of the electrolysis of 60% which corresponds to an hydrogen productivity between 180 mg/m<sup>2</sup>.h (Nordrhein-Westfalen) and 338 mg/m<sup>2</sup>.h (Greece) (HyLog – Project, E. Wahlmüller, Fronius Ltd.). Taking as another benchmark system the production of biogas from energy plants like maize, a production of 2.5 kW electricity out of 1 ha farm land (using a CHP with an electrical efficiency of ca. 39%) is typical. If the same amount of biogas is not converted to electricity but to hydrogen using methane steam reforming a final power of 0.54 W/m<sup>2</sup> can be obtained (assuming an efficiency of the reformer of 85% and neglecting the power demand of the reforming plant) which corresponds to a hydrogen productivity of 16.4 mg/m<sup>2</sup>.h.

The technical and economic feasibility of dark fermentation followed by methane production via anaerobic digestion step have been investigated employing three base cases reflecting the different strategies that can be used when performing dark fermentation: high productivity, high yield, and low productivity-low yield. The production of pure methane was included as a reference case to investigate how the production of hydrogen affected the production cost. The cost estimates ranged from 50 to 340 €/GJ for the three base cases and the reference case for the process alternatives investigated. The capital costs and the nutrients used in the two biological steps were the main contributors to the cost in all base cases and the reference case. Utilization of the mixed biofuel (methane and hydrogen) may increase efficiency and lower environmental impact in terms of lower emissions [156].

Different hydrogen production technologies were evaluated based on renewable raw materials and/or renewable energy such as; alkaline electrolysis, steam reforming of both biogas and gasification gas, the coupled dark and photo fermentation as well as the coupled dark and biogas fermentation [157]. Each technology was investigated with different plant layouts and/or different raw materials. All examined technologies were designed to produce hydrogen in a quality suitable for the use in mobile fuel cells. The reforming of biogas gave good results regarding both hydrogen production and energy efficiency provided that the proper raw material was chosen. The reforming of gasification gas showed good production efficiencies but in contrast the energy efficiencies were low compared to the reforming of biogas. The production efficiency results of the coupled dark and photo fermentation were comparable to those of the reforming of biogas but their energy efficiencies were lower. However, since this technology is in an early stage of development it still has potential for development and might be a real alternative to reforming of biogas. Finally, the best results for the coupled dark and biogas fermentation regarding both hydrogen production and energy efficiency were obtained for the layout with on-site steam reforming of the produced biogas, showing efficiencies comparable to the dark and photo fermentation. The choice of the proper technology will have to be based on the availability of raw materials, since the kind of raw material had a strong influence on the performance of the technology, on the competition between food and energy production and on the development of raw material prices.

## 8. Conclusions

Scientific, project and market strategy is essential to lead in developing biological hydrogen production processes. Plans for future research should be made based on the knowledge and experience gained and techniques developed until now. Although considerable progress has been made, still many basic research questions remain unanswered and new ones were created. Therefore, more fundamental research is necessary besides the need to test several developed techniques at lab- and pilot scale, both as standalone and combined.

Outdoor production of hydrogen with photosynthetic bacteria is strongly affected by fluctuations in temperature and light intensity due to the day-night cycle and due to seasonal, geographic and climatic conditions. In order to forecast the hydrogen productivity at different places throughout the world and based on that to estimate the cost-effectiveness for a certain location, a model describing the dependency of hydrogen production from the natural parameters is necessary.

Operation of photobioreactor in outdoor is an energy requiring process due to the need for temperature control and recirculation. Exploitation of other renewable energy sources (sunlight, wind, geothermal energy, etc.) to supply energy to the PBR to be used for recirculation or for temperature control can be explored and implemented in the design of a biohydrogen plant. Heat economizing is necessary for the plant with the integration of cooling and heating streams in a heat exchange network. The light and substrate conversion efficiencies are low due to problems experienced in the bioreactor. Since the reactor design and materials used are still in research state, it would not be so useful to comment on a factor improvement for economical application of photofermentative hydrogen production by photobioreactors.

## Nomenclature

### *Symbols*

- A Irradiated photobioreactor surface area ( $\text{m}^2$ )
- I Light intensity ( $\text{W}/\text{m}^2$ )
- L Liter
- t Time (h)
- $V_{\text{H}_2}$  Volume of hydrogen gas in liters

### *Greek Letters*

- $\eta$  Light conversion efficiency (%)
- $\rho_{\text{H}_2}$  Density of hydrogen gas ( $\text{g}/\text{L}$ )

### *Acronyms*

- ATP Adenosine tri phosphate
- CBB Calvin-Benson-Bassham
- Hup- Membrane bound uptake hydrogenase deficient (mutant)



- LDPE Low density polyethylene film
- LH Light harvesting
- NADH Reduced nicotinamide adenine dinucleotide
- PBR Photobioreactor
- PMMA Polymethyl methacrylate
- PNSB Purple non sulfur bacteria
- PS Photosystem,
- PU Polyurethane
- PVC Polyvinylchloride

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