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Alessandra Adessi

Hydrogen production using Purple Non-Sulfur Bacteria (PNSB) cultivated under natural or artificial light conditions with synthetic or fermentation derived substrates

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Preface

This thesis is structured as follows.

The introductive section frames the topic of hydrogen production starting from general considerations about energy crisis, then deepening into the actual possibilities of making the use of hydrogen a completely sustainable process by means of microbial processes. A detailed description of the biological hydrogen production process by purple non sulfur (PNS) bacteria is given, with a section dedicated also to combined hydrogen production processes. The final part of the introduction points out what are the issues still open, the problems that arise in hydrogen production with PNS bacteria and some possible answers that need to be proved.

In particular the major aspects that need to be verified and tested concern the use of low cost substrates, the use of sunlight as the source of irradiation for scaled-up processes and the constant need of searching and possibly modifying PNS bacterial strains in order to overcome metabolic hurdles to an efficient hydrogen production.

The aim of the present thesis was to define the feasibility of hydrogen production using PNS bacteria under natural light conditions and growing on low cost substrates, towards an up-scaling of the process. These two aspects were investigated separately: on one side the experimentation on different light conditions was carried out using synthetic substrates, on the other side the experimentation on the use of different substrates deriving from other fermentation processes was conducted under artificial light conditions. As a preliminary activity, a phase of search and screening for new strains of PNS bacteria was conducted in order to identify strains possessing the characteristics needed for the following experimentations.

The Results section is divided in three main Sections that are organized following the three main themes.

Section I is constituted by the work carried out for searching new strains (published on the International Journal of Hydrogen Energy in 2010) that were harvested from a trophic lake (Averno Lake, NA - Italy). Seventeen isolates were phylogenetically classified by 16S rDNA sequencing, and were all qualitatively tested for their hydrogen production capabilities on four of the most common organic substrates used for hydrogen production. The most interesting strains were then tested for quantitative hydrogen production tests, and *Rp. palustris* strain AV33 resulted to be the best hydrogen producer on lactate. This strain was then also tested on a low cost substrate with interesting results.

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Section II is constituted by the works related to the use of substrates deriving from other fermentation processes, in the field of combined sequential hydrogen production processes.

During this thesis different substrates were used, deriving from (i) a mesophilic dark fermentation process (carried out by a microbial consortium), (ii) a thermophilic dark fermentation process (carried out by *Thermotoga neapolitana*) and (iii) the spontaneous dark fermentation of vegetable wastes (carried out by the autochthonous microflora residing on vegetables - no H_2 production in the first stage).

Paragraph 1. is constituted by an experimentation on these three different substrates with two strains of *Rp. palustris*, AV33 and 42OL. Quantitative hydrogen production tests were performed, selecting the best strain for the kind of substrate in use.

The two strains were able to produce hydrogen on the substrates they were tested on, but a consistent cellular growth was observed for every substrate, corresponding to a low substrate-to-hydrogen conversion. This is due to the metabolic shift from H_2 production to cell growth in presence of fixed N sources. In particular, the substrate deriving from the spontaneous fermentation of vegetable residues was rich in ammonium, which is also an inhibitor for hydrogen production, so the medium needed to be diluted to be used for hydrogen production.

From this experimentation results, it came out the need of using strains that are insensitive to fixed nitrogen, in order not to dilute the medium (it would be a further cost in an up-scaled process).

Paragraph 2. is constituted by a work, that is going to be submitted to the International Journal of Hydrogen Energy, regarding the use of a *Rp. palustris* mutant strain that is insensitive to ammonium. The aim of this study was to verify the possibility of using this strain on an actual undiluted waste derived medium .

This experimentation was carried out in collaboration with Prof. Caroline S. Harwood at her laboratory at the University of Washington, Seattle, USA. The mutant strain *Rp. palustris* CGA676 was previously developed at her lab, with a mutation in one of the regulatory genes of nitrogenase expression. This mutant was, then, used in this study, first evaluating its insensitivity at various ammonium concentrations in synthetic medium, and then testing it on the vegetable waste derived medium (also used in the experimentation in Paragraph II.1.). This mutant showed to be insensitive to ammonium at any concentration tested and was able to produce a consistent amount of hydrogen on the vegetable waste derived medium. It was tested at three different dilutions (three-fold, two-fold and undiluted) and it always showed good hydrogen production capabilities. The substrate conversion of the three-fold diluted medium is yet similar to the one obtained in Paragraph II.1. with strain 42OL, but the strain CGA676 could also be used in the undiluted medium with no inhibitory effect by ammonium on nitrogenase.

Section III concerns the experimentations carried out about the use of different light conditions, with the final aim of using sunlight as the light source for the process. This aspect was investigated first under laboratory conditions, where artificial light dark cycles were imposed to *Rp. palustris* 42OL cultures, to verify the feasibility of a long term process in this conditions (that simulate day/night cycles). Then,

the cultures were moved outdoors and it was verified the feasibility of this process under natural light conditions.

In Paragraph 1., it is reported the experimentation about artificial light/dark cycles, where it was not only investigated the behavior of the cultures in these conditions in terms of growth, substrate consumption and hydrogen production, but also the influence of light/dark cycles on the hydrogen production process. A lag phase was observed from the time the light was switched on to the time hydrogen was accumulated in the gas collecting system, but the method chosen for the determination of the culture's lag time was not sensitive enough. Thus, it was concluded that a more sensitive method was required, possibly an electrode for measuring dissolved hydrogen. Another relevant result of this experimentation was that a light/dark cycle of 8 hours of light and 16 h of darkness was not suitable for hydrogen production, thus indicating that (if we consider this a preliminary study for the use of sunlight) there is a part of the year when hydrogen production processes cannot be carried out using only sunlight.

In Paragraph 2., it is described the development of a method for studying the state of the photosystem of PNS bacteria. PAM fluorometry has been chosen as a tool for verifying the performances of the photosystem, a method that up to now has been rarely used with PNS bacteria and only for basic research studies. The aim of developing this method was to provide a tool, finally to be used in massive outdoor cultures, to determine the fitness of the photosynthetic apparatus under different culture conditions. The development of the method and the first results obtained with whole cultures of PNS bacteria grown under lab conditions are reported. This technique showed dramatic changes in the fitness of cultures grown at different temperatures whereas the different light intensities tested did not induced significant changes. In any case, the technique showed to be suitable for measures on outdoor massive cultures, as it is rapid and not invasive, and it was used in the subsequent experiments outdoors.

Both the results collected in Paragraph 1. and 2. were informative and necessary for the outdoor experimentation reported in Paragraph 3.

Paragraph 3. is constituted by a work on outdoor hydrogen production, recently submitted and accepted by the International Journal of Hydrogen Energy. The aim of this study was the assessment of the hydrogen production performances of *Rp. palustris* strain 42OL cultivated outdoors, under sunlight, in a 50 L tubular photobioreactor. Moreover, bacteriochlorophyll *a* fluorescence was used for the first time as a tool for monitoring the physiological status of *Rp. palustris* mass cultures.

The maximum H_2 production rate and the mean rate per illuminated surface obtained resulted the highest so far reported for outdoor experiments carried out with PNS bacteria in tubular photobioreactors of comparable dimensions. The *in-situ* measurement of bacteriochlorophyll *a* fluorescence (F_v/F_m) showed that the photosystem was unaffected by changing light irradiance during the day and remained fully active over the whole period of hydrogen production. Furthermore a dissolved hydrogen measuring electrode was used and resulted to be very informative: in the first place it indicated that the cultures were able to activate nitrogenase-dependent hydrogen production almost as soon as the first sun-beams touched the surface of the reactor; in the second place it was observed that, during the dark periods, the dis-

solved hydrogen was lost and a number of hours in the morning were needed to re-saturate the solution, thus negatively affecting the total amount of gas collectable. Therefore, a light supply to the culture during the night is highly recommendable to achieve a continuous H_2 production process without losses.

At the end of the Results section the General Conclusions are given. As a general conclusion, the process for hydrogen production is ready for a gradual up-scaling from lab conditions; a first step was made in the present theses towards a semi pilot scale H_2 production process with cultures irradiated by natural sunlight. A further step would be the use of low cost substrates in larger scale processes, possibly using PNS bacterial strains that allow to obtain larger H_2 amounts. Furthermore the method for measuring bacteriochlorophyll *a* fluorescence developed in this theses, resulted to be very informative about the photosystem behavior under sunlight irradiation.

Introduction

1. The energy issue

During the last years the discussion about the availability of energy sources has become a relevant matter in public opinion; all the information media are, almost daily, presenting alarming news about the end of the energy sources we are used to, global warming, population increase, future energy demand and other related issues. This testifies a serious and widespread interest of scientists, governants, politicians, economists, all working towards the common objective of finding possible strategies to have an answer to the future energy demand. Possibly with the smallest impact on the Planet.

An important matter is that the energy sources currently used not only are insufficient for our next-future needs, but also their availability is extremely uneven, worldwide. Thus, along with the strategies for alternative ways of producing energy for our Planet also strategies to make them evenly distributed must be designed. The "energy poverty" is not only an economical poverty, but it reflects drastically on life quality, intended as social and cultural conditions**.** The International Energy Agency has assessed the issue of energy poverty in its flagship publication: "modern energy access would improve lives by improving education, achieving gender equality, attaining environmental sustainability, preventing premature deaths from respiratory diseases, and accelerating global economic growth and prosperity" [World Energy Counsil, 2010]

The data about the actual exploitation of the different energy sources available (Figure 1) still point out the relevancy of fossil fuels in the energetic mix. Oil, coal and gas together constitute almost the 90% of the total world primary energy utilization; energy from water is a stable source, nuclear energy has been rising in relative relevancy in the last forty years, while renewable energies (that in these data include biofuels as well) have a shorter life and a smaller slice of relevancy. Anyway only renewables and gas seem to have a rising trend while oil exploitation is decreasing.

The data about the electricity demand and offer (divided by kind of source, shown in Figure 2) for the European Union stress another important issue: the existing energy sources are now sufficient for the energetic demand, but will soon be inadequate to answer to the future energy needs [European Climate Fundation]. According to the US Energy Information Administration, world energy consumption is projected to rise by 49% above 2007 levels by 2035. And the most rapid growth will

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be in non-OECD countries such as China, Brazil and India, where energy consumption is slated to increase 84%.

The future availability of oil sits at the center of a long-running debate: one school believes that global oil production will reach its peak and mid-depletion point in the near future; the other school has a rosier outlook for future hydrocarbon use, extending the oil age well beyond the middle of the century [Dunn, 2001]. Whichever view is more correct, the end of the "oil era" is certain. Nuclear energy has reached a plateau in its contribution to the energetic mix, and presumably will not expand largely its weight in the next years.

Renewable sources seem, at the moment, the only possible answer to fill the gap between the future energy demand and the sources available (Figure 2).

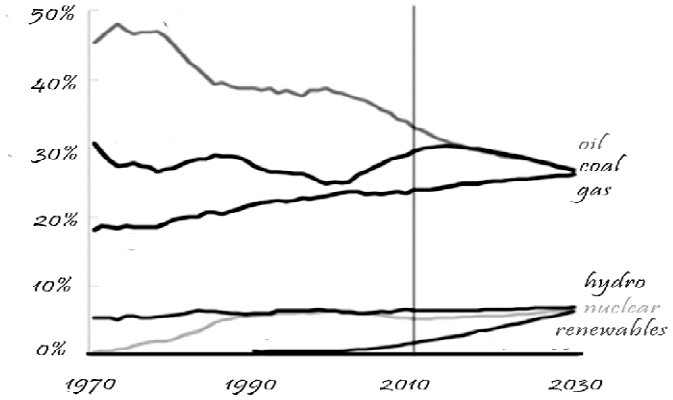

Figure 1. shares of world primary energy in the last decades. * including biofuels. Data from Energy Outlook 2030 [BP p.l.c., 2010].

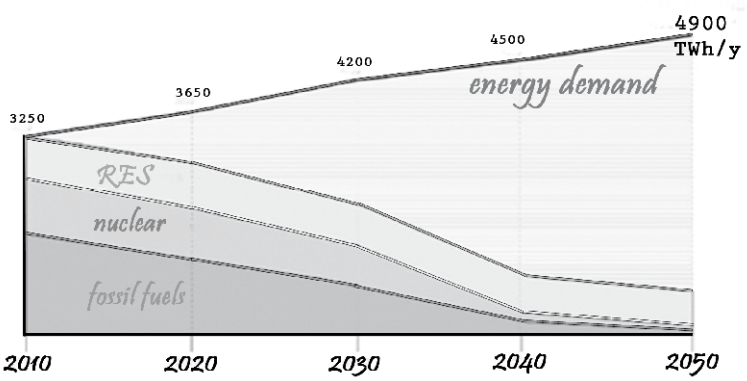

Figure 2. electricity demand (in TWh per year) predictions up to 2050 for the European Union (27 Countries plus Norway and Switzerland) [European Climate Fundation]. RES: renewable energy sources.

2. Sustainable development and renewable energies

With the expression "sustainable development" we refer to a way of development of our society (including economical development, as well as social and environmental) that will not compromise the possibility for the future generations of keeping the positive trend of development, preserving the same availability to the heritage of resources as the previous generations. The main objective is, thus, the preservation and conservation of the natural resources in order to allow the next generations to have the same opportunity of developing in their economy, society and rights.

Though, there is a fundamental disconnect between the ongoing discussion on energy policy and the clear and present energy requirements of the 21st century global economy. A very big attention is nowadays reserved to carbon emissions, which have a worrying rising trend that needs to be arrested in order to slow down the global worming effects that we are assisting to in these days.

Indeed, at Copenhagen conference in 2009, for instance, countries around the world pledged dramatic reductions in carbon emissions well below 2005 levels [World Energy Counsil, 2010].

That's all well and good, if the only issue is carbon emissions. But rarely those reduction goals are compared to the inescapable need to meet what many predict will be a massive increase in energy demand and consumption in the coming decades (see Paragraph 1).

As previously shown (Figure 2), the renewable energies stand as the energy source of our future, mainly because they will be the main energy source available, and not only because of their environmental compatibility.

In 2011, the IPCC [Intergovernmental Panel on Climate Change, 2011] analyzed the potentialities of renewable energies stating that each of any renewable energy has enough or larger potential than the global energy need. An accurate policy of incentive on the development of technologies able to exploit these kinds of sources is not only desirable, but mandatory.

In the scenery of renewable energies exploitation, an efficient and not polluting energy vector will be needed in order to compensate the possible temporary lacks of the single renewable sources.

3. H_2 as an energy vector

Recently there has been international attention on the development of new hydrogen technologies as a potential solution to the current fears and to increase energy and economic security. For example the U.S. Department of Energy has developed a multiyear plan with aggressive milestones and targets for the

development of hydrogen infrastructure, fuel cells, and storage technologies. The targeted hydrogen cost is \$2–4 $kg^{-}1$ (energy equivalent of 1 gallon of gasoline) delivered [U.S. Department of Energy Hydrogen 2006, 2007].

Mostly used for chemical applications, hydrogen has taken center stage on the energy scene. Used in association with fuel cells, it could replace the conventional duo formed by hydrocarbons and combustion systems (engines, turbines, etc.).

The benefits of such a breakthrough would be to:

– Decrease pollution in urban areas;

– Sharply reduce greenhouse gas (GHG) emissions;

– Increase the energy independence of oil consuming countries.

But hydrogen cannot be compared directly with fossil energies, because it is only an energy vector and not an energy source. As such, it simply makes it possible to transmit a given quantity of energy from the place of production to the place of consumption.

3.1 Hydrogen

Hydrogen is the simplest element of our Universe, and constitutes roughly 75% of the Universe's chemical elemental mass. At a molecular state it constitutes a colorless, odorless and non toxic gas. Burnt it reacts with the oxygen in the air to give water (1) with a enthalpy of combustion of −286 kJ/mol [Vargaftik, 1975].

$$H_2 + ½\ O_2 \longrightarrow H_2O \qquad (1)$$

Despite its wide abundance in the Universe, molecular H_2 is scarcely available in our Planet; we find it usually covalently bound in water molecules and in many organic and inorganic compounds in our environment. Hydrogen needs, thus, to be extracted from other compounds with various chemical reactions that all require a large amount of energy (to be treated in paragraph 3.3).

The wide interest in H_2 is due not only to the sustainability of its utilization (no carbon neither toxic emissions), but also to the high calorific power of the molecule (see Table 1). It is important to stress, though, that the amount of energy contained by hydrogen is almost three times the energy contained in gasoline, but only on mass basis. If we convert it into volume units it dramatically decreases, not only for gaseous hydrogen but for liquid hydrogen as well (Table 1).

Table 1. Comparison of energy content and carbon emissions for the main fuels available [Vincenzini et al. 1981].

Fuel	Energy per mass unit (J/kg)	Energy per volume unit (J/m^3)	C specific emission (kg C/ kg fuel)
Liquid hydrogen	141,90	10,10	0,00
Hydrogen gas	141,90	0,013	0,00
Naphtha	45,50	38,65	0,84
Gasoline	47,40	34,85	0,86
Methanol	22,30	18,10	0,50
Ethanol	29,90	23,60	0,50
Bio diesel	37,00	33,00	0,50
Natural gas	50,00	0,04	0,46
Coal	30,00	-	0,50

3.2 Fuel cells

Over the last decade, the tendency to increase the flexibility of electricity generation, and the increase of the world's population have led to an increased interest in the development of more powerful and finely distributed power generation. It is expected that decentralized power plants will reduce both the capital cost for the installer as well as improve the overall efficiency due to the possibility of the co-generation of electricity and heat [Carrette et al. 2001]

One of the major factors that has influenced the development of fuel cells has been the increasing concern about the environmental consequences of fossil fuel use in production of electricity and for the propulsion of vehicles, and their limited availability. More importantly, however, is the increasing global awareness of how human activities influence the environment and how a sustainable development can be achieved with a tremendously increasing world population.

Fuel cells are galvanic cells, in which the free energy of a chemical reaction is converted into electrical energy (via an electrical current). The Gibbs free energy change of a chemical reaction is related to the cell voltage via (2):

$$\Delta G = -nF\Delta U_0 \qquad (2)$$

where n is the number of electrons involved in the reaction, F is the Faraday constant, and ΔU_0 is the voltage of the cell for thermodynamic equilibrium in the absence of a current flow. It has to be said that a small amount of energy is dissipated, generating heat.

The anode reaction in fuel cells is either the direct oxidation of hydrogen or the oxidation of methanol (see Table 2). An indirect oxidation via a reforming step can also occur. The cathode reaction in fuel cells is oxygen reduction, in most cases from air. For the case of a hydrogen/oxygen fuel cell the principle is shown in Figure 3 and the overall reaction is (1).

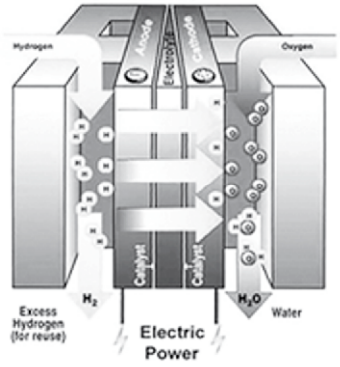

Figure 3. schematic drawing of an hydrogen/oxygen fuel cell and its reactions based on the proton exchange membrane fuel cell (PEMFC-Proton Exchange Membrane Fuel Cell).

Table 2. the different fuel cells that have been realized and are currently in use and development [Carrette et al. 2001].

	AFC (Alkaline)	PEMFC (Proton Exchange Membrane)	DMFC (Direct Methanol)	PAFC (Phosphoric Acid)	MCFC (Molten Carbonate)	SOFC (Solid Oxide)
Operating temp. (°C)	<100	60-120	60-120	160-220	600-800	800-1000 (also 500-600 possible)
Applications	Transportation, Space, Military, Energy storage systems			Combined heat and power for decentralized stationary power systems	Combined heat and power for stationary decentralized systems and for transportation (trains, boats, …)	
Realised power	Small plants 5-150 kW modular	Small plants 5-250 kW modular	Small plants 5 kW	Small-medium plants 50 kW--11 MW modular	Small power plants 100 kW--2 MW	Small power plants 100-250 kW
Charge carrier in the electrolyte	OH-	H+	H+	H+	CO32-	O2-

Fuel cells are usually classified by the electrolyte employed in the cell. An exception to this classification is the DMFC (Direct Methanol Fuel Cell) which is a fuel cell in which methanol is directly fed to the anode. The electrolyte of this cell is not determining for the class.

PEM (Proton Exchange Membrane) fuel cells, also called SPEFC (Solid Polymer Electrolyte Fuel Cells) use a proton exchange membrane as an electrolyte and is schematically represented in Figure 4. They are low-temperature fuel cells, generally operating between 85±105 °C and were the first to be used in Space. PEM fuel cells are considered to have the highest energy density of all the fuel cells, and due to the nature of the reaction have the quickest start up time (less than 1 sec) so they have been favored for applications such as vehicles, portable power and backup power applications [Carrette et al. 2001].

3.3 Hydrogen production methods

As previously introduced, hydrogen is an energy vector as it needs to be produced in order to be used as an eco-compatible fuel. Anyway, at the present time no sustainable process of large-scale production is available.

Most part of the hydrogen currently produced derives from fuel processing technologies that convert a hydrogen containing material into a hydrogen rich stream. Those materials are commonly hydrocarbons, ammonia or methanol. Hydrogen can also be produced from alternative resources such as biomass or water, but the overall production process still results ecologically un-sustainable.

The most extensive hydrogen production method is the steam reforming of hydrocarbons (mainly natural gas) that typically undergo a desulfurization process before they can be used to feed the process.

The steam reforming uses relatively low temperatures (>180°C for methanol, >500°C for other hydrocarbons) if compared with other processes like the partial oxidation (POX) of hydrocarbons (1300-1500°C) and has a higher hydrogen production efficiency (85% from methane, compared to 60-75% of POX). As can be easily understood, though, all of these processes entail large carbon emission, not only CO_2 emissions but also CO. A water-gas-shift process has to be often included to tear down the CO content in the out-coming gas and to increase H_2 content [Holladay et al., 2009].

Non-reforming hydrogen production processes, as previously mentioned, involve biomass or water.

Gasification technology, commonly used with biomass and coal, is very mature and commercially used in many processes. It is based upon partial oxidation of the materials into a mixture of hydrogen, methane, carbon monoxide, carbon dioxide, and nitrogen known as a producer gas. Gasification, even at high temperatures of 800–1000 °C, produces a significant amount of tar in the product gas. Therefore, a secondary reactor, which utilizes calcined dolomite or nickel catalysts, is used to catalytically clean and upgrade the product gas [Asadullah et al., 2002; Demirbas, 2006].

Water splitting in its simplest form uses an electrical current passing through two electrodes to break water into hydrogen and oxygen. Commercial low temperature electrolyzers have system efficiencies of 56–73% (70.1–53.4 kWh/kg H_2 at 1 atm and 25 8C) [Turner et al., 2008]. Currently, electrolysis is more expensive than using large-scale fuel processing techniques to produce hydrogen. And, if non-renewable power generation is used to make the electricity for electrolysis, it actually results in higher emissions compared to natural gas reforming [Bradley et al., 2000; Koroneos et al., 2004].

Thus, no sustainable process is available at the moment to produce hydrogen on a large scale. Researchers is the last decades have been looking for alternatives in biological processes that have a theoretical larger sustainability and lower theoretical costs.

4. Biological H_2 production

Four different groups of microorganisms are involved in biological hydrogen production processes: aerobic green algae (eukaryotes), cyanobacteria (blue-green algae), chemotrophic bacteria and anaerobic photosynthetic bacteria (mostly Gram negative prokaryotes) [Das and Verziroglu, 2008]. The two last processes can

be combined in a multi-step production process. A comparison of the production rates of the different biological production processes (discussed in detail in the following paragraphs) is presented in Table 3.

Table 3. H_2 synthesis rates for the four different kinds of biological production processes [Sasikala et al., 1991]

Production system	Synthesis rate (mmol H_2 g^{-1} h^{-1})
Biophotolysis (microalgae)	0,3 - 2,0
Biophotolysis (cyanobacteria)	1,5 – 2,0
Photofermentation	2,0 – 7,0
Dark fermentation	7,0 – 30,0

4.1 Biophotolysis

Hydrogen evolution by green algae and cyanobacteria can be divided into direct and indirect biophotolysis.

4.1.1 Direct biophotolysis

Hydrogen production through direct biophotolysis is a biological process that splits water to produce hydrogen and oxygen by utilizing solar energy. Green algae contain Photosystem II and Photosystem I for capturing light energy and perform oxygenic photosynthesis like higher plants. In absence of oxygen, electrons from reduced ferredoxin (Fd(red)) can also be used by the hydrogenase to reduce protons and evolve hydrogen (3):

$$2H_2O + h\nu \rightarrow O_2 + 4H^+ + Fd\ (red)\ (4e^-) \rightarrow Fd\ (red)\ (4e^-) + 4H^+ \rightarrow$$
$$\rightarrow Fd\ (ox) + 2H_2 \qquad (3)$$

Partial inhibition of PSII can generate anaerobic condition for the cell within a photobioreactor, as there is less water oxidation activity to evolve O_2 and the residual O_2 is used by respiration [Wykoff et al., 1998]. In a ground breaking work of Melis and his coworkers it was found that sulfur deprivation inhibits PSII activity that led to anaerobic conditions within a photobioreactor [Melis et al., 2000; Melis, 2002]. Kyle et al. showed that photoinhibition is accompanied by selective loss of a 32-kDa protein (later identified as the PSII reaction centre protein D1) followed by activation of the reaction centre through rapid inbuilt repair mechanism [Kyle et al., 1984]. In sulfur deprivation, re-biosynthesis of the D1 protein after loss is inhibited due to the scarcity of cysteine and methionine. Anaerobiosis induces the expression of [FeFe]-hydrogenase in algal cells [Happe and Kaminski, 2002] and sustained hydrogen production can be achieved [Melis et al., 2000; Ghirardi et al., 2000]. Sulfate permease mutant can evolve H_2 without depleting sulphate in the culture media [Chen et al., 2003]. Some photosystem II inhibitors have also been used to inhibit water oxidation activity [Happe and Naber, 1993]. Instead of acetate, which is normally used in the media as a carbon source, CO_2 emitted in industries can be cou-

pled to the system as it will be less expensive in large scale H_2 production as well as helpful for CO_2 removal [Skjanes et al., 2007].

4.1.2 Indirect biophotolysis

Indirect biophotolysis is a very efficient process to separate O_2 and H_2 evolution phases. During the first stage, the photosynthetic processes produce carbohydrates, providing mitochondrial respiration and cell growth. During the second stage, under anaerobic conditions, H_2ase expression is induced. The general reaction is as follows (4-5):

$$12H_2O + 6\,CO_2 \rightarrow C_6H_{12}O_6 + 6O_2 \quad (4)$$
$$C_6H_{12}O_6 + 12H_2O \rightarrow 12H_2 + 6\,CO_2 \quad (5)$$

In anaerobic dark conditions, pyruvate ferredoxin oxidoreductase (PFOR) responsible for decarboxylation (CO_2 evolution) of pyruvate to acetyl-CoA is linked to H_2 production via reduction of ferredoxin. In presence of light, ferredoxin is reduced by NADH produced during catabolism of pyruvate by

the pyruvate dehydrogenase (PDH).

The N_2-fixing cyanobacteria produce hydrogen mainly by nitrogenase (fixing N_2 to NH_3) instead of bidirectional hydrogenase, however in several non-N_2-fixing cyanobacteria, H_2 evolution is also observed through bidirectional hydrogenase [Tamagnini et al., 2002; Tamagnini et al., 2007]. In filamentous cyanobacteria, nitrogenase is located in the heterocyst with a functional PSI (no PSII activity). The electrons are donated to PSI in the heterocyst come from reserve carbon transported from the neighbor vegetative cell.

However, the hydrogen production is energetically burden due to the biosynthesis and maintenance of the heterocysts and the significant ATP requirement of nitrogenase (6-7).

$$N_2 + 8H^+ + Fd\,(red)\,(8e^-) + 16\,ATP \rightarrow 2NH_3 + H_2 + Fd\,(ox) + 16ADP + Pi \quad (6)$$
$$8H^+ + 8e^- + 16\,ATP \rightarrow 4H_2 + 16ADP + 16\,Pi \quad (7)$$

Heterocyst provides spatial separation of O_2 and H_2 evolution. Non-heterocystous cyanobacteria can separate O_2 and H_2 production in time (temporal separation). It has been found that nitrogenase can be inactivated after a sudden and short-term exposure to high oxygen concentrations [Stal and Krumbein, 1985; Stal and Krumbein, 1987].

4.2 Dark fermentation

The production of hydrogen by heterotrophic fermentations can be carried out by a wide variety of microorganisms such as strict anaerobes, facultative anaerobes and aerobes, the latter only under anoxic conditions. A wide variety of organic substrates can be used by these microorganisms for the production of hydrogen.

Much is presently known about the molecular biology and biochemistry of the hydrogen-producing enzymes, reductant generating systems, and physiology of many hydrogen-producing fermentative microorganisms [Hallenbeck, 2001].

The majority of microbial hydrogen production is driven by the anaerobic metabolism of pyruvate, formed during the catabolism of various substrates. The breakdown of pyruvate is catalyzed by one of two enzyme systems (8-9):

1: Pyruvate:formate lyase (PFL)

$$\text{Pyruvate} + \text{CoA} \rightarrow \text{acetyl}-\text{CoA} + \text{formate} \quad (8)$$

2: Pyruvate:ferredoxin (flavodoxin) oxidoreductase (PFOR)

$$\text{Pyruvate} + \text{CoA} + 2\text{Fd (ox)} \rightarrow \text{acetyl}-\text{CoA} + CO_2 + 2\text{Fd(red)} \quad (9)$$

Thus in both these biological systems, the pyruvate generated by glycolysis is used, in the absence of oxygen, to produce acetyl CoA, from which ATP can be derived, and either formate or reduced ferredoxin (Fd (red)), from which hydrogen can be derived. The enteric bacteria derive hydrogen from formate and strict anaerobes derive hydrogen from Fd (red). The overall yields in these metabolisms are relatively low; one to two hydrogen produced per molecule of pyruvate. For one thing, this is a natural consequence of the fact that fermentations have been optimized by evolution to produce cell biomass and not hydrogen. Thus a portion of the substrate (pyruvate) is used in both cases to produce ATP giving a product (acetate) that is excreted. Also, in many organisms the actual yields of hydrogen are reduced by hydrogen recycling due to the presence of one or more uptake hydrogenases, which consume a portion of the hydrogen produced. It is unknown to what extent hydrogen production could be increased through metabolic engineering and manipulation of culture conditions [Hallenbeck and Benemann, 2002].

The major issue is the feasibility of a dark fermentative reaction yielding close to the 12 mol of H_2 stored in each molecule of glucose metabolized. From a thermodynamic perspective, the reaction is unfavorable owing to the positive value of $\Delta G^{0'}$(10).

$$C_6H_{12}O_6 + 6\ H_2O \rightarrow 12H_2 + 6CO_2 \quad (10)$$
$$\Delta G^{0'} = +3.2\ \text{kJ mol}^{-1}$$

Thus, it is only possible a partial oxidation of glucose to acetate, CO_2 and H_2, with a maximum yield of 4 mol hydrogen per mol of glucose and a negative value of $\Delta G^{0'}$: this is described in the reaction (11) and schematically represented in Figure 7.

$$C_6H_{12}O_6 + 2\ H_2O \rightarrow 2C_2H_4O_2 + 4H_2 + 2CO_2 \quad (11)$$
$$\Delta G^{0'} = -206.3\ \text{kJ mol}^{-1}$$

The subsequent oxidation of acetate to CO_2 and H_2 is thermodynamically unfavorable, owing to a very positive value of $\Delta G^{0'}$ (12):

$2C_2H_4O_2 + 4\ H_2O \rightarrow 8H_2 + 4CO_2$ (12)
$\Delta G^{0'} = +104.6$ kJ mol^{-1}

More fundamentally, a near-stoichiometric yield is only achievable under near equilibrium conditions, which implies very slow rates, and/or at very low partial pressures of H_2. Indeed, this was recently demonstrated with an in vitro system using bacterial PPP enzymes by Woodward et al. (2000), who obtained stoichiometric H_2 yields from glucose under such conditions.

Fermentative processes can use either biomass obtained in a first stage light conversion process (e.g. higher plant or microalgae biomass high in starches or other fermentable substrates) or perhaps more attractively, various waste streams. These present an interesting yet largely unexplored avenue for the biological production of hydrogen.

4.3 Photofermentation

4.3.1 Purple Non-Sulfur Bacteria (PNSB): systematics, habitats and main metabolic features.

Purple non-sulfur bacteria (PNSB) are anoxygenic phototrophic bacteria that contain photosynthetic pigments and are able to perform anoxygenic photosynthesis under anoxic conditions; they mainly belong to the taxonomic group *Alphaproteobacteria* , even if many species belong to the *Betaproteobacteria* (respectively 18 and 3 genera have been recognized for each group, as described by Madigan and Jung (2008). The *Alphaproteobacteria* are divided into three subgroups: α-1 for *Rhodospirillum* and relatives, α-2 for *Rhodopseudomonas* and relatives and α-3 for *Rhodobacter* and relatives [Imhoff, 2006]. Indeed, it is a very diverse group as regards morphology, internal membrane structure, carotenoid composition, utilization of carbon sources and electron donors, cytochrome c structures, lipid composition, quinone composition, lipopolysaccharide structure and fatty acid composition [Imhoff, 1995].

PNS bacteria can be found in aquatic environments rich in organic soluble matter as lakes, waste water ponds, costal lagoons. Some representatives can also be found in sediments and moist soils, and some even in marine and hyper saline environments. They usually occur in temperate habitats, but some PNS bacteria reside in thermal springs and in cold polar waters [Imhoff, 2006].

However, eutrophic ponds are the most common habitat where, only occasionally, PNS bacteria can form dense blooms; more frequently they inhabit the anoxic or low-oxygen tension layers of water bodies. At the same time, a suitable light irradiation is preferred even if not strictly necessary.

The publication of the first complete genome sequence of a purple non-sulfur bacterium, *Rhodopseudomonas palustris* [Larimer et al., 2004], pointed out the metabolic versatility of these bacteria. Such a complexity of metabolic pathways requires further discussion.

The specificity of purple bacteria is their ability to form their energy carrier (ATP) in absence of oxygen by using sunlight as a source of energy. All PNS bacte-

ria can grow photoheterotrophically using reduced carbon compounds as electron donors and carbon source; some species can also grow photolithoautotrophically using S^{2-}, H_2 or Fe^{2+} as electron donors and CO_2 as the sole carbon source [Larimer et al., 2004].

The cycle of anoxygenic photosynthesis is presented schematically in Figure 4: a photon stimulates the excitation of bacteriochlorophylls in the reaction center and this energy is used for the release of an electron which reduces the quinone Q. Once the quinone is doubly reduced (i.e. after a second photon is captured) it picks up protons from the cytoplasmic space and translocates through the membrane to reach the cytochrome bc_1 complex: here electrons are addressed to the cytochrome c_2 (Cyt c_2) while protons are released in the periplasmic space. Cyt c_2 is then able to reduce the oxidized primary electron donors in the RC, thus closing the cycle. The protons accumulated in the periplasm form an electrochemical gradient which is used by the ATP-synthase to generate ATP.

The reduced quinones can open the cycle making the NADH dehydrogenase working in the "reversed" way to reduce NAD^+ to NADH, and the succinate dehydrogenase can also work "backwards" reducing fumarate to succinate (processes not shown in Figure 5). The reversed NADH dehydrogenase reaction is also the way to refurnish the cell with NADH reducing equivalents.

In the presence of O_2, anoxygenic photosynthesis in purple bacteria is inhibited and the ATP is synthesized through cellular respiration. Under dark anoxic conditions, electron acceptors other than oxygen can be used for respiration: some conventional substrates, such as sulfur and nitrogen compounds, and some "exotic" substrates like DMSO (dimethylsulfoxide), TMAO (trimethylamine-N-oxide) and even arsenate and halogenated aromatics [Zannoni et al., 2008].

Depending on the metabolic mode PNS bacteria carry out, carbon compounds have different roles being not only a carbon source but also a source of reducing power. In photoheterotrophy they cover both roles, but if some inorganic electron donor is present, carbon is exclusively assimilated. During respiration, carbon compounds are mainly oxidized, and only a small part is assimilated. It has to be stressed that PNS bacteria are also able to fix CO_2 in autotrophic conditions, using RuBisCO to refurnish the cell with organic carbon, but some activity of this enzyme has also been registered in heterotrophic growth in order to equilibrate the redox state [Tabita, 1995].

PNS bacteria are able to use a wide variety of organic carbon compounds, namely the intermediates of the tricarboxylic acid cycle, pyruvate and acetate, organic acids, amino acids, alcohols and carbohydrates; some of them are highlighted in frames in Figure 6. Some species can also use one-carbon atom compounds such as methanol and formate, while some other species grow using aromatic organic compounds such as benzoate, cinnamate, chlorobenzoate, phenylacetate or phenol [Harwood, 2008].

This versatility is not surprising, considering the variety of natural environments in which PSN bacteria have been found, as above reported.

The preferred way to assimilate nitrogen is fixation through nitrogenase that reduces nitrogen to ammonia. The enzyme produces hydrogen as a byproduct, but

also functions in absence of molecular nitrogen using protons as electron acceptors to dissipate the excess of reducing power in the cell.

Hydrogen can also be an electron donor for purple bacteria, oxidized by a membrane bound enzyme, named hydrogenase. The reaction can take place in both directions, depending on the presence or absence of the substrates. A further discussion of these two enzymes is in paragraph 4.3.2.

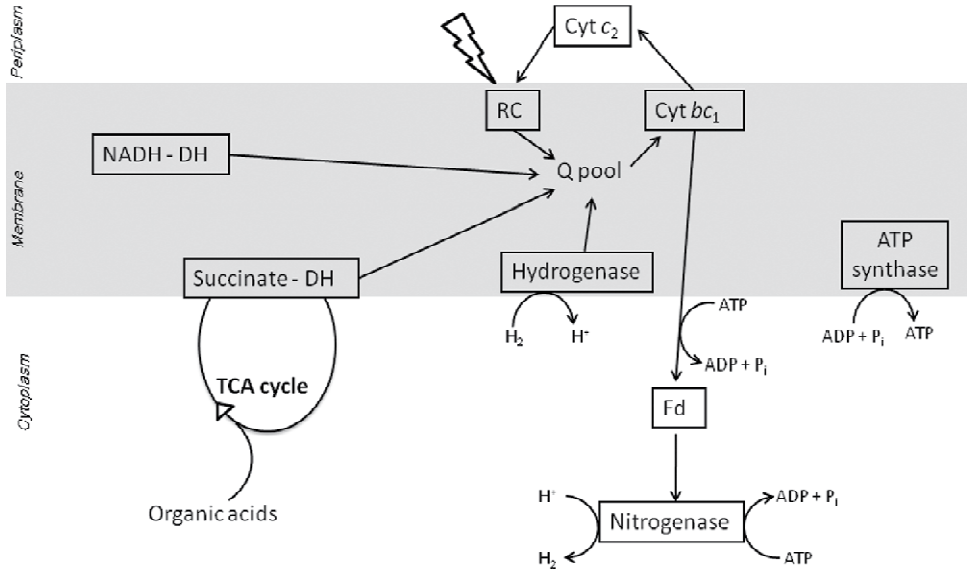

Figure 4. Main processes related to hydrogen production, under photoheterotrophic growth in non-nitrogen fixing conditions: anoxygenic photosynthesis, ATP synthesis, TCA cycle, hydrogenase and nitrogenase activities. The straight black arrows indicate the electron flow. The lightning symbol indicates light excitation. Abbreviations: Cyt bc_1=cytochrome bc_1 complex; Cyt c_2= cytochrome c_2; Fd= ferredoxin; RC= Reaction Center; Succinate – DH= succinate dehydrogenase; NADH-DH=NADH dehydrogenase (Figure from Adessi and De Philippis 2012, with kind permission from Springer Science+Business Media BV).

4.3.2 Enzymes involved in hydrogen production

It is known that nitrogen fixation is related to hydrogen production, for example it was calculated that 1 million tons of H_2 per year is produced by nodule bacteria [Evans et al., 1987] well known as nitrogen fixing microorganisms.

Nitrogenase is the enzyme that, in all the N_2-fixing prokaryotes, including PNS bacteria, is responsible for hydrogen production, catalyzing the reaction (13) that leads to the production of one H_2 molecule per molecule of N_2 fixed.

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP \qquad (13)$$

Nitrogenase is a two-protein complex consisting of a dinitrogenase containing Fe and Mo as cofactors and having a molecular weight of 250 kDa, and of a dinitrogenase reductase (containing Fe) of about 70 kDa. Some alternative

nitrogenases have been described by Larimer et al. (2004), namely a Vanadium nitrogenase and a Fe-only nitrogenase. The three isozymes produce different ratios of hydrogen and ammonia [McKinlay and Harwood 2010a] as shown in reactions (14-16).

Mo-nitrogenase: $N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP$ (14)

V-nitrogenase: $N_2 + 12H^+ + 12e^- + 24ATP \rightarrow 2NH_3 + 3H_2 + 24ADP$ (15)

Fe-nitrogenase: $N_2 + 24H^+ + 24e^- + 48ATP \rightarrow 2NH_3 + 9H_2 + 48ADP$ (16)

Nitrogenase catalyses a very expensive reaction in terms of energy, and thus it is very strictly regulated by the presence of dissolved ammonium ions. The regulation of nitrogenase is described later (paragraph 5.4.1).

As it has been shown in Figure 5, in absence of molecular nitrogen the enzyme, catalyzing the reaction (17), dissipates the excess of reducing equivalents deriving from other metabolic processes.

$8H^+ + 8e^- + 16ATP \rightarrow 4H_2 + 16ADP$ (17)

This is the reaction used for hydrogen production processes; as shown in Figure 5, nitrogenase under non-nitrogen fixing conditions uses ATP and electrons deriving from the cyclic photosynthesis: the electrons are transferred to nitrogenase by ferredoxins that have been previously reduced in an ATP-consuming reaction.

Usually nitrogen fixing microorganisms also possess a mechanism to uptake the hydrogen produced in case of need of reducing power dividing it into electrons and protons (18) through the activity hydrogenase, a membrane bound enzyme, able to catalyze the reaction in both directions.

$H_2 \rightarrow 2H^+ + 2e^-$ (18)

High hydrogenase activities have been observed in cells possessing an active nitrogenase; the hydrogen produced by the nitrogenase stimulated the activity of hydrogenase in growing cells even though the synthesis of hydrogenase is not closely linked to the synthesis of nitrogenase [Colbeau, 1980].

The hydrogenases are iron-sulfur proteins distributed into two main phylogenetically distinct classes, the [NiFe]-hydrogenases and the [FeFe]-hydrogenases, which contain respectively a Ni and a Fe atom or two Fe atoms at their active site. The [NiFe]-hydrogenases are the most studied and the kind most frequently found in photosynthetic bacteria.

The synthesis of these enzymes occurs under anaerobic conditions and it is usually negatively regulated by O_2. [NiFe]-hydrogenases are divided into four groups, according to Vignais (2008), based on the function they have: the most interesting for hydrogen production process is called uptake-hydrogenase. This enzyme is a respiratory enzyme, which recovers the electrons from the H_2 molecule reducing the membrane-soluble quinones; they are involved in anaerobic respiration.

In H_2 production processes, active uptake-hydrogenases are undesirable, as they affect the gas production: in particular an inactivation of such enzymes usually leads to an enhanced hydrogen production [Ooshima et al., 1998; Franchi et al., 2004; Kim et al., 2006a; Öztürk et al., 2006; Kars et al., 2008].

4.3.3 Conversion of substrates to hydrogen

As it has been described, hydrogen production in purple bacteria is related to many metabolic processes that deal with ATP generation (photosynthesis), carbon metabolism (TCA cycle and carbon fixation) and nitrogen fixation (see Figure 4).

As it was mentioned above, the preferred substrates for hydrogen production are the low-molecular weight organic acids that can easily enter the TCA cycle, which is very active during anaerobic photosynthetic growth.

The following scheme (Figure 5.) represents carbon metabolism in purple non-sulfur bacteria, even if not all species and genera follow this scheme: for example *Rp. palustris* doesn't have the Entner-Doudoroff pathway [Larimer et al., 2004].

Figure 5 shows also the presence of Calvin cycle in carbon metabolism. Joshi and Tabita (1996) demonstrated that the absence of the reductive pentose phosphate CO_2 fixation pathway enhances the synthesis of nitrogenase also in presence of ammonium ions, as the reduction of CO_2 is, in photoheterotrophy, just another way to dissipate the reducing power deriving from organic carbon compounds.

An important parameter to evaluate the yield of a hydrogen production process is the substrate conversion efficiency, calculated as the ratio between the moles of hydrogen produced and the moles theoretically obtainable if all the substrate was converted to CO_2 and H_2. Thus, considering the most common organic acids utilized in photofermentation processes [Barbosa et al., 2001], the conversion yields can be calculated from reactions (19-21):

Lactate:	$C_3H_6O_3 + 3H_2O \rightarrow 6H_2 + 3CO_2$	(19)
Acetate:	$C_2H_4O_2 + 2H_2O \rightarrow 4H_2 + 2CO_2$	(20)
Malate:	$C_4H_6O_5 + 3H_2O \rightarrow 6H_2 + 4CO_2$	(21)

It has to be stressed that these reactions are theoretical, because they are neither considering the utilization of the substrate for the growth neither limiting factors occurring in a culture. On the basis of these reactions, the gas expected should be composed of a 66.7% of H_2 and a 33.3% of CO_2 when growing on lactate and on acetate; a 60 % of H_2 and a 40% of CO_2 when growing on malate. Actually the gas phase above the culture is much richer in H_2 then in CO_2, due to a partial solubilization of CO_2 in the culture medium and also to a partial fixation to CO_2 for anabolic reactions [McKinlay and Harwood, 2010a].

The substrate conversion efficiency is strongly affected by the C/N ratio in the culture. Indeed, a high C/N ratio in the culture media usually leads to higher hydrogen production compared with low C/N ratio, where a higher cell growth occurs [Redwood et al., 2009]. In the latter case, the conversion efficiency decreases due to the consumption of the organic acids for cell growth instead that for hydrogen production.

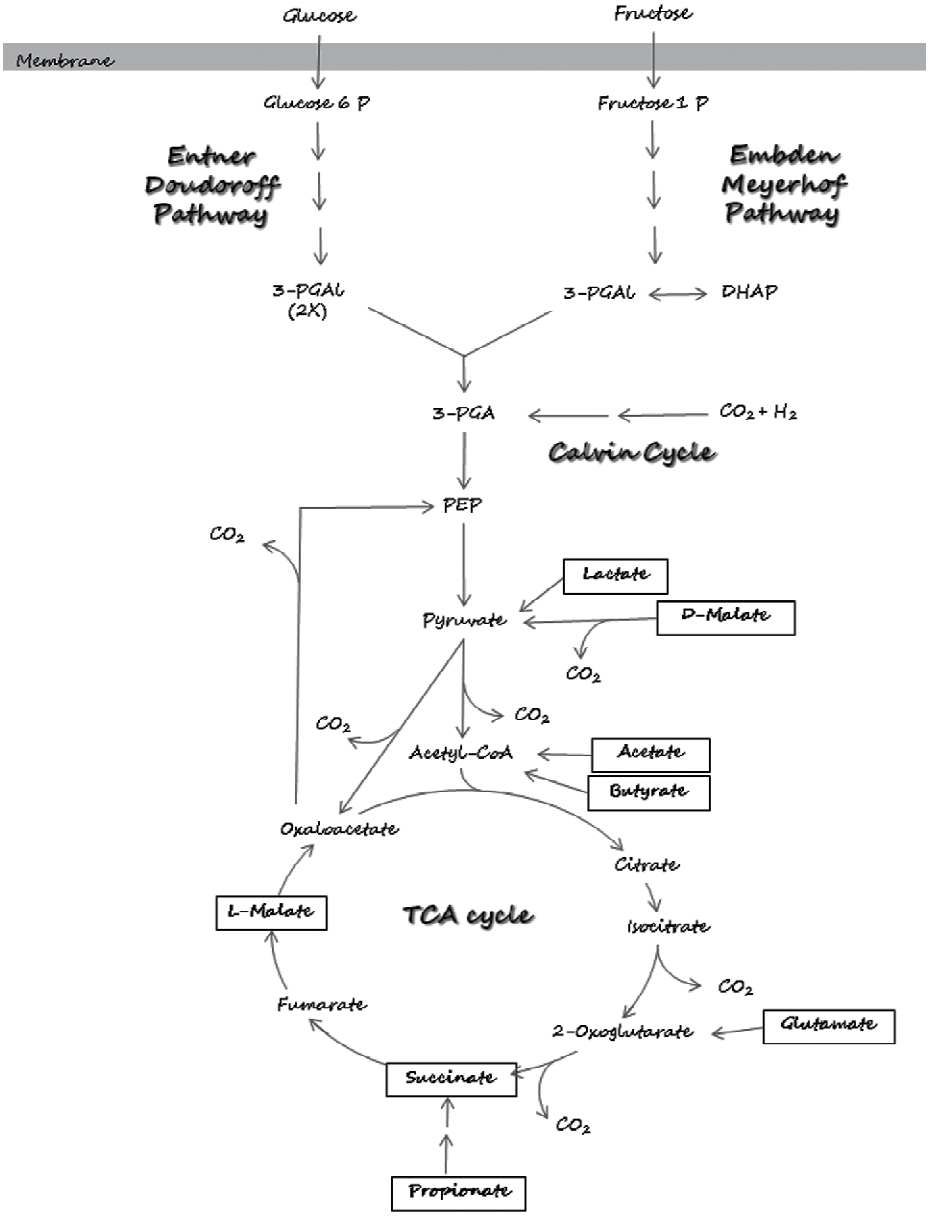

Figure 5. Carbon metabolism in PNS bacteria. Frames highlight some of the most common substrates metabolized by PNS bacteria. Abbreviations: 3-PGAL= glyceraldehydes-3-phosphate; DHAP=di-hydroxy-aceton-phosphate; 3-phospho-glyceric acid; phospho-enol-pyruvate (Figure from Adessi and De Philippis 2012, with kind permission from Springer Science+Business Media BV).

This problem becomes a very relevant matter when wastewaters or liquors deriving from other fermentation processes are utilized for the production of H_2 by means of photofermentation.

4.4 Combined systems

The thermodinamical unfeasibility of the complete oxidation of glucose by chemotrophic bacteria has been already treated in paragraph 4.3. The complete oxidation of glucose, though, becomes possible combining the dark fermentation process with photofermentation of acetate carried out by purple non sulfur bacteria, as the energy required for this endoergonic reaction can be obtained from sunlight. Thus, the combination of a heterotrophic fermentation performing reaction (11) and a photofermentation performing reaction (12) theoretically enables the total conversion of 1 mole of glucose into 12 moles of hydrogen. The combination of these two reactions is schematically represented in Figure 6.

4.4.1 The combined (dark + photofermentation) H_2 production processes

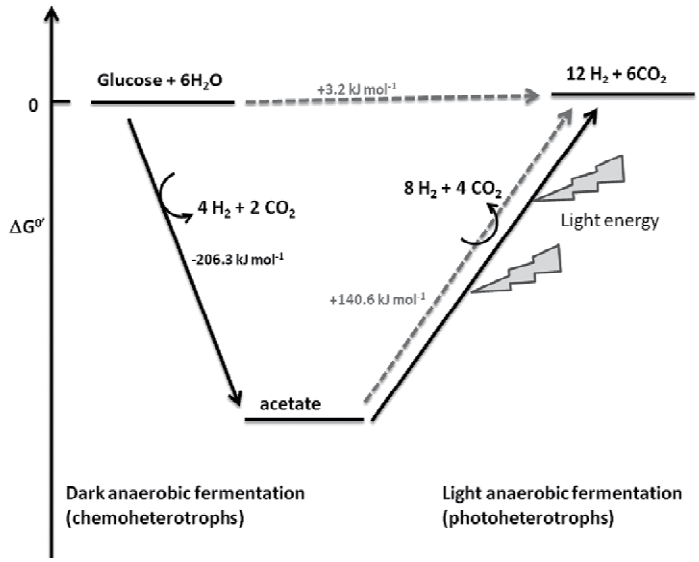

Figure 6. Complete oxidation of glucose by combining dark and light fermentations; on the y axis, Gibbs' free energy at standard conditions; the dark arrows indicate the thermodynamically favorable reactions; the grey dotted arrows indicate the thermodynamically unfeasible reactions [ΔG0' values derived from Thauer et al., 1977] (Figure from Adessi and De Philippis 2012, with kind permission from Springer Science+Business Media BV).

The use of combined processes, i.e. dark fermentation followed by photofermentation, for H_2 production offers the opportunity of exploiting the specific features of different microorganisms for obtaining the best results in terms of H_2 yield. In the following lines, the efforts to combine the dark fermentative process

with photofermentation for obtaining the largest amount of hydrogen possible are discussed. In particular, the attempts done for combining a first stage, where thermophilic or mesophilic chemoheterotrophic bacteria convert carbohydrates into H_2 and organic acids, with a second stage, where the fermentation products deriving from the first stage are converted to H_2 by photoheterotrophic bacteria, are described. A very complete and exhaustive summary of combined systems employed for hydrogen production has been recently given by Redwood et al. (2009).

4.4.2 Processes on synthetic media and related issues

The use of synthetic media gives the opportunity to investigate on some theoretical aspects of the combination and integration of the two different kinds of fermentation. In these studies, glucose was generally used as the starting substrate, but sucrose was used as well (Table 4). The best result so far obtained with combined systems operating with synthetic media was the achievement of an overall productivity of 7.1 moles of H_2 per mole of hexose [Chen et al., 2008], where the most relevant role was played by the photosynthetic bacterium *Rhodopseudomonas palustris* WP3-5, which produced 5.2 moles H_2 per mole of glucose. This result was obtained by using a very complex illumination system, composed of tungsten and halogen lamps in addition to side-light optical fibres and clay particles in the medium, to enhance photofermentation by means of an efficient light distribution in the photobioreactor.

Another interesting issue comes from the comparison of the data reported by Redwood and Macaskie in 2006 and in 2007a,b (Table 4): in the first paper, no H_2 production was observed in the second stage of the combined system tested, as the effluent coming from the dark fermentation was rich in nitrogen sources that inhibited hydrogen production; however, in the following experiments [Redwood and Macaskie, 2007a,b], the NH_4^+ was removed by electro-separation during the passage from the fist to the second stage, and photo-hydrogen production was obtained. These results strongly point out the crucial importance of removing nitrogen sources, and in particular ammonia, from the dark-fermentation effluent for achieving an efficient hydrogen production with combined systems.

Even if the overall productivity was not reported by the Authors, the results obtained by Nath et al. (2005) and Nath et al. (2008) showed a significant increase of hydrogen production in the dark fermentative stage, probably due to the enhancement in the cultivation mode that passed from a 500 ml Erlenmeyer flask to a 600 ml custom designed vertical tubular bioreactor.

It is also worth mentioning an experiment carried out by using the same microorganism (*Rp. palustris*) for the first and the second stage, taking advantage of the metabolic versatility of PNS bacteria capable of chemoheterotrophically growing also under dark conditions. Under these conditions, Oh et al. (2004) demonstrated the feasibility of using only one bacterium for the combined H_2 production, but the production rates obtained were too low for being economically acceptable.

4.4.3 Processes using low cost starting materials and relative issues

From an applicative point of view, the combined process represents an expensive process, even if the combination of the two systems is aimed at reducing the costs as the overall productivity is higher than the single productivities. However, costs can be further reduced by the use of low cost substrates instead of synthetic media.

In Table 5 are reported the combined processes that led to hydrogen production in both stages, utilizing as substrates low cost starting materials. A very interesting result was obtained by Kim et al. (2006b), who reported an overall productivity of 8.3 mol H_2/mol glucose using *Chlamydomonas reinhardtii* biomass as starting material. This process, which use microalgal biomass as starting material, could appear more expensive than the processes that use waste materials as substrate for H_2 production. However, it has to be stressed that the microalga can be grown using as carbon source the CO_2 emitted by some industrial plant, thus reducing the emission in the atmosphere of greenhouse gases. Then, the microalga, which has stored the fixed carbon dioxide in the biomass, can be fermented in a two stage process producing hydrogen, according to the results reported by Kim at al. (2006b), thus coupling the sequestration of CO_2 with the production of a clean energy vector like H_2.

Also the use of sweet potato starch residues [Yokoi et al. 2002] led to a very interesting overall productivity (7.2 mol H_2/mol glucose). Beet molasses have been used as well [Özgür et al. 2010b], obtaining a productivity of 6.85 mol H_2/mol glucose.

Three-phase processes can be performed in the case of using complex matrices due to the need of hydrolyzing the starting material before its use for dark fermentation. In these processes, the first phase can be either chemical (i.e. HCl pretreatment, as in Yang et al. 2010), enzymatic [Su et al. 2009b] or microbial (Lo et al., 2008 used *Caldimonas taiwanensis* On1 to hydrolyze starch), followed by the two fermentative stages.

From a general overview of Table 5, it emerges the occasional need of integrating the starting material with some specific compound in order to start or to speed up the process; in these cases, depending on what kind of modifications are needed for carrying out the process, the costs might increase.

4.4.4 Perspectives of combined H_2 production processes

Multiple organism-systems in two or three stages are realistically the most suitable processes for future biological energy production. As it emerges from the results reported in this paragraph, the microorganisms most frequently utilized to carry out the dark fermentative hydrogen production process are *Clostridia*, some *Enterobacter* (both mesophilic microorganisms) and some thermophilic bacteria such as *Caldicellulosiruptor* and *Thermotoga*. The possibility of working with facultative anaerobes, like *Enterobacter*, offers an easier process management than when working with strictly anaerobes like *Clostridia*; mesophilic are energetically less expensive than thermophilic processes, but the latter processes have on their side the

faster kinetics and the resistance to contaminants, that could balance the energetic costs.

For what concerns the photofermentative stage, purple non-sulfur bacteria are the most suitable organisms due to the high substrate conversion yields that can be achieved, the possibility to use a wide spectrum of the sunlight, and the wide variety of organic compounds that they can metabolize for hydrogen production.

Though the integration of these two processes seems to be the elected solution for biological production processes, some problems have to be overcome.

Actually, in addition to the limitations of the processes taken singularly, the integration brings to light some additional complications: first of all, the cultivation modes (i.e. the use of freely suspended or immobilized cells) have an influence on the integration strategy, as the microbial biomass operating in the first stage needs to be separated from the effluent before starting the second stage. Furthermore, the effluent has to be sterilized, if the aim is to carry out axenic processes with specific microorganisms, and properly treated (e.g. integrated with some limiting factors, or refined in nitrogen composition), thus increasing the operational costs. Finally, in most cases the fermentation product coming from the first stage, in particular when the dark fermentation was carried out with waste-derived substrates, is dark colored and rich in particles in suspensions, thus strongly reducing the light availability for PNS bacteria.

As a conclusion, it has to be stressed that, having a clear outlook of the big potential of the two-stage processes but also of the open problems still to be solved, is a good starting point for addressing future researches aimed at improving current biological systems for hydrogen production.

Table 4. Two stage hydrogen production processes using synthetic (with glucose or sucrose) media; microorganisms utilized, H_2 production in the 1st stage (dark fermentation), H_2 production in the 2nd stage (photofermentation) and overall H_2 production of the combined systems. Data are expressed as moles of H_2 produced per mole of glucose or of hexose consumed.

1st stage – inoculum	1st stage – H_2 production	2nd stage – inoculum	2nd stage - H_2 production	Overall production	Ref.
Mesophilic processes					
Clostridium butyricum NCIB 9576	1.29 mol H_2/mol glucose	*Rhodobacter sphaeroides* E151	0.36 mol H_2/mol glucose	1.65 mol H_2/mol glucose	Kim et al. (2001)
Rhodopseudomonas palustris P4 (dark-adapted)	0.041 mol H_2/mol glucose	*Rhodopseudomonas palustris* P4 (light- adapted)	10% efficiency[(a)]	Doubled dark-fermentation results[(b)]	Oh et al. (2004)
Enterobacter cloacae DM11	1.86 mol H_2/mol glucose	*Rhodobacter sphaeroides* O.U. 001	1.5-1.72 mol H_2/mol	n.a.[(c)]	Nath et al. (2005)

			acetic acid[b]		
Enterobacter cloacae DM11	3.31 mol H_2/mol glucose	*Rhodobacter sphaeroides* O.U. 001	1.5-1.72 mol H_2/mol acetic acid[b]	n.a. [c]	Nath et al.(2008)
Anaerobic bacteria (mixed culture)	1.36 mol H_2/mol glucose	*Rhodopseudomonas capsulata*	3.2 mol H_2/mol glucose	4.56 mol H_2/mol glucose	Shi and Yu (2006)
Escherichia coli HD701	0.4 mol H_2/mol glucose	*Rhodobacter sphaeroides* O.U. 001	No H_2	0.4 mol H_2/mol glucose	Redwood and Macaskie (2006)
Escherichia coli HD701	1.6 mol H_2/mol glucose	*Rhodobacter sphaeroides* O.U. 001	0.83 mol H_2/mol glucose	2.4 mol H_2/mol glucose	Redwood and Macaskie (2007 a,b)
Cattle dung microflora (sucrose as a substrate)	1.29 mol H_2/mol hexose	*Rhodobacter sphaeroides* SH2C	63-70% efficiency[a]	3.32 mol H_2/mol hexose	Tao et al. (2007)
Clostridium pasteurianum (sucrose as a substrate)	1.90 mol H_2/mol hexose	*Rhodopseudomonas palustris* WP3-5	5.20 mol H_2/mol hexose	7.10 mol H_2/mol hexose	Chen et al. (2008)
Cattle dung microflora (sucrose as a substrate)	1.72 mol H_2/mol hexose	*Rhodobacter sphaeroides* ZX-5	4.54 mol H_2/mol hexose	6.26 mol H_2/mol hexose	Zong et al. (2009)
Ethanoligenens harbinense B49	1.83 mol H_2/mol glucose	*Rhodopseudomonas faecalis* RLD-53	4.49 mol H_2/mol glucose	6.32 mol H_2/mol glucose	Liu et al. (2009)
Clostridium butyricum	1.32 mol H_2/mol glucose	*Rhodopseudomonas palustris*	4.16 mol H_2/mol glucose	5.48 mol H_2/mol glucose	Su et al. (2009a)
Thermophilic processes					
Caldicellulosiruptor saccharolyticus	2.25 mol H_2/mol glucose	*Rhodobacter capsulatus* DMS1710	1.33 mol H_2/mol glucose	3.58 mol H_2/mol glucose	Özgür et al. (2010a)

[a] efficiency = substrate conversion efficiency. [b] the conversion efficiency was not referred to the moles of glucose initially present in the substrate. [c]n.a. = data on conversion efficiencies (moles of H_2 per mole of glucose) not available.

Table 5. Two stage hydrogen production processes using low cost starting material; substrates and microorganisms utilized, H_2 production in the 1st stage (dark fermentation), H_2 production in the 2nd stage (photofermentation) and overall H_2 production of the combined systems. Data are expressed as moles of H_2 produced per mole of hexose consumed.

Substrate	1° stage – inoculum	1° stage – H_2 production	2° stage – inoculum	2° stage – H_2 production	Overall H_2 production	References
Mesophilic processes						
Starch + y.e. [(a)] + glutamate	*Clostridium butyricum*	1.9 mol H_2/mol hexose	*Rhodobacter* sp. M-19	1.7 mol H_2/mol hexose	3.6 mol H_2/mol hexose	Yokoi et al. (1998)
Rice-wine wastewater	*Clostridium butyricum* NCIB 9576	1 l H_2/l wastewater in 18 h	*Rhodobacter sphaeroides* E151	0.44 l H_2/l broth/day (for 10 days)	1.44 H_2/l broth/day	Kim et al. (2001)
Tofu wastewater	*Clostridium butyricum* NCIB 9576	0.9 l H_2/l wastewater in 26 h	*Rhodobacter sphaeroides* E151	0. 2 l H_2/l broth/day (for 30 days)	1.1 l H_2/l broth/day	Kim et al. (2001)
Sweet potato starch residue + polypeptone or cornsteep liquor	*Clostridium butyricum* and *Enterobacter aerogenes* co-colture 2:1	2.7 mol H_2/mol hexose	*Rhodobacter* sp. M-19	4.5 mol H_2/mol hexose	7.2 mol H_2/mol hexose	Yokoi et al. (2002)
Algal biomass (*Chlamydomonas reinhardtii*)	*Clostridium butyricum*	2.6 mol H_2/mol hexose	*Rhodobacter sphaeroides* KD131	88% efficiency[(b)]	8.3 mol H_2/mol hexose	Kim et al. (2006b)

Starch hydrolyzate + Endo medium	*Clostridium butyricum* CGS2	5.44 mmol H_2/g COD	*Rhodopseudomonas palustris* WP3-5	10.72 mmol H_2/g COD	16.16 mmol H_2/g COD (3.09 mol H_2/mol glucose)	Lo et al. (2008)
Cassava starch (hydrolyzed)	Activated sludge	11.61 mmolH_2/g starch	*Rhodopseudomonas palustris* WP3-5	5.89 mmolH_2/g starch	2.92 mol H_2/mol hexose	Su et al. (2009b)
Cassava starch (hydrolyzed)	Cattle dung microflora	1.60 mol H_2/mol hexose	*Rhodobacter sphaeroides* ZX-5	4.91 mol H_2/mol hexose	6.51 mol H_2/mol hexose	Zong et al. (2009)
Food waste	Cattle dung microflora	1.77 mol H_2/mol hexose	*Rhodobacter sphaeroides* ZX-5	3.63 mol H_2/mol hexose	5.40 mol H_2/mol hexose	Zong et al. (2009)
Corncob + nutrient stock solution	Heat shocked dairy manure	2.05 mol H_2/mol reducing sugar	*Rhodobacter sphaeroides*	90 % efficiency[(b)]	54.9 % efficiency [(b)] (corncob conversion)	Yang et al. (2010)
Thermophilic processes						
Potato steem peel hydrolyzate	*Caldicellulosiruptor saccharolyticus*	2.9 mol H_2/mol hexose	*Rhodobacter capsulatus* + y.e. [(a)]	45.6 % efficiency[(b)]	5.64 mol H_2/mol hexose	Claassen et al. (2004)
Beet molasses + y.e. [(a)]	*Caldicellulosiruptor saccharolyticus*	2.1 mol H_2/mol hexose	*Rhodubacter capsulatus hup*[(c)] (YO3)	4.75 mol H_2/mol hexose [(d)]	6.85 mol H_2/mol hexose	Özgür et al. (2010b)

Myscanthus	*Thermotoga neapolitana*	2.90 mol H_2/mol hexose	*Rhodobacter capsulatus* DSM155	1.60 mol H_2/mol hexose [(d)]	4.50 mol H_2/mol hexose	De Vrije et al. (2009) and Uyar et al. (2009)

[(a)] y.e. = yeast extract; [(b)] efficiency = substrate conversion efficiency; [(c)] *hup*$^-$ = mutant with uptake hydrogenase knocked out; [(d)] calculated by the Author.

5. Main concerns about photofermentation and questions to be solved

Photobiological H_2 production represents a promise because of the possibility to provide renewable and sustainable energy while using free solar light and managing waste disposal. However, large-scale production is still far from being applicable, as some challenges have to be overcome.
A first aspect is related to light issues, in terms of quality and in terms of quantity of energy spent to sufficiently irradiate the system.
Another aspect that is linked with light issues is the design of photobioreactors; this is a very complex matter, because it integrates many different features: physiological, economical and engineering.
To be taken into account is also the choice of the substrate, as it is not economically feasible to use synthetic media, unless the attainment of very high H_2 production rates; thus the choice is often to use low cost starting materials, that anyway need to be specifically managed.

The last aspect that will be treated here is the kind of microorganism chosen for the process: at the moment a variety of PNS bacterial strains have been selected from natural environments, that respond to some desired characteristics for an efficient H_2 production process, but also the use of genetically engineered strains is widening, in order to selectively manage and solve some hurdles in PNS bacteria metabolism.

5.1 Light issues

One of the most relevant issues to be considered for the optimization of the production of H_2 via photofermentation is the photochemical efficiency of the system. Considering the absorption spectrum of purple bacteria (Figure 7), Miyake (1998) calculated that for the production of a single molecule of H_2 11 photons are required at 860 nm; Akkerman et al. (2002) calculated that 14-15.8 photons are required for a molecule of H_2 at 522 nm. Even though there are no data available on the quantum yields at the other wavelengths utilizable by purple bacteria, it has been estimated that the overall theoretical photosynthetic efficiency (PE) (22) is at least 10%. The details of these calculations can be found in Akkerman et al. (2002).

$$PE = \frac{Combustion\ enthalpy\ of\ H_2 \times H_2\ production\ rate}{Absorbed\ light\ energy \times 100} \quad (22)$$

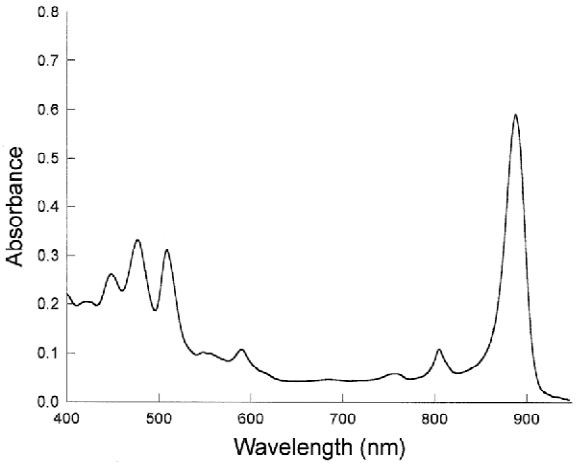

Figure 7. Absorption spectrum typical for PNS bacteria. Absorption maxima at 805 and 875 nm are due to bacteriochlorophyll a (Figure from Adessi and De Philippis 2012, with kind permission from Springer Science+Business Media BV).

Moving from the theory to real applications, the value of light conversion efficiency dramatically drops down not only under natural sunlight but also under artificial irradiation. Indeed, high light conversion efficiencies have only been reached using such low light intensities that the production rates were not enough for being considered interesting for a H_2 production process. Barbosa et al. (2001) observed that higher light intensities may decrease PE, but usually increase hydrogen productivity. Miyake and Kawamura (1987) reported light conversion efficiencies of 7.9 and 6.2% under illumination by a xenon lamp at 50 W m^{-2} and by a solar simulator at 75 Wm^{-2}, respectively: those are very low light intensities to reach a gas evolution significant for a production process.
Light intensity, light quality and sources, light distribution and photobioreactor design are all very important issues for the optimization of H_2 production with PNS bacteria and are thoroughly treated in the following paragraphs.

5.1.1 Light intensity, quality and sources

One of the problems in comparing the data of light efficiency of different H_2-producing systems is due to the lack of homogeneity in the way light intensities are measured and reported in different papers. Indeed, a first cause of un-homogeneity comes from different ways of measuring light, based on different theoretical assumptions, which makes difficult to convert one unit of measurement to another one. Anyway, to give an order of magnitude of the light intensities more frequently used, they range in the thousands of lux, usually around 10 klux, and in hundreds of both μmol(photons) m^{-2} s^{-1} and W m^{-2}.

Another cause of un-homogeneity in the data reported in literature is related with the different light requirements of the organisms utilized and with the characteristics of the light source used (incandescent lamp, LEDs, solar light), which have different spectra of emission as well as different intensities and angle of incidence. It has also to be stressed that in many cases, if not all, the value of the light energy used for calculating the light efficiency with equation (9) is that impinging on the photobioreactor and not the one actually absorbed by the culture.
As an example Uyar et al. (2007) indicate, for *Rb. sphaeroides*, a minimum light intensity of 270 W m^{-2} to obtain high hydrogen production rates; they state that this value is equivalent to 4000 lx and 1370 μmol(photons) m^{-2} s^{-1} .
When using artificial light, the most used light sources are tungsten lamps as their emission spectrum covers the absorption spectrum of PNS bacteria. Particularly important is the near infrared emission, where is located the absorption maximum of bacteriochlorophylls.
As tungsten lamps are energy-expensive light sources some alternatives can be offered by Light Emitting Diodes (LEDs). Kawagoshi et al. (2010) utilized long-wavelength LEDs (LW-LEDs), with a maximum emission at 850 nm, to produce hydrogen by means of an halo-tolerant photosynthetic bacterium. They state that LEDs have a life time ranging between 20000 and 30000 hours, while a tungsten lamp lasts for 1000-2000 hours, and they prefigure a reduction of energy cost by 98% using LEDs instead of tungsten lamps.

At the present state of knowledge in technology and processes, it is possible to prefigure that the best producing system may come from the optimization of a medium scaled, naturally irradiate hydrogen production process [Boran et al., 2010], provided with an artificial light supply switched on as soon as light intensity decreases under a threshold value [Ogbonna et al., 1999] and where the artificial light is given by wavelength-selected LEDs [Kawagoshi et al., 2010].

5.1.2 Solar irradiation and related issues

As it has been above mentioned, light irradiance is very important using photosynthetic bacteria and it has to be stressed that in a cost effective system the best solution would appear the use of natural solar light. However there are a number of problems coming from the use of natural irradiation and they will be presented in the following lines, together with some new possibilities for enhancing the light conversion efficiency.
Even if purple bacteria are able to use a wide range of the solar light spectrum (400-950nm), in fact only the 65.8% is actually part of the PAR (Photosynthetic Active Radiation) for purple bacteria [Akkerman et al., 2002]. Another problem comes from the light saturation of PNS bacteria cultures: Miyake et al. (1999) showed how, in an outdoor experiment, the maximum rates (3.4 l m^{-2} h^{-1}) were obtained 2-3 hours after the maximum of light intensity at noon, while during the period of the day with the highest irradiation (about 1.0 kW m^{-2}) the hydrogen production rates were significantly lower, thus indicating a probable photo-inhibition event.
The same study points out how the intrinsic variability of solar light makes the rates vary along with light intensity during the day: this means that the process is

continuously varying and the rates cannot be constant. It is anyway interesting to observe how after the night period, when gas production ceases, photoevolution of gas starts again after a lag period of 2-4 hours.
Özgür et al. (2009) showed how passing from indoors outdoors the question of temperature fluctuation is very relevant, as temperature fluctuation decreases H_2 production by 50%; the subjection to light/dark cycles further decreases it.
In another photobioreactor irradiated by solar light [Eroğlu et al. 2008], but bigger in volume (8.0 l instead of the 0.550 l of the previous article cited), an average production rate 10 ml l^{-1} h^{-1} was obtained when using malate as a substrate.
Androga et al. (2011) reported what a significant effect has the season of the year in which the process is carried out, stating that the average hydrogen productivity obtained in summer was 7.5 that the one achieved in the startup of the process carried out in winter; it has to be stressed though that the photobioreactor was not thermostated.

In any case, it is evident that an absent or insufficient light irradiation stops hydrogen production, and this has an effect on the total gas production, that is surely lower than the amount that can be produced by continuous illumination.
At this regard, integrated artificial and solar light systems may be taken into consideration; as an example a system has been proposed [Ogbonna et al., 1999] to overcome the solar light variations during the day, the bad weather periods and the night periods: solar light was collected by Fresnel lenses equipped with a light-tracking sensor; the solar light collection device was connected to optical fibers that brought light into light radiators which homogeneously diffused light into the photobioreactor. This system was equipped with a light intensity sensor that in case of insufficient solar light intensity switched on an artificial light, to supply the culture's light needs. This ingenious system can have opened a path aiming at create an homogeneous and continuous hydrogen production process.
However, a careful evaluation of the costs of the use of artificial light and benefits in terms of increased hydrogen production must be done before proposing these technologically complex systems.

5.2 Scaling up of the H_2-producing processes

McKinlay and Harwood (2010a) stressed the relevance of the energetic yield of hydrogen production rates compared with crop-based biofuels if only it was possible to linearly scale up the actual biohydrogen production processes: with a virtual production process using *Rp. palustris* they calculated that it is possible to obtain 23-29 equivalent L of gasoline ha^{-1} day^{-1}, that is much higher than the single equivalent L of gasoline ha^{-1} day^{-1} obtainable with soybean-based biodiesel.

This is actually a wide margin that gives many chances, because even if the scaling up would not be linear, it can probably remain more convenient than crop-based biofuels.

Some recent studies concern this aspect, that is tightly linked with natural irradiation and with the use of low-cost substrates owing to the higher costs of a scaled-up process in comparison with a low volume process.

The passage to a higher culture volume leads to higher amounts of gas production, but the rates and the efficiencies can be significantly lower than with smaller culture volumes.

Eroğlu et al. (2008) carried out a H_2-producing photofermentation in an 8 liter photobioreactor using natural irradiation with *Rb. sphaeroides*. Various substrates have been tested (malate, lactate, acetate and olive mill wastewater) and a 10 ml l^{-1} h^{-1} production rate was obtained with malic acid, while a 3 ml l^{-1} h^{-1} production rate was obtained with olive mill wastewater (4% of olive mill wastewater in distilled water). With the same outdoor reactor Androga et al. (2011) obtained a maximum rate of 11.22 ml l^{-1} h^{-1} during a fed batch process using synthetic medium containing acetate and glutamate with a *hup*$^-$ mutant of *Rb. capsulatus*.

De Philippis et al (2007) scaled up the hydrogen production process starting from a 0.25 l bioreactor to an 11.0 l column photobioreactor using vegetable wastewater (50% of wastewater in distilled water) as a substrate, with *Rp. palustris*. Using an artificial light irradiation, the rates decreased from 16 ml l^{-1} h^{-1} to 11 ml l^{-1} h^{-1} in the scaled up process.

When using much larger volumes, the processes would better pass from a batch to a fed-batch processes, as once the biomass is inoculated it is convenient to use it as longer as possible due to the complex operations needed to manage big volumes of culture.

An example of a fed batch, semi-pilot scale biomass production process was reported by Carlozzi and Sacchi (2001): they used a tubular temperature controlled culture system of 53 l of volume, under solar irradiation with an irradiated area of 1.52 m^2 and a total ground area of 2.0 m^2, growing *Rp. palustris*; even though the process was not aimed at hydrogen production, it highlights the importance of operating at the right biomass concentration when growing phototrophic bacteria. The authors indicated an optimal culture concentration of 1 g of dry weight l^{-1} to obtain the best biomass productivity and also demonstrated that, in order to maintain this cell concentration, a fed batch process is necessary.

Gebicki et al. (2009, 2010) reported an interesting comparison between a flat panel and a tubular photobioreactor: the first was composed of 4 modules of 25 l of volume each, the second had a volume of 65 l. In Gebicki et al. (2010) it was reported a mean hydrogen productivity of 295 ml l^{-1} d^{-1} for the panel reactor and of 152 ml l^{-1} d^{-1} for the tubular reactor, but the most impressive difference between the two is underlined by the ground area productivity where the amount of H_2 produced per ground area per day of the panel reactor is almost 9 times the one of the tubular reactor. Anyway, the authors stress that a more relevant parameter when working outdoors in sunlight is the productivity per illuminated surface; for this parameter the differences between the two reactors dwindle drastically (respectively, 3690 versus 3350 ml m^{-2} d^{-1}).

Boran et al. (2010) have investigated hydrogen production in a solar tubular photobioreactor of 80 l of volume with an illuminated area of 2.0 m^2 and a total ground area of 2.88 m^2, using *Rb. capsulatus*. They artificially illuminated the culture during the exponential phase of growth, than they started the feeding and the natural irradiation. At the end of a period of 32 days, they obtained 80 l of hydrogen, but gas production actually started only after 6 days and after cells reached a

concentration of 0.8 g l^{-1}. A mean rate of 0.31 and a maximum rate of 0.74 mol H_2 m^{-3} h^{-1} were obtained. They calculated a mean light intensity of 90 W m^{-2} during the light hours out of a 13-day period, and on this basis they calculated a conversion efficiency of 1%. However, it has to be stressed that during such a long period of time, the solar light intensity might have undergone many significant variations and thus calculating a mean value seems not to be the best way to evaluate this parameter. They also observed that, when the light intensity was below 90 W m^{-2}, hydrogen production ceased and the substrate was only used for cell growth. However, also considering that the producing system operated under not yet optimized conditions, the results seem promising. Indeed, they have shown the feasibility of the scaling up of the system and the possibility to maintain a culture of PNS bacteria for a long time (about one month) under natural irradiation with a significant production of hydrogen.

5.3 Kind of substrate

5.3.1 Synthetic substrates

The use of synthetic media for hydrogen production process has a very high relevance, as it describes the behavior of the microorganisms in a controlled system, where the culture medium is completely defined. Culture conditions are usually very homogeneous with regard to temperature and pH, respectively around 30°C and around 7.0.

Based on the two reviews recently published by Koku et al. (2002) and Kapdan and Kargi (2006), it is possible to say that acetate, lactate and malate are the most common organic acids used for hydrogen production; only a few data are available for butyrate, and some data refer to the use of sugars as glucose or fructose.

The C/N ratio is quite variable among the studies reported, as it has to be optimized according to the microorganism and the carbon substrate used.

Considering the three most frequently utilized substrates, it appears that their conversion yield is highly dependent on the PNS bacterial strain and on the conditions utilized, showing a high variability even within the data obtained with the same substrate. In any case the best conversion yields are in the range 75 to 88%. In all the studies it was reported the maximum rate of H_2 production but quite frequently the mean production rate, which is a parameter of great interest for practical applications, is not available. The maximum rates reported range from very low values (about 1-2 ml $l^{-1}h^{-1}$) to very high values (103 and 165 ml $l^{-1}h^{-1}$). In particular, the highest maximum production rate so far obtained was achieved by using high light intensity with shacked cultures of *Rb. sphaeroides* ZX-5 [Li et al., 2009].

However, the few data available on the light conversion efficiency show very low values and point out that one of the main critical point to be solved is the light utilization by the PNS bacteria. Indeed, it has been reported that a light conversion efficiency of about 10% should be achieved in order to make economically feasible this process [Akkerman et al., 2002; Basak and Das, 2007].

5.3.2 Substrates deriving from wastes of industrial or agricultural processes

As mentioned above, one of the most interesting feature of PNS bacteria is their capability to use, for the production of H_2, waste residues deriving from industrial or agricultural processes. This characteristic gives two economical potential advantages to this process: (i) the substrate is free or very cheap, being a waste deriving from other processes; (ii) the use of these substrates for H_2 production processes reduces or eliminates the cost of their treatment and/or disposal. Moreover, these substrates are, in many cases, available in large amounts. However it has to be considered that the H_2 production plant must be located close to the site or area of production of wastes, in order to maintain at the lowest level the expenses for transporting this material.

According to Koku et al. (2002), wastes deriving from sugar refineries [Yetis et al. 2000], tofu factories [Zhu et al., 1999a], olive mills [Eroğlu et al., 2002], municipal solid wastes [Fascetti et al., 1998], dairy plants [Türkaslan et al., 1998] and lactate fermentation plants [Sasikala and Ramana, 1991] have been used, at proper dilutions and with proper additions: in particular, as they report, the best waste used with this species was tofu wastewater, considering the high gas production rates and the fact that the wastewater was not diluted and no addition of other nutrients was necessary; they obtained a gas production rate of 15.9 ml l^{-1} h^{-1}. All these processes where carried out using *Rb. sphaeroides*.

Hydrogen production using a *Rhodopseudomonas* sp. strain was investigated by Singh et al. (1994) using as substrates potato starch, sugarcane juice and whey: the best result was obtained with sugarcane juice, which showed a specific rate of hydrogen production of 45 ml per gram of dry weight per hour.

De Philippis et al. (2007) reported the use of a fermentation broth, deriving from the spontaneous fermentation of vegetable wastes, for the production of H_2 with *Rp. palustris*; a mean production rate of 16.0 ml l^{-1} h^{-1} was obtained.

Tao et al. (2008) tested three different wastewaters using *Rb. sphaeroides* ZX5: a wastewater deriving from a succinate producing factory (mixed with a synthetic medium without carbon source), a wastewater of a fuel ethanol manufacturer (with a threefold dilution) and a kitchen waste (diluted twofold) obtaining, respectively, a maximum rate of 55, 48 and 45 ml l^{-1} h^{-1}.

Efforts have been made in order to overcome some of the problems arising from the use of low cost substrates and in particular about the low conversion of substrates and the nitrogenase "switch off" due to the presence of nitrogen sources in the substrate. For solving this problem various approaches have been followed:

- Development of NH_4^+ insensitive strains [Zhinchenko et al. 1991; Zhinchenko et al. 1997; Drepper et al., 2003; Rey et al. 2007].
- Electroseparation of NH_4^+ from the culture medium [Redwood and Macaskie 2007a].
- Use of immobilized cultures in anion selective matrices [Zhu et al., 1999b, Zhu et al., 2001].

However it has to be stated that most of the above mentioned studies have been done at a lab scale, and their real applicability needs to be verified at a pilot scale.

5.4 The appropriate strain

The choice of the more appropriate strain for the process that one is intended to carry out is a crucial step. A multitude of strains isolated from natural environments have been reported to be suitable for consistent hydrogen production processes, so the harvest of new strains is a never stopping activity.

On the other hand, though, not necessarily the most suitable strain is available in nature, or maybe it has not yet been found, so it becomes useful to genetically manipulate well known strains in order to overcome some hurdles for an efficient hydrogen production process.

5.4.1 Use of engineered strains

The efficiency of photobiological hydrogen production catalyzed by the nitrogenases in PNS bacteria is inherently limited. In addition to the inherent characteristics of nitrogenases, as previously mentioned, the total hydrogen production is constrained due to several reasons such as consumption of hydrogen by uptake hydrogenase, limited electron flow to the nitrogenase due to the presence of alternative electron sinks such as production of poly-β-hydroxybutyrate (PHB) or CO_2 fixation, inhibition of the nitrogenase from ammonium, light requirement of the nitrogenase, etc all schematically illustrated in Figure 8. These limitations can be overcome by several genetic manipulation approaches. In the following lines the approaches for the enhancement of the H_2 production will be investigated in details.

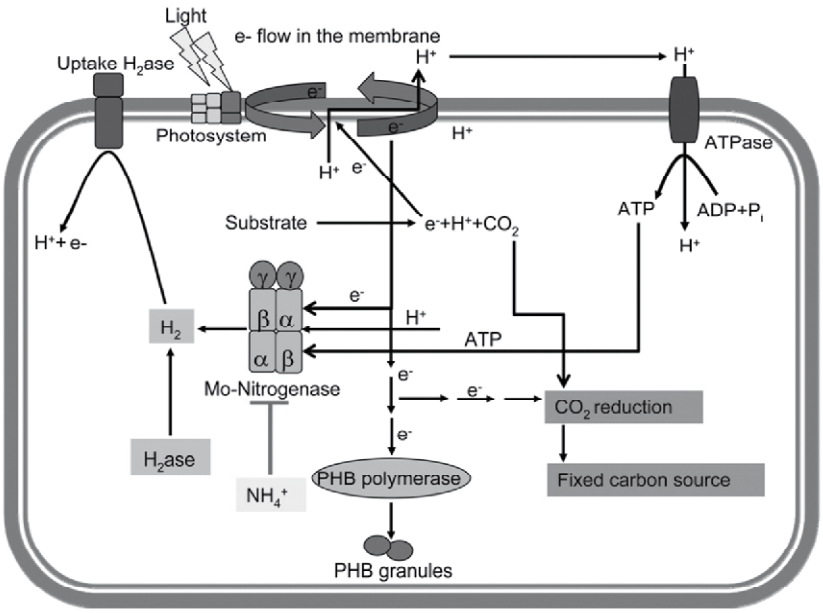

Figure 8. General view of H_2 related pathways in a PNS bacterium (Figure from Kars and Gunduz, 2010; with kind permission of the International Journal of Hydrogen Energy].

- Elimination of uptake hydrogenase

The hydrogen respiration let the cells recapture the electrons from molecular hydrogen and deliver it to the electron carriers in the membrane while making proton motive force which is then used to produce ATP. In this way, the uptake hydrogenase helps to maintain a redox balance [Vignais et al., 1985; Appel and Schulz, 1998]. Since uptake hydrogenase decreases the efficiency of H_2 production, it was targeted to be eliminated in many PNS bacteria either by antibiotic resistance gene insertion into the *hup* genes or by deletion of *hup* genes [Kern et al. 1994; Ooshima et al. 1998; Franchi et al. 2004; Kim et al. 2006a; Ozturk et al., 2006; Kars et al. 2008; Kars et al. 2009]. It was shown that the inactivation of uptake hydrogenase resulted in significant increase in total hydrogen production in these bacteria.

- Inhibition of the synthesis of PHB

The biosynthesis of storage material such as poly-β-hydroxybutyrate is a way that competes with hydrogen production, as it has the same function of dissipating the excess of reducing power [Vincenzini et al., 1997; Koku et al., 2002]. PNS bacteria spend much energy for the production of PHB especially when grown on acetate as carbon source [Kemavongse et al., 2007, 2008]. The development of mutants with an inactivation of the PHB biosynthetic pathway resulted in improved hydrogen production rates, but only in addition to the deletion of the uptake-hydrogenase activity [Franchi et al. 2004; Kim et al., 2006a]; the strain with only the deletion of the PHB biosynthetic pathway did not exhibit increased rates [Franchi et al., 2004].

- Enhanced energy flow to nitrogenase

The charge or redox status of the cells is very important for biohydrogen production since the nitrogenase needs electrons and ATPs to reduce protons to hydrogen. Furthermore, an enhancement in the electron and ATP flow to the nitrogenase would certainly lead to a burst of H_2. In the photosynthetic bacterium *Rb. sphaeroides* and *Rb. capsulatus*, a putative membrane-bound complex encoded by the *rnf*ABCDGEH operon is thought to be dedicated to electron transport to nitrogenase [Jeong et al., 2000]. It was shown that over-expression of *rnf* operon in *Rb. capsulatus* enhanced in vivo nitrogenase activity. In another study done by Ozturk et al. (2006), it was shown that a loss of function in the electron carriers in the membrane of *Rb. capsulatus* resulted in significant decrease in H_2 production [Ozturk et al., 2006].

- Ammonium insensitivity

As previously mentioned, nitrogenase is finely regulated. The regulation has been modeled as a three-level control mechanism, as described by Masephol et al. (2002a) for *Rhodobacter capsulatus* , but the regulative cascade described for this microorganism might not be applicable to all PNS bacteria, due to the differences

existing also in the presence or absence of three different isozymes. Some bacteria, including *Rhodobacter capsulatus* and *Rhodospirillum rubrum* as well as various cyanobacteria and *Clostridium pasteurianum*, encode, in addition to the most common molybdenum nitrogenase (Mo nitrogenase), either an iron nitrogenase (Fe nitrogenase) or a vanadium nitrogenase (V nitrogenase) [Eady RR, 1996; Masephol et al., 2002b]. Alternative nitrogenases have been proposed to serve as a route for nitrogen fixation in situations where molybdenum is limited in the environment. Most rarely bacteria posses all three isozymes, as described for *Rp. palustris* [Larimer et al., 2004; Oda et al., 2005].

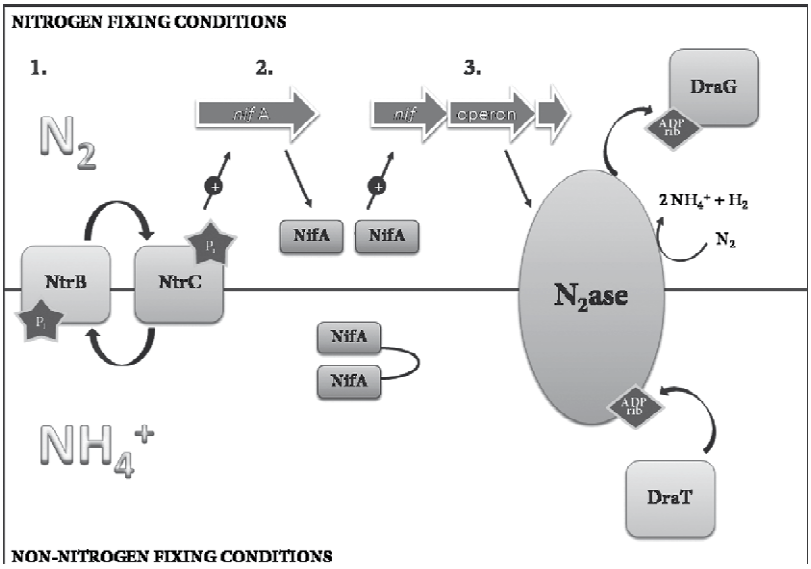

Figure 9. Regulation of nitrogenase in response to available nitrogen is a complex and multileveled process. 1. In the presence of ammonium, PII proteins (not shown) respond to high levels of glutamine and prevent phosphorylation of NtrC by NtrB. When only N_2 is available as a nitrogen source, α-ketoglutarate levels rise, leading to the uridylylation of PII proteins and allowing NtrB to phosphorylate NtrC. NtrC-P then promotes the transcription of various genes (e.g., *nifA*) involved in nitrogen fixation. 2. PII proteins also respond to the nitrogen status of the cell either allowing or preventing NifA to activate the transcription of nitrogenase-encoding genes by influencing its structure. 3. After nitrogenase is expressed, its activity can be switched off in response to addition of ammonium by DraT, which adds ADP-ribosyl groups to nitrogenase, preventing nitrogenase activity. When ammonium is depleted, DraG removes the ADP-ribosyl groups allowing nitrogenase activity to continue [source: McKinlay and Harwood, 2011].

Despite all differences, the main regulative levels at which ammonium acts are represented in Figure 9: at a first level fixed nitrogen acts on NtrC-B system repressing the transcription of *nif*A, a gene encoding for an RNA polymerase sigma 54-dependent transcriptional activator; at a second level it acts on NifA, inducing structural changes that prevent the transcriptional activator from binding to its binding site and activate nitrogenase genes (*nif* genes) transcription; at a third level it acts on nitrogenase itself causing a reversible "switch-off" of the enzyme through ADP-

ribosylation on nitrogenase mediated by DraT [Masephol et al., 2002a, Rey 2007, McKinlay and Harwood 2010b].

It was shown that in *gln*B-*gln*K double mutant strain; ammonium regulation of Mo-nitrogenase was completely abrogated leading to an active Mo-nitrogenase in the repressive concentrations of ammonia [Drepper et al., 2003].

In a study done by Rey et al. (2007), constitutive hydrogen production in the presence of repressive concentrations of ammonia by *Rp. palustris* having mutations in the four different sites in the NifA transcriptional regulator was reported. This mutation made the bacteria escape from the repressive effect of ammonium.

- Reduced pigment content

The photophosphorylation capacity is also slightly greater in cells grown under high light intensity than in cells grown under low light intensities [Steinborn and Oelze, 1989]. Therefore, it could be proposed that high ATP production rate under well-illuminated conditions results in higher hydrogen production activity. Although this type enhancement of H_2 production is not a result of genetic manipulation but it proves that H_2 production could possibly be increased by just adjusting the light conditions. In addition, there are genetic manipulations by which the enhancement of H_2 production was recorded through alterations in light harvesting complexes (LH) [Kondo et al., 2002; Vasilyeva et al., 1999]. In a study done by Kondo et al. (2002), a mutant strain of *Rb. sphaeroides* with reduced LH1 complex produced 50% more hydrogen than its wild type parent. This finding is of special importance since it shows the negative effect of shading due to the high pigment concentration on H_2 production process. Reducing pigment content of the bacteria by genetic manipulations increased the light penetration inside the bioreactor, thereby increasing the H_2 production.

- Carbon fixation depletion

One of the interesting findings about the relationship of three fundamental biological processes; photosynthesis, biological nitrogen fixation, and carbon dioxide assimilation was well documented by Joshi and Tabita (1996) and Qian

and Tabita (1996). Previously it was known that the RegA (PrrA)/RegB (PrrB) system controls the ability of *Rb. sphaeroides* and *Rb. capsulatus* to respond to different intensities of light for the anoxygenic photosynthesis gene expression [Sganga and Bauer, 1992; Mosley et al., 1994; Eraso and Kaplan, 1995; Eraso and Kaplan, 1994], however, it was proved that this two component regulatory system also influences the nitrogen fixation in addition to photosynthesis and carbon dioxide reduction [Joshi and Tabita, 1996; Qian and Tabita 1996]. It was shown that mutations in the regB (PrrB) gene of *Rb. sphaeroides* blocked transcription of the *cbb* regulon which contains genes that encode two forms of RubisCO and the enzymes in CBB cycle. And, inactivation of the *reg*B (PrrB) gene of *Rb. sphaeroides* resulted in the production of active nitrogenase in the normally repressive concentrations of ammonia [Qian and Tabita, 1996]. Similar result was obtained when the CBB route is blocked by mutation in the genes coding for form I and II RubisCO in *Rb.*

sphaeroides and *Rs. rubrum* [Joshi and Tabita, 1996]. The explanation for why the *nif* system is on even in the presence of normally repressive levels of ammonia is that organisms seek alternative mechanisms to dispense the large amounts of reducing power generated via

photosynthesis and the oxidation of organic carbon (such as malate) when the CBB route is blocked. In other words, CO_2 is no longer capable of functioning as the major electron sink when the CBB route is blocked, and the large amount of reducing power is forced to be dissipated through nitrogenase action [Joshi and Tabita, 1996; Qian and Tabita, 1996].

In a recent study carried out by McKinlay et al. (2010a), the evidences of the competition between the H_2 production route and the carbon fixation route is reported for *Rp. palustris*.

5.5 *Rhodopseudomonas palustris*

Rp. palustris, which is widespread and usually isolated from waterlogged soils and stagnant bodies of water, is a nearly deal model organism for studying biological production of H_2.

It has been already mentioned the wide metabolical versatility of purple bacteria, and of *Rp. palustris* in particular [Larimer et al. 2004, Oda et al. 2008]. This organism can grow in all different types of metabolism, as shown in Figure 10.

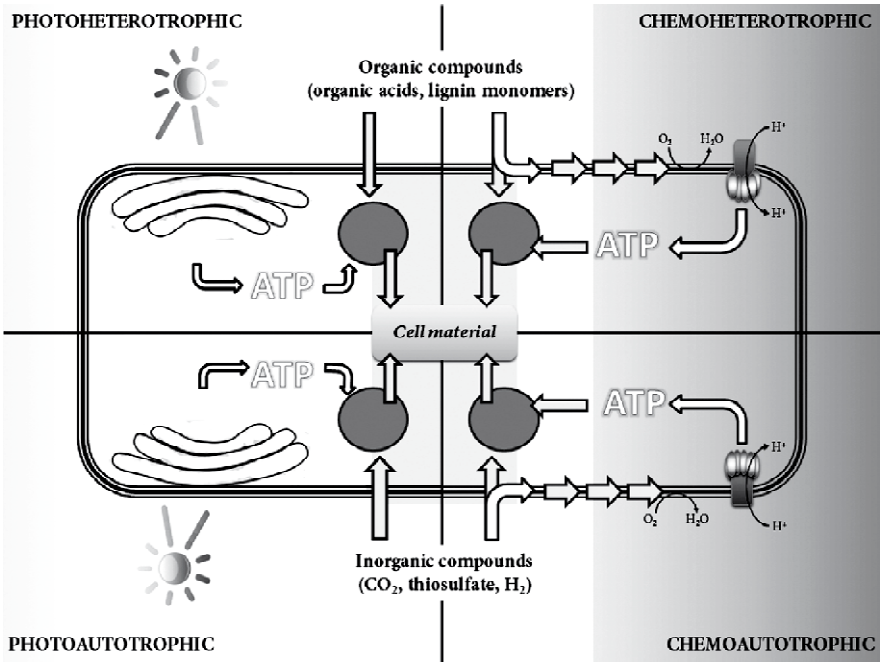

Figure 10. Overview of the physiology of *Rp. palustris*. Schematic representations of the four types of metabolism that support its growth are shown. The blue circle represents the enzymatic reactions of central metabolism.

It grows with or without oxygen and uses many alternative forms of inorganic electron donors, carbon and nitrogen. It degrades plant biomass and chlorinated pollutants and it generates hydrogen as a product of nitrogen fixation. Thus, *Rp. palustris* is a model organism to probe how the interconnection of metabolic reactions within a single cell adjusts itself in response to changes in light, carbon, nitrogen and electron sources that are easily manipulated experimentally.

Its genome sequence reveals that *Rp. palustris* has additional capabilities, not shared by other purple bacteria, that enhance its potential for use in biotechnological applications. These include modulating photosynthesis according to light quality [Evans, 2008] and degrading complex (also aromatic) compounds [Harwood, 2008] that are typically found in agricultural and industrial wastes.

Rp. palustris has physical attributes that are well suited for process development. It undergoes asymmetric cell division and produces a cell surface adhesin at one end of the cell that causes cells to stick to solid substrates or the cells one to another as shown in Figure 11.

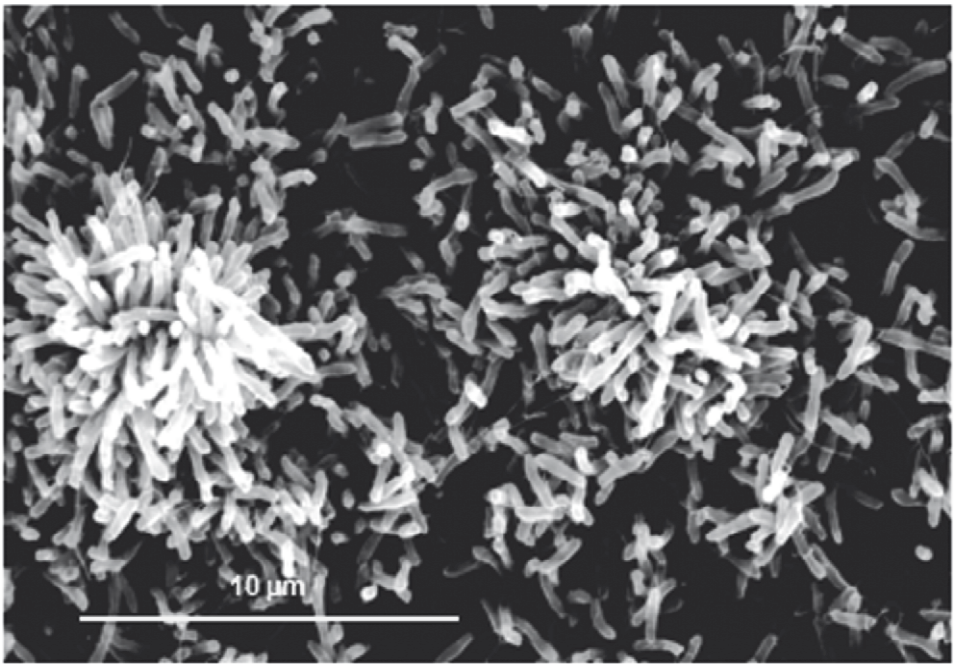

Figure 11. *Rp.palustris* cell-aggregates under growth with p-coumarate forming the typical "star" conformation [Image kindly provided by Yasuhiro Oda,:University of Washington].

Rp. palustris has especially good potential for use as a biocatalyst for hydrogen production. It is unique among purple phototrophic bacteria in encoding a vanadium-containing nitrogenase that catalyzes the production of approximately three times as much hydrogen as do molybdenum-containing nitrogenases [McKinlay and Harwood, 2010b].

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Aim of the Thesis

The aim of the present thesis was to define the feasibility of hydrogen production using PNS bacteria, and in particular the versatile microorganism *Rhodopseudomonas palustris*, in natural light conditions and growing on low cost substrates, towards an up-scaling of the process.

These two aspects were investigated separately:

- The experimentation on the use of different substrates deriving from other fermentation processes was conducted indoors, in artificial light conditions.
    - A medium derived from a mesophylic dark fermentation process was used in order to produce hydrogen in a combined H_2 production process.
    - A medium derived from a thermophylic dark fermentation process was used in order to produce H_2 in a combined H_2 production process.
    - A vegetable waste derived medium was used in order to produce hydrogen with PNS bacteria, in a two stage process with no H_2 production in the first (dark fermentative) stage; different *Rp. palustris* strains were used, and also an ammonium insensitive mutant strain.

- The experimentation regarding light conditions was carried out using synthetic substrates both outdoors and indoors under different light conditions.
    - An experimentation on the effect of different light/dark cycles was conducted indoors with artificial irradiation;
    - An experimentation on the use of solar light for up-scaled H_2 production processes was conducted outdoors, in a tubular photobioreactor sunlight-irradiated.

As a preliminary activity, a phase of search and screening for new strains of PNS bacteria was conducted in order to identify strains possessing the characteristics needed for the subsequent experimentations.

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Results – Section I

1. Hydrogen-producing purple non sulfur bacteria isolated from the trophic lake Averno (Naples, Italy)

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Abstract

Seventeen purple non sulfur bacterial strains, isolated from the trophic lake Averno, Naples, Italy, were phylogenetically classified and their H_2-producing performances were tested utilizing various synthetic substrates and the fermentation broth derived from the spontaneous fermentation of vegetable residues. All the strains showed the capability to produce hydrogen on at least one of the four carbon substrates tested (malic, lactic, acetic and succinic acid). On lactate, *Rhodopseudomonas palustris* strain AV33 showed the best maximum production rate (50.7 ± 2.6 mL (H_2) L^{-1} h^{-1}), with a mean rate, calculated on the whole period of production, of 17.9 mL ± 0.7 (H_2) $L^{-1}h^{-1}$. In the presence of acetate, AV33 produced only few mL of H_2, but intracellularly accumulated poly-β-hydroxybutyrate up to a concentration of 21.4 ± 3.4 % (w/w) of cell dry weight. *Rp. palustris* AV33 also produced H_2 on the fermentation broth supplemented with Fe, with a maximum production rate of 16.4 ± 2.3 mL (H_2) L^{-1} h^{-1} and a conversion yield of 44.2%.

1.1 Introduction

Biological hydrogen production is generally considered a promising process owing to both its low impact on the environment and the possibility to use organic wastes as substrate for the production of the gas [1, 2].

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Amongst the microorganisms that can be utilized for biological hydrogen production, purple non sulfur bacteria (PNSB) are generally considered as good candidates, being characterized by a very versatile metabolism [3, 4] and by a high substrate to H_2 conversion efficiency [1]. This feature can be profitably exploited for producing H_2 from organic wastes or from agricultural residues [5, 6, 7, 8] , thus making this process economically more favourable. Furthermore, PNSB are also capable to produce poly-β-hydroxybutyrate (PHB) [9, 10], a valuable by-product which is a biodegradable thermoplastic having industrial and medical interest [11], even if it was demonstrated a competition between the metabolic pathways involved in the production of H_2 and PHB [9, 12].

In order to make the process of biological hydrogen production economically feasible, PNSB strains capable of efficiently utilizing the reducing equivalents coming from the oxidation of the organic substrates and the energy coming from the solar light for synthesizing hydrogen are needed. Thus, an intensive effort for searching new PNSB strains in environments suitable for harbouring this kind of microorganisms is highly desirable.

PNSB are widely distributed in nature in all the aquatic environments characterized by low oxygen concentration and by the availability of light and soluble organic matter [13]. These very common environmental conditions and the extreme metabolic versatility of PNSB bacteria have as consequence that these bacteria are widely distributed not only in freshwater and marine habitats but also in sediments and moist soils. However, the largest number of PNSB species has been found in eutrophic lakes, both in the water column and in the sediments [13].

Averno Lake is a trophic lake located in a volcanic basin in the area named *Campi Flegrei*, close to Naples (Italy). It is occasionally polluted by urban waste waters coming from the overflows of the pipelines connecting the city sewer system to the local depuration plant [14]. The lake presents stratification during the warmer season, whereas the water column is occasionally overturned in winter by the cooling of epilimnion due to weather conditions [15]. When the stratification occurs, it is possible to discriminate three zones through the water column, an upper aerobic zone in the first six meters, a second zone (from - 6 to -15 m) characterized by a deep decrease of oxygen concentration and a third anaerobic zone (from -15 to the bottom of the lake) with high concentration of H_2S, CO_2 and CH_4 and strongly affected by anoxic microbial activity both in the water column and in the sediments [15]. In particular, this third zone, characterized by high concentrations of organic substances, anoxic conditions and a residual amount of light sufficient for phototrophs, was considered to be an environment suitable for harbouring PNSB capable of efficiently utilizing the low MW fatty acids, produced by the anaerobic chemotrophic microbial community, for producing H_2.

This research was aimed at finding, in the water column and in the sediments of the trophic Averno Lake, new PNSB strains possessing good hydrogen producing properties. The newly isolated strains were phylogenetically classified and their H_2-producing performances were tested utilizing various synthetic substrates and the fermentation broth derived from the spontaneous fermentation of vegetable residues.

1.2 Materials and methods

1.2.1 Sampling and isolation procedures.

The samples were withdrawn from the Averno Lake (geographical coordinates: 40°50'18"N 14°04'30"E) at nine different depths of the water column (-1, -3, -5, -6, -9, -15, -21, -27, -32 m) and from the sediment, at -33 m, using a Niskin bottle. The samples were shacked in tubes containing the enrichment medium (RPN, see below). In order to create anaerobic conditions, the tubes were incubated at 30°C in the darkness, thus favoring the consumption of the oxygen by PNSB. After 1 hour, the tubes were exposed to a continuous light with an intensity of 20 μmol (photons) $m^{-2}sec^{-1}$. After 24 h, the light intensity was increased to 40 μmol (photons) $m^{-2}sec^{-1}$. The enriched cultures that showed, after four days of incubation, a red or pink pigmentation were serially diluted and the PNSB were isolated by the streak plate method. The plates were aerobically incubated at 30°C for one hour in the dark and then anaerobically under a light intensity of 20 μmol (photons) $m^{-2}sec^{-1}$, for 24 h, and of 40 μmol (photons) m^{-2} sec^{-1} for the following days. After six to ten days incubation, the red colonies formed were individually transferred into screw cap tubes filled with RPN nutrient medium and incubated as above reported.

1.2.2 Cultivation media

The cultures were carried out in RPN medium containing (g L^{-1}): DL-malic acid, 2.0; NH_4Cl, 0.5; K_2HPO_4, 0.5; KH_2PO_4, 0.3; $MgSO_4.7H_2O$, 0.4; NaCl, 0.4; $CaCl_2.2H_2O$, 0.075; Ferric citrate, 0.005; yeast extract, 0.4. Trace elements were provided by adding 10 mL per liter of a solution containing (mg L^{-1}): $ZnSO_4.7H_2O$, 10; $MnCl_2.4H_2O$, 3; H_3BO_3, 30; $CoCl_2.6H_2O$, 20; $CuCl_2.2H_2O$, 1; $NiCl_2.6H_2O$, 2; $Na_2MoO_4.2H_2O$, 30. The pH of the medium was adjusted at 6.8 with NaOH before autoclaving.

The hydrogen production medium (RPP) contained (g L^{-1}): organic substrate (malic acid, 4.0 , lactic acid, 3.6, acetic acid, 3.6, or succinic acid, 3.6, alternatively used); Na glutamate, 0.4; K_2HPO_4, 0.5; KH_2PO_4, 0.3; $MgSO_4.7H_2O$, 0.4; NaCl, 0.4; $CaCl_2.2H_2O$, 0.075; Ferric citrate, 0.005. The trace elements were added as above reported. The vitamins necessary for bacterial growth were supplied as a solution (1 mL per liter of medium) containing (mg per 100mL): biotin, 10; niacin, 35; thiamine dichloride, 30; p-aminobenzoic acid, 20; pyridoxolium hydrochloride, 10; Ca-pantothenate, 10; Vitamin B12, 5.

The fermentation broth was obtained by the spontaneous fermentation of vegetable residues carried out by the microflora residing on the vegetables, as previously described [6]. The main products contained in the fermentation broth, depending on the stock, were: acetic acid (in the range 2.0 to 3.0 g L^{-1}), lactic acid (in the range 8.0 to 9.0 g L^{-1}) and ammonia (80 to 100 mg L^{-1}). Before being utilized for the experiments with PNSB, the fermentation broth was diluted 1:3 with distilled water and the pH was adjusted from 3.2 to 6.8 with NaOH.

1.2.3 Enzymatic amplification of 16S rDNA

Bacterial isolates were identified by 16S rDNA sequencing. Total DNA was extracted from cultures anaerobically grown on solid media under continuous light for six-seven days.

DNA was extracted from samples by using the Instagene matrix according to the manufacturer's protocol (Instagene DNA Matrix, Bio-Rad Laboratories, USA). PCR amplification of 16S rDNA was performed under the same conditions for all strains using universal primers: primer forward 27 and primer reverse 1392. PCR amplicons were directly sequenced, forward and reverse, with their respective oligonucleotide primers. Sequencing was performed by the BMR Genomics (Padova, Italy). 16S rDNA sequences were compared with sequences of 9 validly described PNSB species from the NCBI database GenBank. These species and their accession numbers are: *Rhodobacter capsulatus* ATCC 11166 (D16428); *Rhodobacter blasticus* ATCC 33485 (DQ342322); *Rhodopseudomonas palustris* ATCC17001 (D25312); *Rhodobacter sphaeroides* ATCC 17023 (DQ342321); *Rhodospirillum rubrum* ATCC 11170 (D30778); *Rhodobacter azotoformans* KA 25 (D70846); *Rhodovulum sulfidophilum* ATCC 35886 (DQ342323); *Rhodospirillum molischianum* ATCC 14031 (M59067); *Rhodospirillum salinarum* ATCC 35394 (M59069).

Sequences were aligned using Clustal X (Version 1.81) and dissimilarities were converted to evolutionary distances according to Jukes and Cantor [16]. The construction of neighbouring joining trees and bootstrap analysis of 1000 resamplings were performed using the software package TREECON for Windows Version 1.3b [17], including *Escherichia coli* (K02555) 16S rDNA as the single-sequence (forced) outgroup.

1.2.4 Qualitative tests for H_2 production.

All the experiments for assessing the H_2 production capability of the strains were carried out with RPP medium alternatively containing one of the above reported carbon sources. The hydrogen production experiments were carried out under anaerobic conditions at 30°C in 100 mL rubber-stopped vessels equipped with syringes for the detection of hydrogen. The illumination was supplied by an incandescent lamp giving a light intensity of 150 μmol (photons) m^{-2} sec^{-1}.

1.2.5 Quantitative tests for H_2 production.

The PNSB used in the quantitative tests were *Rhodopseudomonas palustris* AV32a, AV32b and AV33, which showed the most promising features in the qualitative tests. The experiments were carried out with synthetic medium (RPP with lactic acid as organic substrate) and also, in the case of strain AV33, with a fermentative broth obtained from vegetable residues, as above reported. The fermentation broth was used with and without the addition of Fe (III) citrate (0.005 g L^{-1}), according to the procedure reported by Zhua et al. [18].

For preparing the inocula, the strains were grown in RPP medium containing 1g L^{-1} of Na-glutamate at 30°C under a light intensity of 150 μmol (photons) m^{-2}

sec^{-1}. After seven days, the precultures were centrifuged (15' at 4500 rpm) and the pellets re-suspended in the RPP medium or in the fermentation broth. The experiments were carried out under anaerobic conditions at 30°C in 250 mL rubber-stoppered vessels connected with a tygon tube to a trap where the evolved gas was stored. The light was supplied by an incandescent lamp giving a light intensity of 200 μmol (photons) m^{-2} sec^{-1}. All the experiments were done in triplicate and the data are reported as the mean value ± standard deviation.

1.2.6 Analytical methods.

Cell dry weight was determined in triplicate on 5 mL culture samples: after filtration through 0.45 μm membranes, the cells were washed twice with distilled water and dried at 60 °C 12 h.

The photosynthetic photon flux density at the culture surface was determined with a quantum/radiometer/photometer model DO9721 equipped with a quantum sensor model LP9021 (Delta Ohm, Italy).

The concentration of lactic and acetic acid and of ethanol in the culture medium and/or in the fermentation broth was determined with an HPLC chromatograph (1100 series, Agilent Technologies, USA) equipped with a 8 mm (I.D.) ×300 mm Shodex-SH1011 packed column (Denko K. K., Tokyo, Japan). A diode array detector (DAD) was used for the determination and quantification of the organic acids while a refractive index (RI) detector was used for ethanol. The column was maintained at 50◦C and the eluent was 0.01 N H_2SO_4, at a flow rate of 0.6 mL min^{-1}.

The intracellular content of PHB was determined according to De Philippis et al. [19].

The amount of hydrogen in the gas phase of the trap was determined by gas chromatography using a GC3800 Varian (Agilent Technologies, USA) equipped with a Molecular Sieve column, a Hayesep Q 80-100 column and a TCD filament detector with He as carrier gas.

The ammonia content of the fermentation broth was determined with Nessler method [20].

Efficiencies of substrate and energy conversion to hydrogen.

The conversion yield of the carbon substrate (lactate) to hydrogen was calculated as the percentage of the actual amount of hydrogen produced on the maximum amount of hydrogen theoretically obtainable from the complete conversion of the substrate to hydrogen, according to the following reaction:

$$C_3H_6O_3 + 3H_2O = 6H_2 + 3CO_2$$

The efficiency of light energy conversion to hydrogen (E_{eff}) was calculated according to the following equation (23)[21]:

$$E_{eff} = \frac{Combustion\ enthalpy\ of\ H_2 \times H_2\ production\ rate}{Absorbed\ light\ energy \times 100} \quad (23)$$

assuming that all the incident light was absorbed by the culture.

1.3 Results

1.3.1 Identification of the purple non-sulfur bacteria isolated from the Averno lake

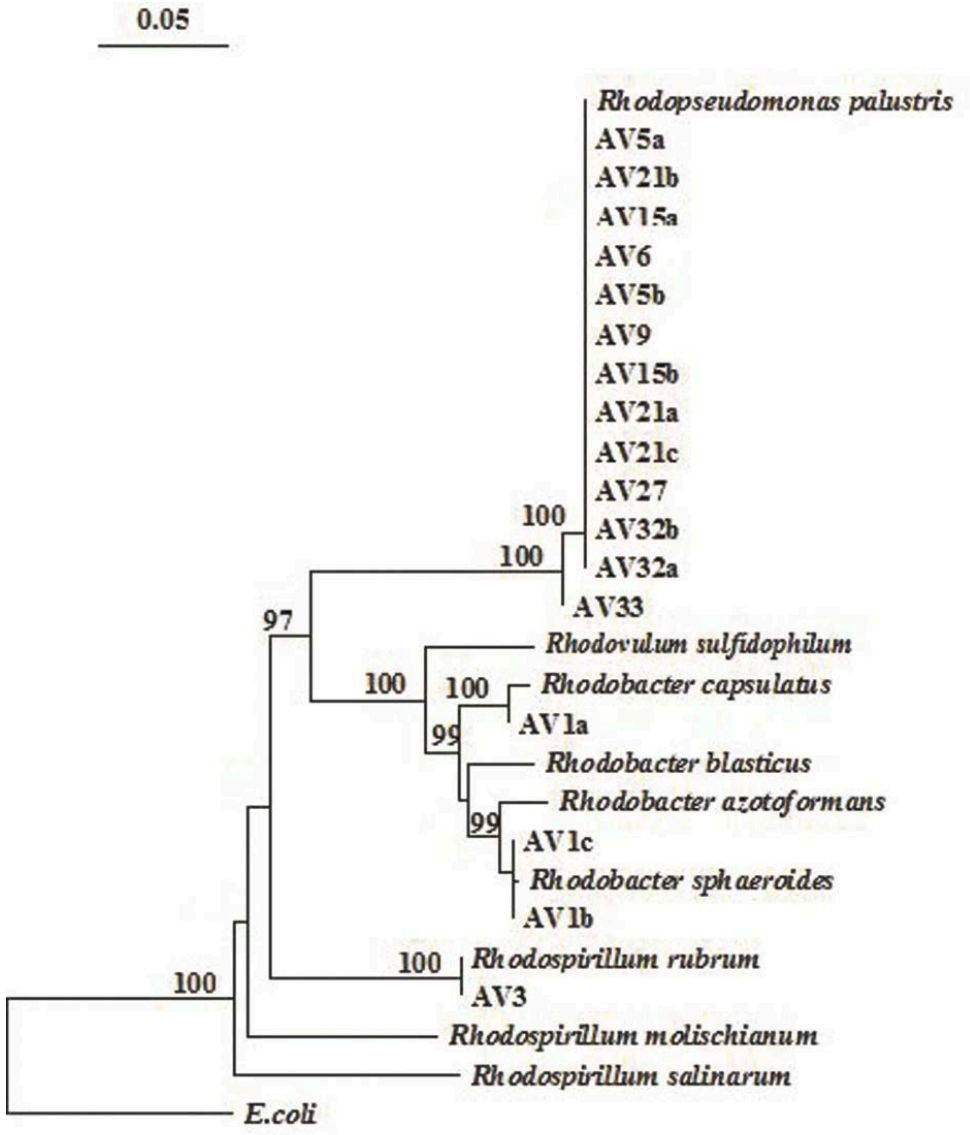

Figure 12. Neighbour-joining phylogenetic tree for the isolates and related strains of PNSB bacteria based on the 16s rDNA gene sequences. Bar = 0.05% nucleotide substitutions. The numbers at the nodes are bootstrap values (only values above 90% were indicated).

From the water samples taken at various depths in the Averno Lake, seventeen PNSB strains, at least one from each sample, were isolated by means of the streak plate method.

The bacterial isolates were identified by sequencing 16S rDNA and the sequences obtained (about 800 bp) were aligned by Clustal X with non-sulfur α-*Proteobacteria* groups; 16S rDNA from *Escherichia coli* (k02555) was used as reference sequence. For the alignment, a neighbour-joining phylogenetic tree (Figure 12) was build up. The phylogenetic analysis of the isolates, carried out by comparing 16S rDNA sequences, pointed out that the isolates belong to three different genera: three isolates were identified as members of the genus *Rhodobacter*, two belonging to *Rb. sphaeroides* and one to *Rb. capsulatus*, thirteen were identified as belonging to *Rhodopseudomonas palustris* and one as belonging to *Rhodospirillum rubrum*.

With regard to the vertical distribution of these isolates, the two strains of *Rb. sphaeroides* and the strain of *Rb. capsulatus* were isolated from the water sampled at -1 m depth, the strain of *Rs. rubrum* from the water sampled at -3 m depth and the *Rp. palustris* strains from the samples taken from a depth of -5 m to the sediment of the lake (-33 m).

1.3.2 Screening of the hydrogen producing capability of the isolates utilizing various electron donors.

Table 6. Hydrogen production by the PNSB strains, isolated from the Averno Lake, grown on four distinct organic substrates (malic acid, lactic acid, acetic acid, succinic acid). Notes: + = presence of H_2 production; - = absence of H_2 production.

Strains	Species	Malic acid	Lactic acid	Acetic acid	Succinic acid
AV1a	*R.capsulatus*	+	+	-	+
AV1b	*R.sphaeroides*	+	+	-	+
AV1c	*R.sphaeroides*	+	+	-	+
AV3	*R.rubrum*	+	+	-	-
AV5a	*R.palustris*	+	+	-	+
AV5b	*R.palustris*	+	+	-	-
AV6	*R.palustris*	+	+	-	-
AV9	*R.palustris*	+	+	-	+
AV15a	*R.palustris*	+	+	-	+
AV15b	*R.palustris*	+	+	-	+
AV21a	*R.palustris*	+	+	-	+
AV21b	*R.palustris*	+	-	-	-
AV21c	*R.palustris*	+	+	-	-
AV27	*R.palustris*	+	+	-	-
AV32a	*R.palustris*	+	+	-	-
AV32b	*R.palustris*	+	+	-	+
AV33	*R.palustris*	+	+	+	-

The hydrogen producing capability of the strains was tested by using four different substrates, namely acetic, succinic, lactic and malic acids. This first set of experiments was carried out growing the strains on each organic substrate and qualitatively evaluating the presence or absence of hydrogen production. All the seventeen strains showed the capability to produce hydrogen on at least one substrate (Table 6): ten of them were capable of utilizing three different substrates, six two different substrates and one, AV21b, only malic acid. None of the strains showed the capability to produce H_2 with all the four substrates. Malic acid was utilized by all the strains, lactic acid by sixteen and succinic acid by nine of them while acetic acid was only utilized by strain AV33.

1.3.3 H_2 production with *Rp. palustris* strains AV33, AV32a, AV32b

In a following set of experiments, aimed at quantitatively measuring the hydrogen production performances of a selected number of isolates, three *Rp. palustris* strains (AV33, AV32a, AV32b) were chosen on the basis of their origin from the deepest water layers of the lake, where the processes of decomposition of the organic matter are very active due to the activity of chemoheterotrophic microorganisms, with the consequent presence of large amounts of volatile organic acids suitable for the metabolism of purple non sulfur bacteria.

Two different carbon sources were used for the hydrogen production assays, acetic acid and lactic acid, which are among the typical products of the H_2-producing fermentations carried out by anaerobic chemotrophs. Acetate was only supplied to *Rp. palustris* AV33, the strain that showed, in the qualitative tests, the capability to produce hydrogen using this substrate (Table 6).

All the three *Rp. palustris* strains confirmed their capability to use lactate as substrate for producing detectable and stockable amounts of H_2. The production of hydrogen started after few hours, about 5 h for AV33 and 20 h for the other two strains (Figure 13), and at the end of the production phase, corresponding to the time when lactate was exhausted, the total amount of H_2 produced was 721 ± 33 mL, 585 ± 28 mL and 415 ± 39 mL, respectively for strains AV33, AV32a and AV32b. The production rates, calculated over periods of 24 hours, steadily increased in the first days of production, reaching the maximum values of 50.7 ± 2.6 mL (H_2) L^{-1} h^{-1} (at time = 75 h) for the strain AV33, of 17.8 ± 2.1 (at time = 75 h) for the strain AV32a and of 10.8 ± 0.3 (at time = 145 h) for the strain AV32b (Table 7 and Figure 14). The highest specific H_2 production rate, calculated as mmoles of hydrogen produced per g of cell dry weight per hour, was 1.03 ± 0.12, 0.58 ± 0.07 and 0.34 ± 0.01 mmol (H_2) g^{-1} h^{-1} for strains AV33, AV32a and AV32b, respectively. The mean H_2 production rate, calculated on the whole period of H_2 production, was 17.9 mL ± 0.7 (H_2) $L^{-1}h^{-1}$ for strain AV33, 11.1 ± 1.1 mL (H_2) $L^{-1}h^{-1}$ for strain AV32a and 7.1± 0.2 mL (H_2) $L^{-1}h^{-1}$ for strain AV32b. During the same period, it was observed an increase in the cell dry weight of the cultures of 232 ± 11 mg L^{-1}, 644 ± 20 mg L^{-1} and of 605 ± 15 mg L^{-1} for AV33, AV32a and AV32b, respectively.

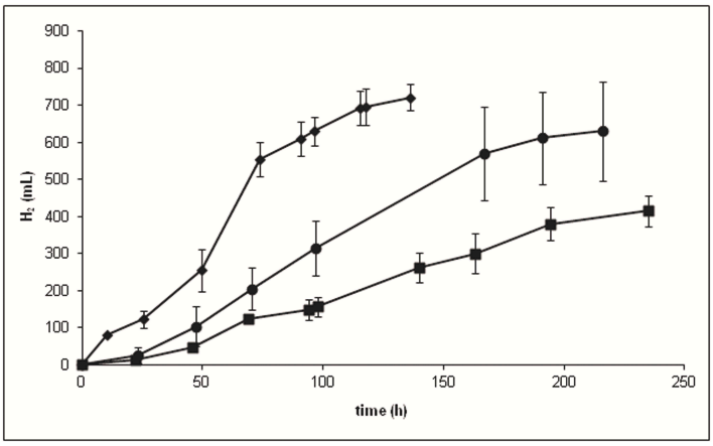

Figure 13. Time course of hydrogen production by *Rp. palustris* strain AV32a (●), AV32b (■), AV33 (◆) grown on synthetic medium containing lactate as electron donor. Data are mean values of at least three independent experiments and bars represent the standard deviation.

Table 7. Hydrogen production rates, conversion yield of substrate and light efficiencies of *Rp. palustris* strains AV32a, AV32b and AV33 grown on synthetic medium containing lactate as carbon substrate.

	Mean H_2 production rate mL (H_2) L^{-1} h^{-1}	Maximum H_2 production rate mL (H_2) L^{-1} h^{-1}	Conversion yield of substrate (%)	Light efficiency (%)
AV32a	11.1 (±2.1)	17.8 (±2.1)	42.8	1.44
AV32b	7.1 (±0.7)	10.8 (± 0.3)	28.1	0.97
AV33	17.9 (± 4.7)	50.7 (± 2.6)	55.4	2.6

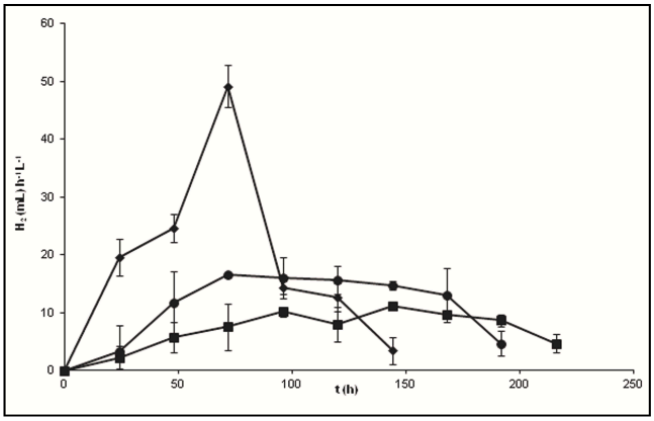

Figure 14. Time course of hydrogen production rates by *Rp. palustris* AV32a (●), AV32b (■), AV33 (◆) grown on synthetic medium containing lactate as electron donor. Data are mean values of at least three independent experiments and bars represent the standard deviation.

The strain AV33 was also grown with acetate as substrate, but it showed a very low activity of hydrogen production and the gas was only accumulated in the head space of the bioreactor, where it reached at the end of the experiment (250 h), a concentration of 30% (v/v), roughly corresponding to a volume of H_2 produced of 8-10 mL. In the same period, an increase in the cell concentration of 805 ± 30 mg L^{-1} was observed; the cells grown for 250h on acetate showed an intracellular concentration of poly-β-hydroxybutyrate (PHB) of 21.4 ± 3.4 % (w/w) of cell dry weight, whereas in the cells grown with lactate a PHB concentration of 10.9 ± 3.3 % (w/w) was found.

1.3.4 Hydrogen production on fermentation broth derived from vegetable residues

Rp. palustris AV33 was also tested for its capability to produce hydrogen with a substrate obtained from the spontaneous fermentation of vegetable residues carried out by the autochthonous microflora residing on the residues. The fermentation broth utilized contained, as main products, lactic and acetic acids (2.8 and 0.8 g L^{-1}, respectively), together with a small amount of ammonia (0.03 g L^{-1}) and of ethanol 0.12% (v/v).

Under the conditions above described, *Rp. palustris* AV33 produced hydrogen at an average production rate, calculated on the whole period of production, of 6.6 ± 1.4 mL (H_2) L^{-1} h^{-1}, with a maximum H_2 production rate of 9.7 ± 0.7 mL (H_2) L^{-1} h^{-1}. The addition of Fe (III) to the fermentation broth significantly enhanced the mean H_2 production rate, which reached the value of 10.8 ± 2.6 mL (H_2) L^{-1} h^{-1}, with a maximum production rate at the second day of 16.4 ± 2.3 mL (H_2) L^{-1} h^{-1} (Table 8, Figure 15). In the presence of Fe (III), the lactic acid present in the fermentation broth resulted completely consumed at the end of the H_2 production phase, whereas in the absence of Fe (III) the production of the gas ceased when part of the lactate (0.5 g L^{-1}) was still present in the fermentation broth. Under both conditions, the acetate proved to be completely exhausted at the end of the experiments (275h) and the cells showed a concentration of PHB of 21.5 ± 2.6 %.

Table 8. Hydrogen production rates, total amount of H_2 produced, conversion yield of substrate and light efficiencies of Rp. palustris AV33 grown, with or without addition of Fe citrate, on the fermentation broth derived from the spontaneous dark fermentation of vegetable residues.

	Mean H_2 production rate mL (H_2) L^{-1} h^{-1}	Maximum H_2 production rate mL (H_2) L^{-1} h^{-1}	Total H_2 produced mL	Conversion yield of substrate (%)	Light efficiency (%)
w/o Fe addition	6.6 (± 1.4)	9.7 (± 0.7)	349 (± 74)	30.0	0.7
with Fe addition	10.8 (± 2.6)	16.4 (± 2.3)	605 (± 61)	44.2	1.2

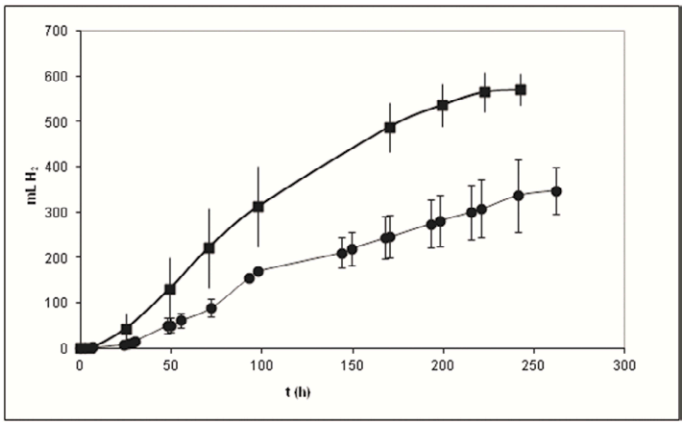

Figure 15. Time course of hydrogen production by *Rp. palustris* strain AV33 grown on the fermentation broth derived from the spontaneous dark fermentation of vegetable residues without (●) or with (■) the addition of Fe (III) to the substrate. Data are mean values of at least three independent experiments and bars represent the standard deviation.

1.4 Discussion

The phylogenetic analysis based on 16S rDNA gene sequences of the PNSB strains isolated from the water of Averno Lake pointed out that these isolates belong to the genera *Rhodospirillum*, *Rhodobacter* and *Rhodopseudomonas*, and are phylogenetically related to the known species of *Rhodospirillum rubrum*, *Rhodobacter capsulatus*, *Rhodobacter sphaeroides* and *Rhodopseudomonas palustris*. *Rhodobacter* and *Rhodospirillum* strains were only isolated from the samples taken in the first three meters of the water column, while the strains isolated at all the other depths were identified as belonging to *Rp. palustris*. The results obtained, also supported by the DGGE data obtained on the same water samples by a research group of ENEA (Italian National Agency for New Technologies, Energy and Sustainable Economic Development) (Giulio Izzo, personal communication), point out that PNSB were present all along the water column, independently of the depth. These findings are a further confirmation of the capability of these bacteria to grow in habitats characterized by large differences in their main characteristics owing to their great metabolic versatility [13, 22]. In particular, it has to be stressed that most of the isolates are phylogenetically related to *Rp. palustris* species, which is one of the most metabolically versatile microorganism known. Indeed, the recently published complete genome sequence of *Rp. palustris* showed the presence in the genome of genes involved in biochemical pathways supporting four different modes of metabolism, photoautotrophy, photoheterotrophy, chemoheterotrophy and chemoautotrophy [3]. More recently, the complete genome sequences of three other *Rp. palustris* strains, isolated from closely related microenvironments in lake sediments, pointed out that the three ecotypes evolved in a way that gave them the capability to successfully adapt to the distinctive features of these microhabitats [4]. The presence of *Rp. palustris* strains capable to grow in the Averno lake at different depths, under completely different conditions of light and nutrient availability and of oxygen concen-

tration [15], is a further confirmation of the great metabolic versatility of this species.

All the strains isolated from the Averno Lake showed the capability to produce hydrogen, confirming the hypothesized suitability of this environment to harbour H_2-producing PNSB. However, the strains showed a different behaviour with regard to their capability to utilize different electron donors for the production of the gas, even if all of them were capable to produce H_2 at least with one of the four acids tested. In any case, these differences are related neither with the species the strain belongs to nor with the depth of their habitat.

Rp. palustris AV33, the best H_2-producing strain on lactate, showed a maximum specific production rate, calculated over a period of 24 h, of 1.03 ± 0.12 mmol (H_2) g^{-1} (dry cell) h^{-1}, a value well comparable with the production rates obtained with most of the other H_2-producing PNSB so far tested, that mainly range between 0.9 and 2 mmol (H_2) g^{-1} (dry cell) h^{-1}, with some best results around 4.7 and 5.9 mmol (H_2) g^{-1} (dry cell) h^{-1} [21]. From the H_2-production rate of strain AV33, a volumetric daily production of about 1.2 L of H_2 per L of culture per day was calculated for the period of maximal activity. Even if the volumetric productivity is a parameter not completely satisfactory for evaluating the performances of photosynthetic microorganisms, for which the illuminated surface of the bioreactor should be taken into consideration, this parameter is frequently used for comparing the different H_2-producing systems from an engineering point of view. The value of 1.2 volume of gas produced per 1 volume of bioreactor obtained with *Rp. palustris* AV33 can be considered interesting when compared with most of dark fermentative processes of hydrogen production which, with few exceptions, were reported to range between 0.5 and 3.3 L (H_2) L^{-1} d^{-1} [23].

Considering the conversion yield of the substrate in hydrogen and the light efficiency of the process, the best values obtained with *Rp. palustris* AV33, 55.4 % and 2.60 % respectively, are quite interesting in comparison with most of the data reported in literature, that range mainly between 38-84% and between 1.1-4.8%, respectively [24, 25]. However, it has been reported that a light conversion efficiency of about 10% should be reached in order to consider economically feasible this process [21, 24], a goal that is considered achievable only by reducing the intensity of the incident light or by reducing the light harvesting antenna size of the PNSB utilized [1, 8].

Rp. palustris AV33 was the only strain that showed the capability to produce H_2 growing on acetate, but the amount of gas produced at the end of the experiment (250 h) was very low. On the other hand, a much higher intracellular PHB concentration was found in AV33 cells grown on acetate in comparison with those grown on lactate, pointing out that also in this strain acetate is more suitable for the synthesis of PHB than for the production of H_2, as it was previously reported for other PNSB [26]. It was also previously demonstrated that the two processes, PHB synthesis and H_2 production, compete for the reducing power, which in the presence of an active nitrogenase is preferentially channelled towards the synthesis of hydrogen [9].

When *Rp. palustris* AV33 was grown on a substrate derived from the spontaneous fermentation of vegetable residues, it showed a good capability to produce hy-

drogen, but the conversion yield in H_2 of the lactate contained in the fermentation broth was 30.0%, a value much lower than that observed using the synthetic medium containing lactic acid. On the other hand, in the fermentation broth the growth of the cells was 2.2 times higher (510 against 232 mg L^{-1}) in comparison with that observed with the synthetic medium owing to the presence of ammonia and other nitrogen sources which directed the utilization of the acids solubilized in the broth towards the biosynthesis of new biomass instead of H_2 production. It is also worth stressing the importance of the addition of Fe for obtaining good efficiencies in the conversion of the substrate in H_2, as it was previously found in *Rhodobacter sphaeroides* [27]. In this connection, it is worth mentioning that the structural genes encoding a large number of Fe-siderophore receptors and three different nitrogenases, including a Fe-nitrogenase, were shown to be present in the genome of *Rp. palustris* [3], thus confirming the great importance of Fe for its metabolism.

1.5 Conclusions

The results obtained in this study confirm the validity of searching, in natural environments having the suitable characteristics for harbouring PNSB, new strains to be exploited for the biological production of hydrogen. In particular, the good performances obtained with *Rp. palustris* AV33, even under not optimized growth conditions, suggest carrying out further studies aimed at improving the H_2-production performances of the strain. Moreover, the capability of *Rp. palustris* AV33 to produce H_2 in the presence of lactate is rather promising for the utilization of this strain in integrated H_2- producing systems combining dark- and photofermentation. This capability, in fact, is very useful when chemoheterotrophic bacteria operating in the dark phase produce large amounts of lactate due to metabolic conditions that channel the reducing equivalents derived from the oxidation of the carbon substrate in the reduction of pyruvate instead of in the reduction of protons to H_2. Finally, the preferential use of acetate for PHB synthesis by strain AV33 might be usefully exploited for recovering this economically valuable product from the exhausted cells in a two stage process where, at the end of the H_2-producing phase, the cultures are subjected to P- [28] or S- deprivation [29] for inducing the iperaccumulation of PHB.

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Results - Section II

1. The use of low cost substrates and their implementation

1.1 Introduction

The use of combined processes, i.e. dark fermentation followed by photofermentation, for H_2 production offers the opportunity of exploiting the specific features of different microorganisms for obtaining the best results in terms of H_2 yield [Redwood et al., 2009; Keskin et al., 2011].

The complete oxidation of the substrate is obtained combining the dark fermentation process with photofermentation carried out by purple non sulfur bacteria, thus theoretically enabling the total conversion of 1 mole of glucose into 12 moles of hydrogen.

Usually those processes integrate a first stage, where thermophilic or mesophilic chemoheterotrophic bacteria convert carbohydrates into H_2 and organic acids, with a second stage, where the fermentation products deriving from the first stage are converted to H_2 by photoheterotrophic bacteria. To these two kind of processes a third one can be added, with no H_2 production in the first dark fermentative stage [Kapdan and Kargi, 2006; Redwood et al., 2009].

This experimentation had the aim of verifying the feasibility of all these three kind of integrated processes, testing PNS bacteria performances on substrates deriving: i) from a mesophilic dark fermentation process (carried out by a microbial consortium), indicated as MDF; ii) from a thermophilic dark fermentation process (carried out by *Thermotoga neapolitana*), indicated as TDF; iii) from the spontaneous dark fermentation of vegetable wastes (carried out by the autochthonous microflora residing on vegetables - no H_2 production in the first stage), indicated as VDF.

The purple non sulfur bacterial species chosen for the experimentation is *Rp. palustris*, due to its wide versatility and ability of utilizing unconventional and complex substrates [Larimer et al., 2004; Oda et al., 2008; Harwood, 2008].

1.2 Materials and methods

1.2.1 Bacterial strains and culture conditions

Two *Rhodopseudomonas palustris* strains were used for this study, namely strain AV33 and 42OL, both part of the collection of the Department of Agricultural Biotechnology (University of Florence, Italy). The cultures were activated in RPP

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medium [Bianchi et al. 2010] containing malate 4 g L^{-1} and glutamate 1 g L^{-1}. The substrates deriving from the three fermentation processes were used as the production media, with the modifications decribed below. Actively grown cells were used as an inoculum and were centrifugated (10' 4500 rpm) and resuspended in the H_2 production medium to reach an initial OD_{660} of 0.8. The hydrogen production experiments were carried out under anaerobic conditions at 30°C in 250 mL rubber-stopped vessels equipped a hydrogen gas collecting system (water displacement). The illumination was supplied by an incandescent lamp giving a light intensity of 200 μmol (photons) m^{-2} sec^{-1}.

1.2.2 Analytical methods

The concentration of the organic acids in the culture medium was determined with an HPLC chromatograph (1100 series, Agilent Technologies, USA) equipped with a 8 mm (I.D.) ×300 mm Shodex-SH1011 packed column (Denko K. K., Tokyo, Japan). A diode array detector (DAD) was used for the determination and quantification of the organic acids while a refractive index (RI) detector was used for ethanol. The column was maintained at 50◦C and the eluent was 0.01 N H_2SO_4, at a flow rate of 0.6 mL min^{-1}.

The amount of hydrogen in the gas phase of the trap was determined by gas chromatography using a GC3800 Varian (Agilent Technologies, USA) equipped with a Molecular Sieve column, a Hayesep Q 80-100 column and a TCD filament detector with He as carrier gas.

The conversion yield of the carbon substrate to hydrogen was calculated as the percentage of the actual amount of hydrogen produced on the maximum amount of hydrogen theoretically obtainable from the complete conversion of the substrate to hydrogen. As the main components of the VWD medium were lactate and acetate, the theoretical reactions used were (24-25):

$$C_3H_6O_3 + 3H_2O \rightarrow 6H_2 + 3CO_2 \quad (24)$$

$$C_2H_4O_2 + 2H_2O \rightarrow 4H_2 + 2CO_2 \quad (25)$$

1.2.3 Substrates used and modifications applied

The exhaust medium deriving from the mesophilic dark fermentation (MDF) was obtained from the incubation of an hydrogen producing microbial consortium collected in the Averno Lake (Naples, Italy); the medium was kindly provided by Dr. Giulio Izzo (ENEA, Casaccia – RM). The medium contained 0.8 g L^{-1} of acetate and 2.7 g L^{-1} of lactate. Fe(III) in the form of ferric citrate (5 mg L^{-1}) was added to the medium where indicated.

The exhaust medium deriving from the thermophilic dark fermentation (TDF) was obtained from the incubation of *Thermotoga neapolitana* DSMZ 4359^T; the medium was kindly provided by Dr. Angelo Fontana (ICB-CNR, Pozzuoli – NA). The medium contained 2.1 g L^{-1} of acetate and 0.3 g L^{-1} of lactate. Fe(III) in

the form of ferric citrate (5 mg L^{-1} or 30 mg L^{-1}) was added to the medium where indicated.

The medium deriving from the fermentation of vegetable wastes (VDF) was obtained by the spontaneous fermentation of vegetable residues carried out by the microflora residing on the vegetables, as previously described [De Philippis 2007].The main products contained in the fermentation broth, were: acetic acid (2.19 g L^{-1}; 36.4 mM) and lactic acid (7.71 g L^{-1}; 85.6 mM). The medium also contained 110 mg L^{-1} of ammonium. Fe(III) in the form of ferric citrate (5 mg L^{-1}) was added to the medium where indicated and the medium was diluted with 2 parts of distilled water.

1.3 Results and discussion

1.3.1 Two stage process: mesophilic dark fermenting microbial consortium + *Rhodopseudomonas palustris* AV33

The experimentation was conducted with the strain AV33, as it was isolated from the same lake of origin of the microbial consortium (Bianchi et al. 2010). H_2 production with *Rp. palustris* could be obtained only after the addition of Fe(III), a component that often results necessary or improving the H_2 production process in PNS bacteria [Kars et al. 2006, Zhu et al. 2007, Uyar et al. 2009, Bianchi et al. 2010]. A consistent cell growth was observed (4.5 g d.w. L^{-1} of increment), and this negatively affected the substrate conversion rate [Redwood et al., 2009], that is noticeably low (Table 9). This elevated cell growth could be due to the abundance of N containing molecules that can be uptaken by *Rp. palustris*, such as NH_4^+ or aminoacids and switch its metabolism towards cell growth instead of H_2 production.

Table 9. Results for photofermentation on MDF derived medium [with the addition of Fe-citrate5 mg L^{-1}] by *Rp. palustris* AV33

	Rp. palustris AV33
Mean hour rate* mL (H_2) L^{-1} h^{-1}	1.9
Mean daily rate* L (H_2) L^{-1} d^{-1}	0.05
Max hour rate ** mL (H_2) L^{-1} h^{-1}	3.3
Max daily rate ** L (H_2) L^{-1} d^{-1}	0.08
% substrate conversion	7.1 (lactate)***
Moles lactate + acetate in moles of H_2	1 mol lactate*** = 0.42 mol H_2

* mean rate calculated on the whole period (6-7 days)
** max rate, generally obtained during the 2nd-3rd day, calculated on 24 hours
*** AV33 uses only lactate to produce H_2
Two stage process: *Thermotoga neapolitana* DSMZ 4359T + *Rhodopseudomonas palustri*s 42OL.

The experimentation was carried out with the *Rp. palustris* strain 42OL, due to the high concentration of acetate in the medium, as our previous data (not shown) indicate this strain as the best producer of H_2 from acetate among the strains in our possess. Similarly to the above cited results, H_2 production with *Rp. palustris* could be obtained only after the addition of Fe(III); the addition of a more concentrated

Fe(III) solution brought to higher production rates (slope of the curves in Figure 16), but no relevant increase in the total amount of gas produced was observed. Molibdenum was also added (at the concentration present in RPP medium [Bianchi et al. 2010]) as there were found evidences of the effectiveness of this element on hydrogen production [Kars et al. 2006], but in the present tests it did not bring any improvement on hydrogen production neither when added alone or in combination with Fe(III). A significant increase in cell dry weight was observed (2.5 – 3.6 g d.w. L^{-1} of increment), bringing to a low substrate conversion rate (Table 10). However, cell growth was smaller and the substrate conversion higher than in the above reported experimentation, indicating this substrate as a better medium than the other.

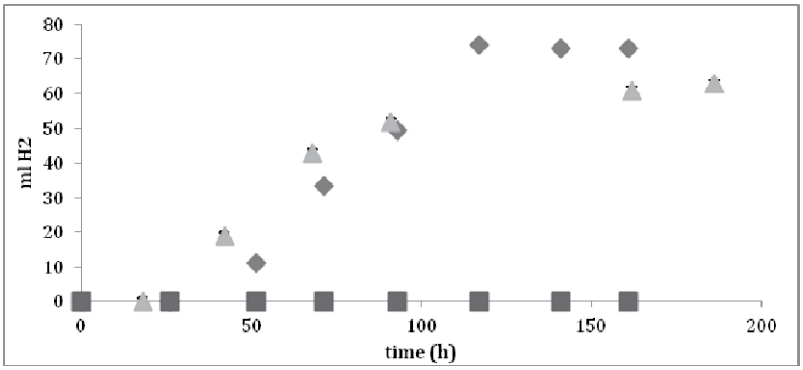

Figure 16. time course of hydrogen production by *Rp. palustris* 42OL on TDF derived medium with no Fe-citrate added (squares), 5 mg L-1 Fe-citrate added (triangles), with 30 mg L-1 Fe-citrate added (rhombs).

Table 10. Results for photofermentation on TDF derived medium [with the addition of Fe (III) 30 mg L-1] by *Rp. palustris* 42OL

	Rp. palustris 42OL
Mean hour rate* mL (H_2) L^{-1} h^{-1}	3.9
Mean daily rate* L (H_2) L^{-1} d^{-1}	0.09
Max hour rate ** mL (H_2) L^{-1} h^{-1}	8.2
Max daily rate ** L (H_2) L^{-1} d^{-1}	0.20
% substrate conversion	16.5
Moles lactate + acetate in moles of H_2	0.3 mmol lactate+3.3 mmol acetate=2.5 mmol H_2

* mean rate calculated on the whole period (6-7 days)
** max rate, generally obtained during the 2nd-3rd day, calculated on 24 hours

1.3.2 Two stage process: spontaneous dark fermentation of vegetable wastes + *Rhodopseudomonas palustris* 42OL and AV33.

The substrate obtained from the dark fermentation of vegetable wastes carried out by the autochthonous microflora was used for photofermentation with both the strain AV33 and 42OL, but at a three-fold dilution due to the dark color of the substrate and to the presence of a high concentration of ammonium (that is an inhibitor

fro hydrogen production by nitrogenase [Ludden and Roberts, 2002]). The addition of Fe (III) was not necessary for hydrogen production, but an addition of Fe-citrate 5 mg L^{-1} significantly improved the rate and the amount of hydrogen produced (Figure 17). Cell dry weight increments were smaller than with the other two substrates (1.7 – 1.9 5 g d.w. L^{-1}) and this positively affected the substrate conversion (Table 11) that is the highest obtained in this study.

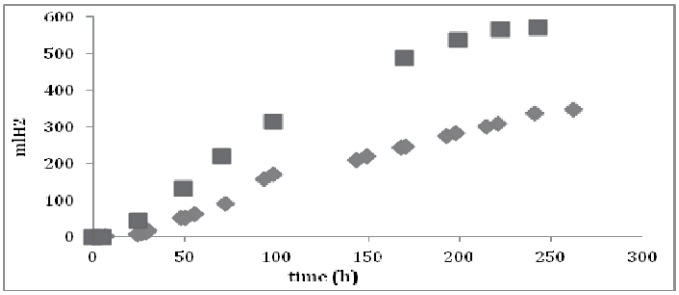

Figure 17. Time course of hydrogen production by *Rp. palustris* 42OL on VDF derived medium with no Fe-citrate added (rhombs) and 5 mg L-1 Fe-citrate added (squares).

Table 11. Results for photofermentation on VDF derived medium [with the addition of Fe (III) 5 mg L-1] by *Rp. palustris* AV33 and 42OL.

	Rp. palustris AV33	*Rp. palustris* 42OL
Mean hour rate* mL (H_2) L^{-1} h^{-1}	10.75 (+/- 2.6)	15.6 (+/- 1.0)
Mean daily rate* L (H_2) L^{-1} d^{-1}	0.26	0.37
Max hour rate ** mL (H_2) L^{-1} h^{-1}	16.4 (+/- 2.3)	n.d.
Max daily rate ** L (H_2) L^{-1} d^{-1}	0.39 ((+/- 0.06)	n.d.
% substrate conversion	54.7 % (lactate)***	43%
Moles lactate + acetate in moles of H_2	1 mol lactate***=3.3 mol H_2	6 mmol lactate+ 2.7 mmol acetic =22.6 mmol H_2

* mean rate calculated on the whole period (6-7 days)
** max rate, generally obtained during the 2nd-3rd day, calculated on 24 hours
*** AV33 uses only lactate to produce H_2

1.4 Conclusions

Photo-fermentation of vegetable residues or of effluents derived from previous dark fermentations showed to be experimentally feasible, thus giving the possibility to recover energy from organic wastes by coupling the production of H_2 with waste disposal or to integrate microbial processes to get a higher total hydrogen yield than with the single independent processes.

The addition of nutrients, like Fe (III), resulted to be in some cases necessary for hydrogen production, in other cases it improved the rates and/or the total amount of gas produced.

However, the utilization of this kind of substrates still needs to be optimized, in particular for minimizing the shift of the microbial metabolism from H_2 production to cell growth. Possible solutions stand in the removal of fixed N sources from the medium [Redwood and Macaskie, 2007] or in the use of strains insensitive to fixed nitrogen [Redwood et al., 2009; Kars and Gunduz 2010]. Furthermore, a insensitiveness to ammonium could prevent the need of diluting the medium with water, that would constitute a further cost in terms of money, energy and management of a possible up-scaled process. This aspect was investigated using mutant strains impaired in N regulation of nitrogenase (see the following Section II.2).

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2. A *Rhodopseudomonas palustris* nifA* mutant produces H_2 from NH_4^+-containing vegetable wastes

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Abstract

Research on photobiological H_2 production processes is pointing towards the use of low cost substrates as sources of reduced carbon for H_2 generation.

Those substrates (either wastewaters or effluents derived from other fermentation processes) are often rich not only in carbon, but also in fixed nitrogen. NH_4^+ is an inhibitor of nitrogenase-mediated H_2 production in purple non sulfur bacteria.

We used a *Rhodopseudomonas palustris* mutant strain (NifA*), which constitutively expresses nitrogenase genes, to test the use of NH_4^+-containing fermentation products for photobiological production of H_2. The strain was grown on both synthetic and waste-derived NH_4^+ containing media.

The NifA* mutant produced H_2 in the presence of high concentrations of NH_4^+, both in a synthetic medium and in a real vegetable waste-derived medium resulting in higher H_2 levels than the wild-type strain. Thus, this study demonstrates that the NifA* strain is well suited to overcome the effects of inhibitory naturally occurring NH_4^+ as it converts agricultural waste into biofuel.

2.1 Introduction

The role of H_2 gas in the future energy market is expected to be crucial, as it is a clean energy vector [1]. When H_2 reacts with oxygen, energy and water are generated, with minimal emissions or even none when using fuel cells. It also has the highest gravimetric energy content of any potential fuel [2].

However, in order to consider H_2 as a sustainable energy vector it has to be produced via sustainable processes. None of the currently available renewable H_2 production technologies, such as photovoltaic-electrolysis or gasification of biomass, is economically affordable [3].

This is the reason for the widespread interest in investigating biological routes to H_2 production that involve microbial activity. Among the various opportunities, photofermentation is frequently cited as a promising process, as it has the potential to use waste material as a substrate source, thus combining the remediation of waste effluents with the simultaneous production of clean energy [4]. The use of wastes as substrates is one option to achieve an economically viable process.

Most research looking at the production of H_2 from food and vegetable waste has focused on dark fermentation [5]. However, fermentations produce large quantities of electron-rich products that cannot be fermented further into H_2. Purple non-sulfur bacteria (PNSB) can naturally convert fermented waste into H_2 using the enzyme nitrogenase. Thus, photofermentation often stands as a downstream process, following other fermentation processes where carbohydrates are converted to organic acids. Indeed PNSB can overcome the thermodynamic barrier of converting organic acids into H_2 by using light energy [6].

These materials, either wastewaters or effluents derived from other fermentation processes, are frequently rich not only in desired carbon sources but also in amino acids and other compounds that are broken down to NH_4^+. NH_4^+ acts at several levels of regulation to inhibit H_2 production by PNSB via nitrogenase.

The regulation has been modeled as a three-level control mechanism, as described by Masephol et al. [7] for *Rhodobacter capsulatus*. This regulatory cascade is often used as a general model for the regulation of nitrogenase in PNSB though there are many variations on this regulatory scheme. The general scheme is as follows: at the first level fixed nitrogen signals (e.g., NH_4^+) are sensed through the NtrBC two-component system to prevent the transcription of *nif*A, a gene encoding for an RNA polymerase sigma 54-dependent transcriptional activator. At the second level, the presence of NH_4^+ affects NifA, inducing structural changes that prevent the transcriptional activator from binding to its binding site and activating nitrogenase (*nif*) gene transcription. At the third level, the presence of NH_4^+ affects nitrogenase itself causing a "switch-off" of the enzyme through ADP-ribosylation mediated by DraT [7-10].

Recently Redwood et al. [6] and Kars and Gunduz [11] reviewed the use of mutants insensitive to NH_4^+, indicating that genetic manipulation is a potential route to overcome nitrogenase inhibition by fixed-nitrogen-containing compounds including NH_4^+. A number of strains bringing mutations in *nif*A have been obtained [12, 13], all bringing single point mutations. The NifA* phenotype of the strain used in this study is attributed to a 48 nucleotide deletion in *nif*A, thus it is expected to more stable in a practical setting than mutants with single nucleotide changes.

The aim of this study was to use a *Rhodopseudomonas palustris nif*A mutant strain that is insensitive to NH_4^+, for phototrophic conversion of substrates in vegetable waste to H_2.

2.2 Materials and methods

2.2.1 Bacterial strains, growth media and culture parameters

All experiments were conducted with *Rp. palustris* strains CGA009 and its derivative *nif*A* mutant strain CGA676 constructed as described [9]. The strain CGA009 is defective in uptake hydrogenase activity [14]. CGA676 has constitutive nitrogenase activity allowing it to produce H_2 in the presence of NH_4^+ [9]. *Rp. palustris* was grown anaerobically in front of a 60-W light bulb at 30 °C in 16 mL volumes in sealed 27-mL anaerobic culture tubes (Bellco) with an argon (Ar) headspace. Cultures were grown in PM mineral medium [15] with $(NH_4)_2SO_4$ at the concentration indicated in the text, under an Ar headspace, or with vegetable waste derived (VWD) medium (described below) at dilutions and with additions as indicated in the text. Sodium acetate was supplied at a final concentration of 20 mM, when using PM mineral medium.

The VWD medium was obtained by the spontaneous fermentation of vegetable residues carried out by the microflora residing on vegetables, as previously described [16].The main products contained in the fermentation broth, were: acetic acid (2.19 g L^{-1}; 36.4 mM), lactic acid (7.71 g L^{-1}; 85.6 mM) and NH_4^+ (110 mg L^{-1}; 6.1 mM).

Before being used for the experiments with PNSB, the VWD medium was (where indicated) diluted with distilled water; it was supplemented with Fe(III) as ferric-citrate (5 mg L^{-1}) and the pH was adjusted from 3.2 to 6.8 with NaOH as previously described in [17]. After H_2 production stopped SO_4^{2-} was added to VWD medium in the form of Na_2SO_4 (0.124 mM, as in NF medium [18]), and Fe (III) was added in the form of ferric-citrate; this addition of citrate resulted in only in a small addition of carbon (0.020 mM of citrate) in comparison with the concentrations of acetate and lactate present in the VWD medium.

2.2.2 Analytical methods

H_2 was quantified by gas chromatography as indicated in [19]. Organic acids and culture supernatants were analyzed by HPLC as in [19].

The NH_4^+ content of VWD medium was determined with Nessler method [20]. The concentration in the various dilutions was estimated to be half and a third respectively for the two and the three fold dilutions.

Cell growth was quantified by optical density at 660 nm; as a blank, the medium without cells was used for the tests with VWD medium (Abs_{660} undiluted 0.77; two-fold diluted 0.39; three fold diluted 0.27). A calibration curve for optical density (OD) versus dry weight (d.w.) was built (R^2=0.974) and the equation resulted to be d.w. = 462.4*OD.

The intensity of the light reaching the front of the culture tubes was 35 µmol (photons) m^2 s^{-1} measured using a quantum meter (Spectrum Technologies, Plainfield, IL, USA).

2.2.3 Substrate conversion and photosynthetic efficiency

The conversion yield of the carbon substrate to H_2 was calculated as the percentage of the maximum amount of H_2 theoretically obtainable from the complete conversion of the substrate consumed to H_2 that was actually produced as H_2. As the main components of the VWD medium were lactate and acetate, the theoretical reactions used were (26-27):

$$C_3H_6O_3 + 3H_2O \rightarrow 6H_2 + 3CO_2 \quad (26)$$
$$C_2H_4O_2 + 2H_2O \rightarrow 4H_2 + 2CO_2 \quad (27)$$

The photosynthetic efficiency (PE), i.e. the efficiency of conversion of light energy to H_2 energy, was calculated assuming that all the incident light was absorbed by the culture; the following equation (28), based on [21] was applied:

$$PE = \frac{Combustion\ enthalpy\ of\ H_2 \times H_2\ production\ rate}{Absorbed\ light\ energy \times 100} \quad (28)$$

2.3 Results

2.3.1 Effect of the NH_4^+ ion on H_2 production by the *Rp. palustris* CGA009 wild type strain and *Rp. palustris* CGA676 *nif*A* mutant strain

In order to assess the effect that NH_4^+ has on H_2 production, the two strains were compared for their cumulative H_2 production when grown in PM mineral medium containing different concentrations of NH_4^+.

We found that whereas addition of NH_4^+ ion to the medium inhibited H_2 production by wild type cells, the NifA* mutant strain produced H_2 regardless of the concentration of the NH_4^+ ion in the medium. Starting with a same concentration of carbon (20 mM acetate), the total amount of H_2 produced was constant for the three NH_4^+ concentrations tested: 142.1 ± 20.8 μmoles of H_2 produced in presence of 2.5 mM NH_4^+, 137.3± 6.3 μmol H_2 produced in presence of 5.0 mM NH_4^+, 138.7 ± 1.5 μmol H_2 produced in presence of 10.0 mM NH_4^+. Indeed, two-way ANOVA showed an extremely significant difference between the two strains ($P=0.0009$), but no significant effect of the ammonium concentration in terms of total amount of H_2 produced. Bonferroni post-test indicated a significant difference between the two strains for 5.0 and 10.0 mM NH_4^+ ($P<0.05$); the difference between the two strains was not significant at 2.5 mM NH_4^+ ($P>0.05$) (Figure 18).

NH_4^+ concentrations of 5.0 mM and 10.0 mM completely inhibited H_2 production by the wild type strain. At 2.5 mM NH_4^+ some wild-type replicates were able to produce the same amount of H_2 as the NifA* mutant (162.6± 26.5 μmol H_2), while other replicates were completely inhibited and no gas was produced (Figure 18). Even in the cases where the wild type was able to produce as much H_2 as the mutant, the growth behavior of the wild type was significantly different from that of the mutant, as shown in Figure 19. The production of H_2 by the NifA* mutant was not influenced by NH_4^+, all the data referring to H_2 production at any NH_4^+ concentration

tested fitting one linear correlation with a positive slope (R^2=0.9769, Figure 19) to the cellular growth of the culture. The wild-type strain generally did not produce H_2, thus the data referring to all the NH_4^+ concentrations tested fit a line very close to the x-axis. However, two wild-type replicates out of five showed a different behavior at 2.5 mM NH_4^+. In those cases (Figure 19), a period of growth for 48 h occurred, followed by a period of 120 h of H_2 production with a modest increase in growth.

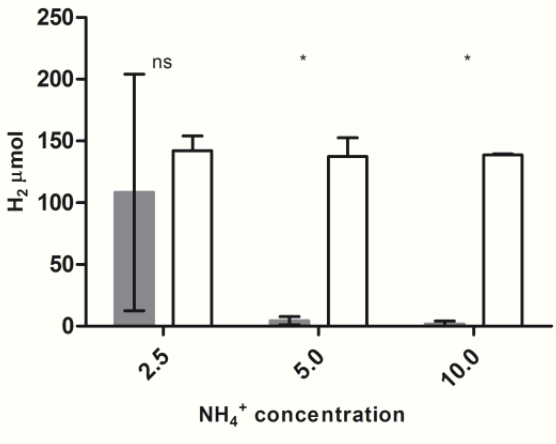

Figure 18. Total amount of H_2 (µmoles) accumulated by *Rp. palustris* CGA009 (■) and *Rp. palustris* CGA676 (□) in the headspace of the culture tubes upon the depletion of acetate for three different concentrations of NH_4^+ in PM culture medium. The data are the mean of at least three replicates and bars indicate SD. Two-way ANOVA and Bonferroni post-test were conducted, comparing the differences between the two strains in each condition (*=significant difference – P<0.05; ns= not significant difference - P>0.05).

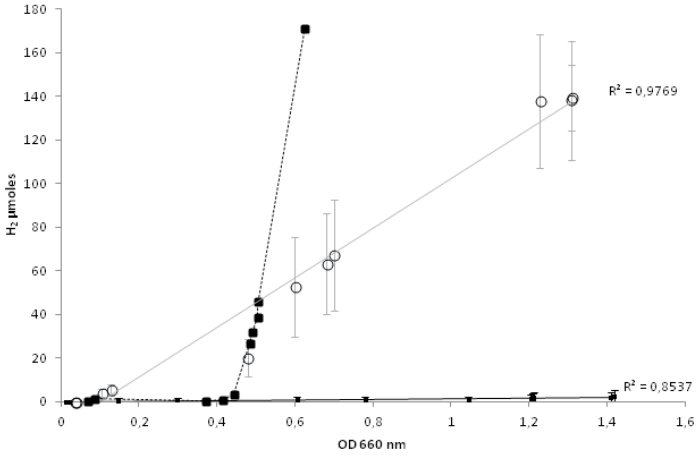

Figure 19. H_2 accumulated in the headspace versus cellular growth expressed as optical density at 660nm. Data for all NH_4^+ concentrations (2.5-10.0 mM) are included for *Rp. palustris* CGA009 wild type (■) and CGA676 mutant strain (○), except for the two wild-type replicates that eventually produced H_2 with 2.5mM NH_4^+ (dotted line). Error bars indicate SD.

2.3.2 H_2 production using vegetable waste derived (VWD) medium

The two strains of *Rp. palustris* used in this study normally grow poorly on lactate, the main constituent of the VWD medium. Thus, the strains were first adapted to grow on lactate by incubating them in PM medium with an argon atmosphere and 20 mM of lactate. After 190 h of adaptation and multiple transfers to fresh media, both the wild type and the mutant strain showed improved growth using lactate (data not shown).

The lactate-adapted strains were then tested for their H_2 production capability using the VWD medium. They both produced H_2 gas when grown in undiluted medium. However, the wild-type strain produced significantly less H_2 (432 ± 172 µmol H_2) than the NifA* mutant strain (1375±127 µmol H_2) and at a lower rate (1.34 ±0.53 ml H_2 l^{-1} h^{-1} for the wild type strain; 3.92 ± 0.82 ml H_2 l^{-1} h^{-1} for the mutant strain) (Figure 20).

After about 350 hours, H_2 production stopped for both the wild type and the mutant strain, but its reactivation was tested upon the addition of various mineral medium elements.

Phosphate buffer addition was ineffective, while the addition of NH_4^+ had an effect only on cell growth but not on H_2 production (data not shown). Sulfate and Fe (III) both had the effect of restoring H_2 production after it stopped. Sulfate was able to restore H_2 production at a rate of 2.47 ±1.55 ml H_2 l^{-1} h^{-1} for the NifA* mutant strain and no significant OD increase was detected. Fe(III) was added at the same concentration added at the beginning; it was more effective in restoring H_2 production than sulfate, as the rate after the addition was 6.36 ± 2.58 ml H_2 l^{-1} h^{-1} for the NifA* mutant strain, while no significant cellular growth was detected.

Organic acids (lactic and acetic) were present in excess throughout all the process, having at the end (770 h) a concentration of respectively 69.6 ±11.7 mM and 16.5±1.2 mM for the wild type strain and 38.8±3.9 mM and 1.85±0.5 mM for the mutant strain.

Both the strains were tested for H_2 production using the VWD medium at three different dilutions (Figure 21). The un-inoculated medium itself showed a relatively high absorbance at 500 nm (1.49 for the undiluted medium; 0.77 for the two-fold diluted medium; 0.53 for the three fold diluted medium), and at 800 and 875 nm (respectively: 0.49 and 0.42 for the undiluted medium; 0.24 and 0.21 for the two-fold diluted medium; 0.16 and 0.15 for the three fold diluted medium). Data on H_2 production showed a high variability, due to the complexity of the substrate.

Two-way ANOVA showed an extremely significant difference between the two strains ($P=0.0005$), but no significant effect of the dilution in terms of total amount of H_2 produced. Bonferroni post-test indicated a significant difference between the two strains in the undiluted medium ($P<0.05$) and at a 2-fold dilution ($P<0.05$). The difference between the two strains was not significant for the 3-fold diluted VWD medium ($P>0.05$). The data of the undiluted medium reported in Figure 21 refer to the total amount of H_2 produced before the additions of Fe(III) and SO_4^{2-}.

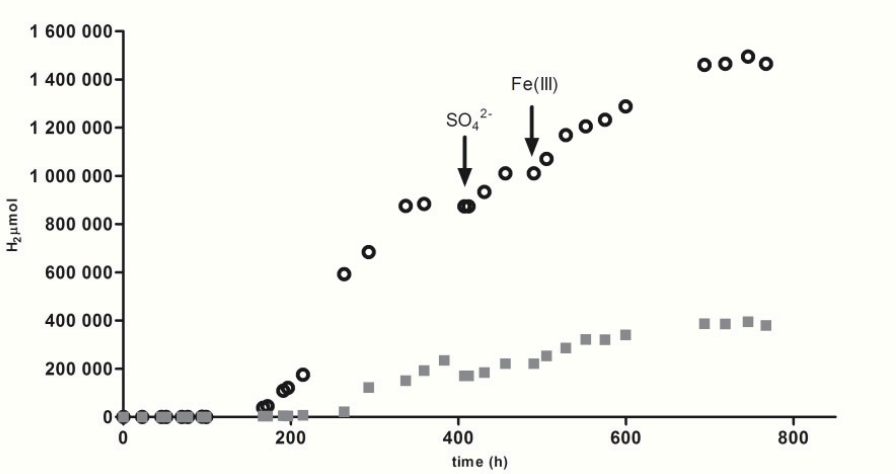

Figure 20. Time course of H_2 production for *Rp. palustris* CGA009 (■) and *Rp. palustris* CGA676 (○) growing on undiluted VWD medium. For the sake of clarity, the figure shows only one of the three replicates for each strain. Similar reactivation of H_2 production by the addition of sulfate or ferric iron was observed in the other replicates but at different times (data not shown). Lactate and acetate were present in excess throughout all the process, having (for this replicate) at the end (770 h) a concentration of, respectively, 66.4 mM and 17.2 mM for the CGA009 strain and 41.5 mM and 3.7 mM for the CGA676 strain.

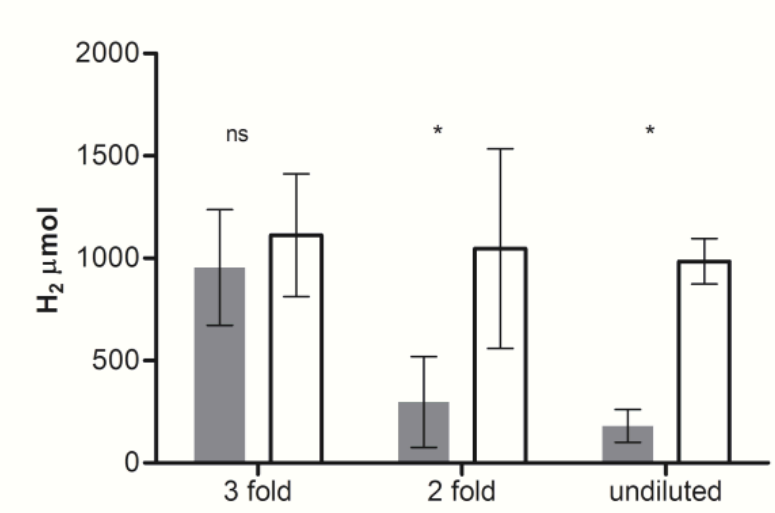

Figure 21. Total H_2 µmoles accumulated by *Rp. palustris* CGA009 (■) and *Rp. palustris* CGA676 (□) in VWD medium at three different dilutions (NH_4^+ concentrations were: 2.03 mM for the 3-fold dilution, 3.05 mM for the 2-fold dilution and 6.1 mM for the undiluted VWD medium). Error bars indicate SD of three replicates. Two-way ANOVA and Bonferroni post-test were conducted, comparing the differences between the two strains in each condition (*=significant difference – $P<0.05$; ns= not significant difference - $P>0.05$).

Table 12. H_2 production with the *Rp. palustris* NifA* strain grown on three different dilutions of VWD medium; ± indicates SD of three replicates.

	Rate (ml l^{-1} h^{-1})	Substrate conversion(%)[a]	PE (%)[b]
Three-fold	9.6 ± 2.6	41.5 ± 14.6	1.93 ± 0.48
Two-fold	6.3 ± 3.7	33.3 ± 10.2	1.05 ± 0.61
No dilution	3.9 ± 0.8	20.5 ±0.6	0.64 ± 0.06

[a] Substrate conversion is the percentage of maximum theoretical yield in H_2 obtainable from the amount of organic acids consumed.

[b] PE=photosynthetic efficiency, calculated as the amount of energy obtained as H_2 per amount of energy absorbed.

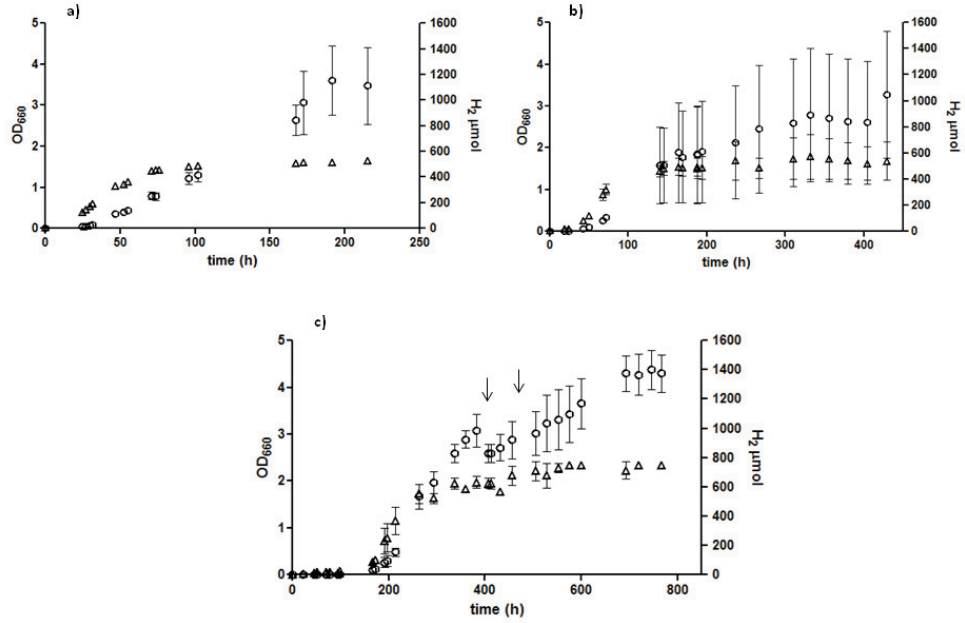

Figure 22. Time course of H_2 production (○) and cell growth, measured as OD at 660 nm (Δ), of *Rp. palustris* CGA676 growing on VWD medium: a) at a three-fold dilution, b) at a two-fold dilution, c) undiluted (with the additions of SO_4^{2-} and Fe (III), indicated by the black arrows). Error bars indicate SD of three replicates. Time scales are different for the three graphs.

2.3.3 H_2 production parameters by the *Rp. palustris* NifA* mutant strain in VWD medium

The volumetric H_2 production rate, substrate conversion and photosynthetic efficiency were evaluated for the *Rp. palustris* NifA* mutant strain at the three different dilutions (Table 12); differences were analyzed by one-way ANOVA and found to be not significant with regard to substrate conversion to H_2 ($P > 0.1$), but significant with regard to production rates ($P=0.065$) and with regard to photosynthetic efficiency ($P=0.0184$). Bonferroni post-test showed a significant difference ($P<0.1$)

between the three-fold dilution and the undiluted VWD medium for both production rates and photosynthetic efficiency.

When the mutant strain was grown with VWD medium, H_2 gas could be detected in the tubes from the very beginning for every dilution tested, but a consistent gas accumulation was registered only after cell growth had started. The dilution of the medium shortened this lag phase as well as the total period of the growth (Figure 22).

2.4 Discussion

2.4.1 Effect of the NH_4^+ ion on H_2 production by the *Rp. palustris* CGA009 wild type strain and the *Rp. palustris* CGA676 NifA* mutant strain

The NH_4^+ concentration range for these tests was chosen in order to include the NH_4^+ concentration present in the VWD medium to be used in the subsequent tests.

This first set of tests demonstrated the efficacy of the *nifA** mutation, as it ensured NH_4^+ insensitivity for any NH_4^+ concentration tested (2.5 mM, 5 mM and 10 mM). This is supported by two-way ANOVA results which indicate a significant effect of the strain used, but no significant effect of the NH_4^+ concentration.

While 5.0 and 10.0 mM of NH_4^+ completely inhibited H_2 production by the wild type strain, the concentration of 2.5 mM appeared to be a threshold concentration: out of five replicates, three did not produce any H_2, while two showed the behavior illustrated in Figure 19 by the black dotted curve. This behavior is most likely driven by nitrogen metabolism. That is, when the cells grew presumably used all the available fixed nitrogen (ammonium) for growth. During this time nitrogenase was inhibited and no H_2 could be produced. However, as soon as the fixed nitrogen source was consumed, cellular growth almost stopped, and at the same time the repressive effect on nitrogenase was removed and H_2 started to be produced (as already shown by Zheng et al. [22]), provided that reduced carbon was still available [19]. The amount of H_2 produced by the wild type in this condition was noticeably higher than the amount produced by the mutant; this can be attributed to the fact that NH_4^+ had been consumed at the moment H_2 production started, so the electrons deriving from acetate were all diverted to H_2 rather than biosynthesis, as previously described [19].

The observed value of 2.5 mM of NH_4^+ as the limit value for H_2 production is in agreement with other data reported in literature with other wild type strains of PNSB. Yokoi et al. [23] reported 2 mM of NH_4^+ as a limiting concentration for *Rhodobacter* spp., and the same concentration was reported for *Rb. sphaeroides* as well [24]. Akkose et al. [25] reported that a wild type strain of *Rb. sphaeroides* was able to produce H_2 in the presence of up to 3 mM NH_4^+, but did not produce H_2 at 5 and 10 mM NH_4^+.

2.4.2 H_2 production using the vegetable waste derived (VWD) medium

The VWD derived medium (containing 6.1 mM of NH_4^+) proved to be suitable for H_2 production by both *Rp. palustris* wild type and *Rp. palustris nifA** cells. However the NifA* strain produced more H_2 than the wild type. The delay in and

lower efficiency of H_2 production by the wild type in comparison with the NH_4^+-insensitive mutant, can be attributed to the presence of NH_4^+. Indeed, the wild type strain, before producing H_2, consumed both the carbon substrate and NH_4^+ converting them into biomass. As a consequence, NH_4^+ concentration decreased to a value below the inhibiting value for H_2 production. In further support of this, diluting the medium resulted in more H_2 production by the wild type strain, while the mutant produced the same amount of H_2 for all three dilutions tested (Figure 21).

A large variability was observed for several measurements in the VWD medium (Table 12), highlighting that it is a complex medium with some components that are not homogeneously distributed. Statistical analysis showed that there was a very significant effect of the strain used on the total amount of H_2 produced, but a not significant difference among the three dilutions; the wild type strain produced significantly less H_2 than the mutant at lower dilutions. This constitutes a further demonstration of the effectiveness of the *nif*A* mutation, even in such a complex substrate.

Diluting the VWD medium did not result in significant changes to H_2 production by *Rp. palustris* CGA676 in terms of substrate conversion, but it had a significant effect on photosynthetic efficiency and production rates. Those differences are most probably due to the greater clarity of the diluted medium compared with the undiluted one, as it is evidenced by the absorbance of the three dilutions of the VWD medium at the wavelengths corresponding to the absorption peaks of the culture. The VWD medium particularly absorbed at the wavelengths at which the carotenoids of these strains absorb (470, 492, 518 nm). Indeed, the absorbance of 1.49 measured at 500 nm for the undiluted medium means that carotenoids are mostly obscured by the absorbance of the medium. The clarity of the medium is a preeminent issue when working with photosynthetic microorganisms; a higher light penetration could be obtained increasing the intensity of the incident light [4].

The limited irradiation of cells in undiluted VWD medium may have reduced the H_2 production rates as well, according to the previously described proportional increase of H_2 production and cell growth with light intensity [26].

Substrate conversion was not significantly different among the three dilutions, so, taking into account all the data obtained, it is possible to conclude that about one third of the substrate was converted to H_2 by *Rp. palustris* CGA676 mutant strain. This phenomenon can be attributed to the presence of NH_4^+, whose presence, even if not inhibitory for H_2 production, induced a large biomass production, deviating part of the substrate from H_2 production to cellular growth [27]. However, the substrate conversion we observed was higher than the one obtained with the same strain (10.5%) when grown in synthetic medium with only acetate [28]. Such a higher conversion can be explained by the fact that, in the present study, most of the H_2 was produced during stationary growth phase, when the carbon substrates can only be converted to H_2 as they are not used for biosynthesis. In the other study, only H_2 that accumulated during the exponential growth phase were reported [28].

Whereas H_2 yields were higher during stationary phase, H_2 production rates were higher during the linear growth phase than in stationary phase (see Figure 22) as previously reported for the wild type strain [29].

The dilution of the VWD medium had a relevant effect on the duration of H_2 production. Indeed, when undiluted VWD medium was used the total duration of the experiment was 800 hours (with almost 200 h of lag phase), while with the two and the three-fold diluted VWD medium both the total duration and the initial lag phase were noticeably shorter (Figure 22). Both these phenomena can be explained considering that the dilution decreases both the amount of carbon substrate available to the cells and the amount of NH_4^+ to be consumed before reaching the concentration not inhibitory for H_2 production.

During the experiments carried out with the undiluted VWD medium, H_2 production was observed to cease after about 350 h (Figure 20). In order to investigate the reason for why H_2 production stopped a number of potentially limiting components of the mineral medium were added. The addition of phosphate didn't have any detectable effect on H_2 production. Also NH_4^+ addition was ineffective at stimulating H_2 production, but stimulated cell growth. This trend indicates that the cessation of H_2 production was not due to a lack of nitrogen needed for protein turnover (e.g., to repair nitrogenase), which was shown to be necessary for prolonged H_2 production [30]. A reversible "switch-off" of nitrogenase has to be taken into account [31], but the NifA* strain was reported to escape ADP-ribosylation of nitrogenase in presence of NH_4^+ [10]. Furthermore, H_2 production did not start even after cell growth when NH_4^+ was added, thus pointing out the lack of some components for H_2 production other than nitrogen. On the contrary, both sulfate and iron additions induced the re-establishment of H_2 production, but did not have any significant effect on cell growth. Actually, a decrease in H_2 production upon sulfur deprivation of PNSB cultures was previously reported [32], as well as the effect of low Fe concentrations on the expression levels of *nif*D, one of the nitrogenase genes [33]. As nitrogenase is an enzyme containing Fe-S clusters [34], our results seem to indicate a need of turnover of these inorganic clusters.

2.5 Conclusions

Considering our results it is possible to draw some conclusions:

- The *nif*A* mutation makes the *Rp. palustris* mutant strain insensitive to NH_4^+ in a concentration range between 2.5 and 10 mM.
- Both the wild type and the NifA* strain were able to produce H_2 on the undiluted VWD medium, but the *nifA** mutation allowed higher H_2 production at a faster rate.
- The mutant strain started earlier and produced more H_2 than the wild type.
- Dilution of the VWD medium sped up the process, shortening the H_2 production lag time, and increased the photosynthetic efficiency.

The major conclusion is that the NifA* strain of *Rp. palustris* is capable of producing H_2 independently from NH_4^+ concentration, both in a synthetic medium and in an actual waste derived medium, and thus it can be considered suitable for being used in the production of H_2 with substrates derived from previous fermentation of vegetable wastes.

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Results - Section III

1. Light/dark cycles under laboratory conditions

1.1 Introduction

Most of the experimentations about photobiological hydrogen production are conducted under continuous artificial light conditions. However, the energy for photofermentation can be also derived from sunlight in order to decrease the energetic and economical costs of the process.

Sunlight is free and largely abundant, but is subject to day/night cycles, so an assessment on the feasibility of the hydrogen production process with PNS bacteria in such conditions is desirable before stepping to an actual outdoors experimentation [Gadhamshetty et al. 2010].

PNS bacteria are able to survive in the absence of light but they are not able to produce hydrogen, as it is a light dependent process [Uffen 1970, Larimer et al. 2004]. However, there is evidence of the ability of make hydrogen production start again after a dark period, as soon as the culture is irradiated by light [Miyake et al. 1999, Uyar et al. 2007].

In our previous experience with hydrogen production processes in light/dark cycles, we observed the presence of a lag time from the moment the culture was irradiated (after the period of darkness) and the time we could detect H_2 gas accumulation.

The main interest of this experimentation was to determine the lag time that daily takes the cultures to start producing H_2 from the moment the light is switched on. Cells were subject to cycles with different light/dark ratios, in order also to determine if there is a limiting number of hours of darkness for the process to start again in light.

1.2 Materials and methods

1.2.1 Bacterial strains and culture conditions

The *Rhodopseudomonas palustris* 42OL strain used for this study is part of the collection of the Department of Agricultural Biotechnology (University of Florence, Italy). The cultures were activated in RPP medium [Bianchi et al. 2010] containing malate 4 g L^{-1} and glutamate 1 g L^{-1}. The same medium without glutamate was used as the H_2 production medium. Actively grown cells were used as an inoculum and

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were centrifuged (10' 4500 rpm) and resuspended in the H_2 production medium to reach an initial OD_{660} of 0.8. The hydrogen production experiments were carried out under anaerobic conditions at 30°C in 250 mL rubber-stopped vessels equipped with equipped a hydrogen gas collecting system (water displacement method). The illumination was supplied by an incandescent lamp giving a light intensity of 180 μmol (photons) m^{-2} sec^{-1}. Cultures were subject to three different light/dark cycles: 16 hours of light and 8 of darkness; 12 hours of light and 12 of darkness; 8 hours of light and 16 of darkness.

1.2.2 Analytical methods

The concentration of malic acid in the culture medium was determined with an HPLC chromatograph (1100 series, Agilent Technologies, USA) equipped with a 8 mm (I.D.) ×300 mm Shodex-SH1011 packed column (Denko K. K., Tokyo, Japan). A diode array detector (DAD) was used for the determination and quantification of the organic acids while a refractive index (RI) detector was used for ethanol. The column was maintained at 50◦C and the eluent was 0.01 N H_2SO_4, at a flow rate of 0.6 mL min^{-1}.

The amount of hydrogen in the gas phase of the trap was determined by gas chromatography using a GC3800 Varian (Agilent Technologies, USA) equipped with a Molecular Sieve column, a Hayesep Q 80-100 column and a TCD filament detector with He as carrier gas.

The conversion yield of the carbon substrate (malate) to hydrogen was calculated as the percentage of the actual amount of hydrogen produced on the maximum amount of hydrogen theoretically obtainable from the complete conversion of the substrate to hydrogen, according to the following reaction (29):

$$C_4H_6O_5 + 3H_2O \rightarrow 6H_2 + 4CO_2 \quad (29)$$

1.3 Results and discussion

Cellular growth was monitored taking samples any time the light was switched on or off. In Figure 23 is shown the time course of the OD_{660} for the test with 16 hours of light and 8 of darkness; no cell growth was observed during the dark periods (grey bands) [Koku et al. 2003]; after 150 hours a slight decrease of OD during the dark period was observed and this course was the same for all the light/dark cycle tested (data not shown). The decrease in OD occurs during growth stationary phase, where the culture is more sensitive to stress, so cellular death might have occurred in anaerobic conditions and in the absence of light.

The consumption of the organic substrate (malate) was also monitored and is shown in Figure 24. No organic acid consumption was observed during the dark periods.

Hydrogen production occurred after a 40-hours lag time, probably because of the need for the acclimation to the light/dark conditions (Figure 25). The culture stopped producing H_2 gas with the absence of light because in these conditions (anaerobiosis) the only source for ATP generation is cyclic photosynthesis, and 16

molecules of ATP are needed to generate 4 molecules of H_2. As long as the organic substrate was available, however, at every new period of light the culture was able to start producing hydrogen again.

Comparing the three light/dark cycles tested, the 12/12h test showed the best results in terms of production rate and substrate conversion (Table 13). This is consistent with the results obtained by Wakayama et al. 2000. It is not possible to exclude, though, a not optimal condition of the inoculum used for the 16h light/8h dark conditions, as the replicates showed a bigger variability. The results for the 8h light/16h dark cycle tests showed that this cycle is stressing the cultures, that could not recover during the light period from the damage suffered during the dark period.

The daily mean lag time for hydrogen production was measured observing the moment the first gas bubble passed through the gas collecting system; the tests with 16h light/8h dark and the tests with 12/12h didn't show any statistically significant difference in lag time, while the lag phase for the 8h light/16h dark cycle tests was much longer (Table 13).

However, from a qualitative observation gas bubbles could be seen in the culture right after light was switched on, while a larger amount of time was needed to measure the first gas bubbles in the accumulation system.

These first tests revealed the need of developing more sensitive methods to determine the exact moment at which H_2 production begins.

1.4 Conclusions

The results obtained in this study demonstrate the ability of the *Rp. palustris* strain 42OL to operate under light/dark cycles conditions, ensuring hydrogen production at every new light period, as long as the substrate was available. However, a light/dark cycle of 8h light/16h dark showed not to be suitable for an efficient hydrogen producing process.

A lag time in hydrogen production was observed, but the method chosen for the determination of the starting point is not enough sensitive. A more sensitive method, as an electrode for measuring dissolved H_2 in the culture, can be a good tool to answer this question and it was utilized in the subsequent experiments outdoors (see Section III.3).

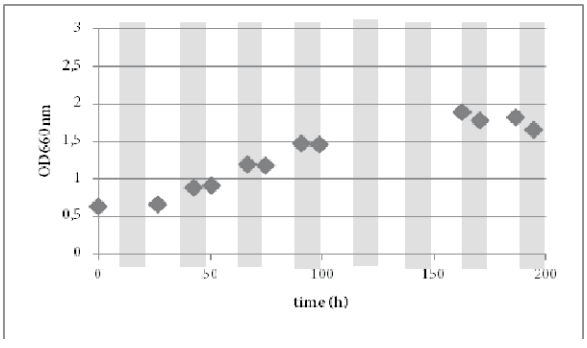

Figure 23. time course of OD660 for cultures of *Rp. palustris* 42OL under light/dark cycles of 16h light/8h dark (grey bands indicate dark periods).

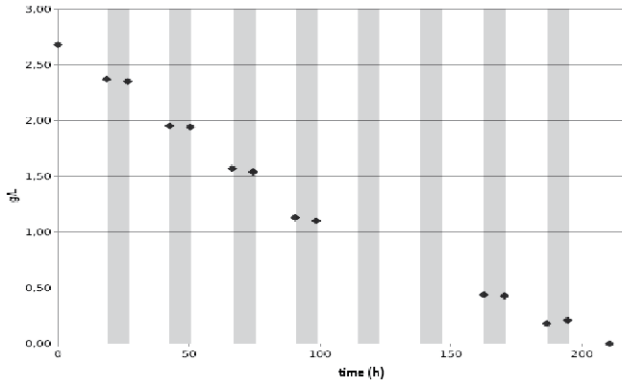

Figure 24. time course of malate consumption for cultures of *Rp. palustris* 42OL under light/dark cycles of 16h light/8h dark (grey bands indicate dark periods).

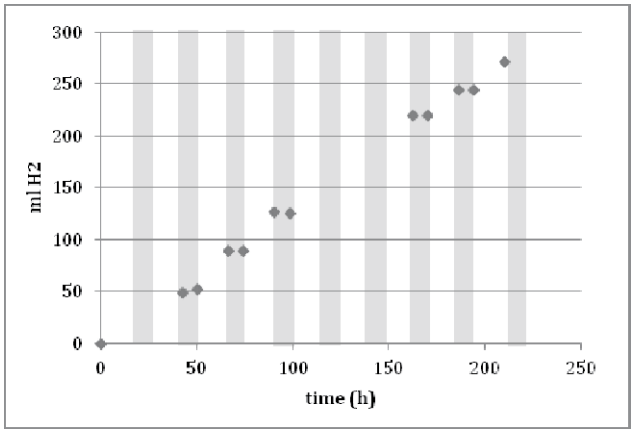

Figure 25. time course of H_2 accumulation for cultures of *Rp. palustris* 42OL under light/dark cycles of 16h light/8h dark (grey bands indicate dark periods).

Table 13. hydrogen production with *Rp. palustris* 42OL under light/dark cycles.

	16h light – 8h dark	12h light – 12h dark	8h light – 16h dark
Daily lag time (min)	98.5 ± 30.3	74.9 ± 10	212.3 ±53
Mean rate (ml l^{-1} h^{-1})	4.12 ±2.02	10.47 ±0.40	2.76*
Substrate conversion (%)	17.15 ± 6.12	49.12 ± 6.48	9.97*

*SD not determinable

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2. PAM fluorometry on purple non sulfur (PNS) bacteria

2.1 Introduction

Purple non sulfur (PNS) bacteria convert light energy into electrochemical potential through the directional proton translocation connected to a cyclic electron transfer between the reaction center (RC) and the cytochrome *bf*1 complex, mediated by mobile carriers cytochrome c_2 and ubiquinone/ubiquinol redox pair [Drews and Golecki 1995, Adessi and DePhilippis, in press]. Structural and functional homology between the purple bacterial RC and the RC of the photosystem II (PSII) of oxygenic photosynthesis allows the use of variable bacteriochlorophyll (Bchl *a*) fluorescence to investigate the energy transfer and electron transport within the photosynthetic apparatus in photosynthetic bacteria in a manner similar to plants and algae [Kolber et al. 2001, Strasser and Ghosh 1995]. Today there is much evidence that confirms the applicability of chlorophyll fluorescence to photosynthetic bacteria [Koblizek et al. 2005, Maroti 2008, Asztalos et al. 2010]. Those studies, though, all concern photosynthetic kinetics and other basic research studies.

Aim of this study is to use PAM fluorometry in order to measure the photosynthetic activity evaluating the efficiency of light capturing and utilization of the incident radiation by the bacterial photosystem as an estimate of the physiological

status of the culture. The interest is to develop a sensitive and ready-to-use method that could then be applicable to massive outdoors cultures.

2.2 Materials and methods

2.1.1 Instruments

Chlorophyll fluorescence was recorded using a portable pulse-amplitude-modulation fluorometer (PAM-2100, H. Walz, Effeltrich, Germany) operated by an external PC using PamWin software. For F_v/F_m determination, 0.5-ml samples were taken from the bottles, and dark-adapted for 15 minutes. Minimum BChl a fluorescence, F_o, was measured by modulated light (< 0.5 µmol photons m^{-2} s^{-1}) coming from a light-emitting diode (peak wavelength at 655 nm, 600 Hz). A single high-intensity flash (6000 µmol photons m^{-2} s^{-1}, 0.8 s in duration) provided by a miniature 8V/20W halogen lamp (Bellphot, Osram) was used to raise the fluorescence yield to the maximum, F_m. The effective quantum yield of PSII was measured as $(F'_m-F_s)/F'_m$, where F'_m and F_s were the maximum and the steady-state fluorescence under light exposure, respectively. The fluorescence nomenclature follows Van Kooten and Snel (1990). Fluorescence data collected by Pam-2100 were exported into a spread sheet program (Excel). The fraction of absorbed light utilized in electron transport was given by the effective PSII quantum yield (EQY), $\Delta F/F_m' = (F_m'-F_s)/F_m'$ (Genty et al., 1989).

2.2.2 Setup of the best measuring conditions

Cellular concentration of the culture

It was evaluated the answer of samples at different cell concentration, balancing the need of obtaining an intense signal (obtainable with a more concentrated sample) and the risk of self-shading of the cells. The best measuring concentration resulted to be in the range of 1.4-1.8 OD_{660}.

Stirring

For measures with microalgae and cyanobacteria the stirring of the sample during the measurements is necessary as the cells rapidly sediment; for PNS bacterial cultures no cell sedimentation could be observed during measuring time, so no stirring was needed.

Temperature

Measures were done at room temperature in order to simplify as much as possible the measuring conditions. As the measuring time is relatively short, it was assumed that small temperature variations could not affect the measures.

Dark adaptation

Since it is necessary to dark adapt cultures before taking the measures, different dark adaptation times were compared in terms of intensity of the signal obtainable

with the following measure; for artificial irradiation, a 5 min dark adaptation time was sufficient, while for outdoor cultures a longer time might be necessary.

2.2.3 Set up of the measuring method

Measures were taken on cultures of growing *Rhodopseudomonas palustris* strain 42OL, belonging to the collection of the Department of Agricultural Biotechnology- University of Florence.

To evaluate the efficacy of the method, significantly different growth conditions were imposed to the cultures and we evaluated the fluorescence answer, both qualitatively and quantitatively. The choice of the growth parameters to impose to the cultures fell on the most important for what concerns PNS bacteria:

- Light conditions
- Growth temperature

Furthermore, as the interest of developing this technique is the utilization of the same in outdoors massive cell cultures, those two parameters are outwardly the most relevant.

All measures were taken on three samples and for every sample three measures were taken. Data have been statistically elaborated using the t-test ($\alpha<0.05$): for the values of F_v/F_m the test was calculated between the mean couples; for the Effective Quantum Yield (EQY) curves the test was calculated for each point of the mean curve, in couples as specified in the results.

2.3 Results and discussion

2.3.1 Exposure to different temperatures

The signal of fluorescence, intended as F_v/F_m, resulted significantly different (Figure 26). In particular it could be observed a decrease in the F_v/F_m ratio with the increasing temperature.

In Figure 27 the EQY curves are reported for each condition; it has to be noticed that the EQY for the cultures grown at 28°C is higher than the EQY for cultures grown at 17°C or 45°C. As the EQY is a measure of the capability of the photosystems to function when stimulated by photons [Masojìdek et al. 2004], it makes sense that a culture grown at its optimal growth temperature has the best quantum yield. This seems inconsistent with the F_v/F_m, but it has to be stressed that this parameter measures the number of photosystems available in the sample, and not their activity.

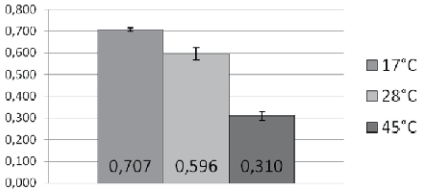

Figure 26. F_v/F_m ratio at three different temperatures for growing cultures of *Rp. palustris* 42OL; t-test ($\alpha<0.05$).

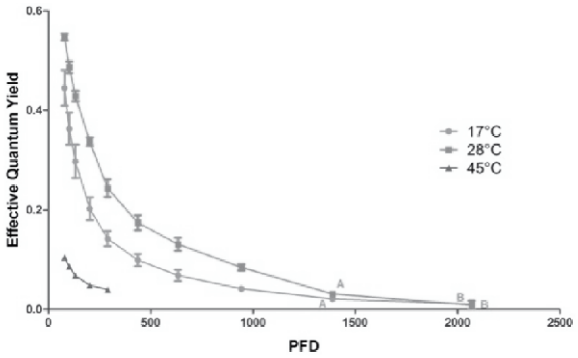

Figure 27. *Effective Quantum Yield. PFD= Photon Flux Density* (µmol photons m^{-2} s^{-1}). The curves obtained for the three conditions are significantly different from each other, except for the last two points. t-test was performed for each PFD value in couples (17°C vs 28°C; 28°C vs 45°C; 17°C vs 45°C)

2.3.2 Exposure to different light intensities

Three light intensities were tested. The F_v/F_m values (Figure 28) were significantly different, but it cannot be observed a relationship with the increase of light intensity, at least in the range tested.

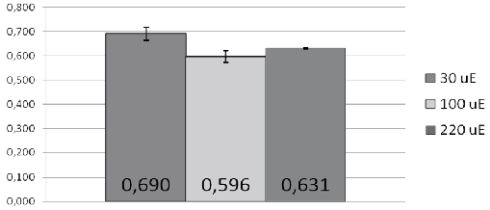

Figure 28. Fv/Fm ratio at three different light intensities for growing cultures of *Rp. palustris* 42OL; t-test (α<0.05).

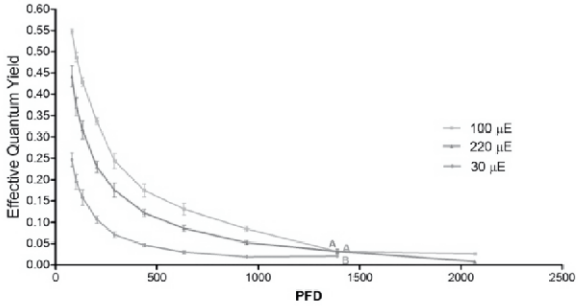

Figure 29. The curves obtained for the three conditions are significantly different from each other, except for the second last point. t-test was performed for each PFD value in couples (100 µE vs 220 µE; 220 µE vs 30 µE; 100 µE vs 30 µE).

In Figure 29 are reported the EQY curves for the three light conditions tested. The efficiency of the photosystem is higher for the culture irradiated with a light intensity of 100 µE, a little lower for the culture irradiated with a light intensity of 220 µE and noticeably lower for the culture irradiated with a light intensity of 30 µE.

2.3.3 Comparison of cultures in H_2 production and not in H_2 production

In Figure 30 are reported the values of F_v/F_m ratios for a growing culture and a hydrogen producing culture. The difference between the two is significant but not very marked, having the H_2 producing culture a slightly higher value. The same thing can be said for the EQY curves (Fig 31).

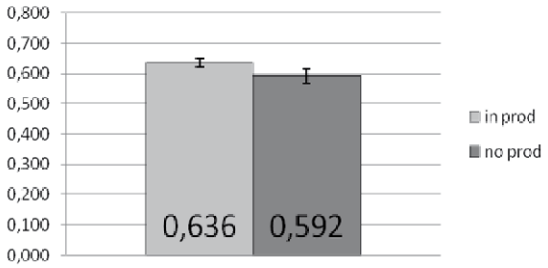

Figure 30. Fv/Fm ratio for growing cultures (no prod) and hydrogen producing cultures (in prod) of *Rp. palustris* 42OL; t-test (α<0.05).

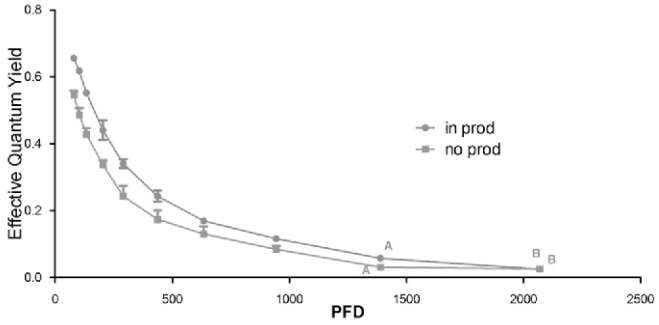

Figure 31. *Effective Quantum Yield. PFD= Photon Flux Density* (µmol photons m^{-2} s^{-1}). The curves obtained for the two conditions are significantly different from each other, except for the last two points. t-test was performed for each PFD.

2.3.4 Exposure of H_2 producing cultures to different temperatures

The F_v/F_m values (Figure 32) obtained with the H_2 producing cultures at 18°C and 30°C were not significantly different, while 45°C showed to be a temperature not compatible with healthy and functioning photosystems during hydrogen production. Surprisingly the EQY curve (Figure 33) of the culture maintained at 18°C is higher than the curve corresponding to the culture grown at 30°C. Again, 45°C are not compatible with the functioning of the photosystem.

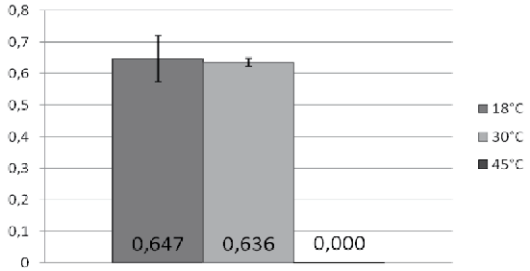

Figure 32. Fv/Fm ratio at three different temperatures for growing cultures of *Rp. palustris* 42OL; t-test (α<0.05).

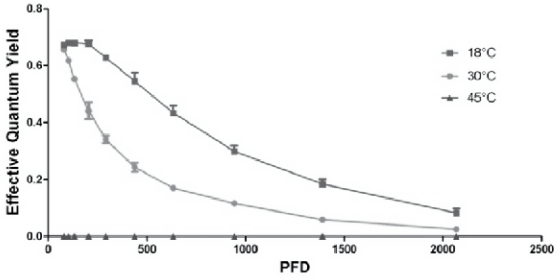

Figure 33. *Effective Quantum Yield. PFD= Photon Flux Density* (µmol photons m^{-2} s^{-1}). The curves obtained for the three conditions are significantly different from each other. t-test was performed for each PFD value in couples (18°C vs 30°C; 30°C vs 45°C; 18°C vs 45°C)

2.4 Conclusions

PAM fluorometry showed to be an efficient technique for measuring fluorescence emitted by PNS bacterial cultures with the instruments available. No modification of the wavelenght of the emitting light resulted to be necessary to get a good signal from the cultures (the measuring light emitted by the instrument is at 665 nm; this wavelength corresponds to the tail of carotenoid absorption area, not to the bacteriochlorophyll *a* peaks).

From these results it can be stated that the method can be useful to evaluate the physiological state of the cultures, in particular for the stress imposed by temperature variations, both for growing and hydrogen producing cultures.

It is interesting how the cultures seem to be more sensitive to temperature stress under hydrogen producing conditions than in growing conditions. This is however confirmed by many studies about hydrogen production, where it is stated that controlling the operating temperature is crucial for the process [Ozgur 2009].

The method showed not to be sensitive enough to discriminate between cultures grown at light intensities comprised in the range 30-220 µE, with F_v/F_m values. However the corresponding EQY curves were more informative, as a bigger difference could be seen between the 100-220 µE and the 30 µE irradiated cultures. It

cannot be excluded, though, that larger difference in fluorescence parameters could be recorded for cultures grown at light intensities above the range tested.

As a major conclusion the method, with the measuring conditions set up in this study, results to be applicable to cultures of PNS bacteria both in growing and in hydrogen producing conditions. An application with outdoors massive cell cultures can be foreseen of the method developed is reported in the following Section III.3.

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3. Sustained outdoor H_2 production with *Rhodopseudomonas palustris* cultures in a 50 L tubular photobioreactor

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Abstract

Sunlight represents an important choice for low cost photosynthetic cultures irradiation, especially in up-scaled processes. The aim of this study was the assessment of the hydrogen production performances of *Rhodopseudomonas palustris* strain 42OL cultivated outdoors, under sunlight, in a 50 L tubular photobioreactor. Moreover, bacteriochlorophyll *a* fluorescence was used for the first time as a tool for monitoring the physiological status of *Rp. palustris* mass cultures.

The maximum H_2 production rate of 27.2 ml L^{-1} h^{-1}, and the mean rate per illuminated surface of 3.54 ± 1.53 L m^{-2} d^{-1} obtained resulted the highest so far reported for outdoor experiments carried out with purple bacteria in tubular photobioreactors of comparable dimensions. The *in-situ* measurement of bacteriochlorophyll *a* fluorescence (F_v/F_m) showed that the photosystem was unaffected by changing light irradiance during the day and remained fully active over the whole period of hydrogen production.

3.1 Introduction

H_2 can be considered as the energy vector of the future, as is a clean, renewable and energy-efficient fuel. Among the questions still open about its use as an effective renewable fuel is its production in an environmental friendly, economical and sustainable way [1].

Processes for H_2 production that involve purple non-sulfur (PNS) bacteria are considered promising sustainable strategies as these bacteria are characterized by a very versatile metabolism [2, 3], by a high substrate to H_2 conversion efficiency [4] and are able to use a wide spectrum of the solar light [5].

Yet, at the present state of the art, the energy input for the processes is still larger than the energy output in the form of H_2 gas. A possibility for increasing this ratio lies in reducing the electricity consumption, particularly for illumination and mixing of the cultures [6]. Relaying on this, the use of solar light seems to be a first step to-

wards the reduction of energy input, as it is free and largely abundant [7]. In particular, the use of solar light is mandatory for the scaling-up of the photobiological hydrogen production process which is desirable and very promising for reaching as industrial scale level with PNS bacteria [8], but research on the topic of sunlight utilization for hydrogen production is still needed [9, 10].

When using natural light, the choice of the proper geographical site where to install the photobioreactor represents a *sine qua non* condition for an economical successful cultivation of photosynthetic microorganisms. Though solar light cannot be controlled in wavelength selection, solar photobioreactors (PBRs) can be oriented to receive long wavelength radiation at sunrise and sunset in order to better fit PNS bacteria peaks in the absorption spectrum [11,12].

PNS bacteria convert light energy into electrochemical potential through the directional proton translocation connected to a cyclic electron transfer between the reaction center (RC) and the cytochrome *bf*1 complex, mediated by mobile carriers cytochrome c_2 and ubiquinone/ubiquinol redox pair [13,14]. Structural and functional homology between the purple bacterial RC and the RC of the photosystem II (PSII) of oxygenic photosynthesis allows the use of variable bacteriochlorophyll (Bchl *a*) fluorescence to investigate the energy transfer and electron transport within the photosynthetic apparatus in photosynthetic bacteria in a manner similar to plants and algae [15, 16]. Today there is much evidence that confirms the applicability of chlorophyll fluorescence to photosynthetic bacteria [17-19].

Since the mid-1990s, a significant contribution to microalgal biotechnology has been made by the introduction of modern photobiochemical methods, mainly chlorophyll (Chl) fluorescence. Indeed, it was demonstrated that it is possible to monitor the photosynthetic activity of microalgal cultures in outdoor systems by using this method [20, 21].

Amongst PNS bacteria, *Rhodopseudomonas palustris* in particular is a very versatile microorganism [2]; this wide versatility gives it the opportunity to adapt to very different growing conditions. In particular, the utilization of light is a very complex process in *Rp. palustris* and it is finely regulated, as pointed out by the huge number of regulatory bacteriophytochromes present for the acclimation to different environmental conditions in terms of light wavelength and intensity [22].

Although *Rp. palustris* has been grown outdoors for biomass production [23, 24], the information on outdoor hydrogen production with this PNS bacterium still lacks. Furthermore, only a very limited number of papers are available on H_2 production processes using PNS bacteria in photobioreactors of order of magnitude of tens of liters and up to now they all involve the use of *Rhodobacter capsulatus* [25-28].

The aim of this study was the assessment of the hydrogen production performances of a *Rhodopseudomonas palustris* strain cultivated outdoors, under solar illumination, in a 50 L tubular photobioreactor. This study was also aimed at developing and testing a new method, based on the fluorescence of Bchl *a*, as a tool for monitoring the physiological status of *Rp. palustris* cells during the process of outdoor hydrogen production.

3.2 Materials and methods

3.2.1 Bacterial strain and culture medium

The *Rhodopseudomonas palustris* 42OL strain used for this study is part of the collection of the Department of Agricultural Biotechnology (University of Florence, Italy). The cultures were activated in RPP medium [29] containing malate 4 g L^{-1} and glutamate 1 g L^{-1}. The same medium was used in the 50 L tubular photobioreactor as the H_2 production medium. The presence or absence of glutamate (1g L^{-1}) is indicated in the text. Actively grown cells were used as an inoculum in the photobioreactor.

3.2.2 The 50 L photobioreactor

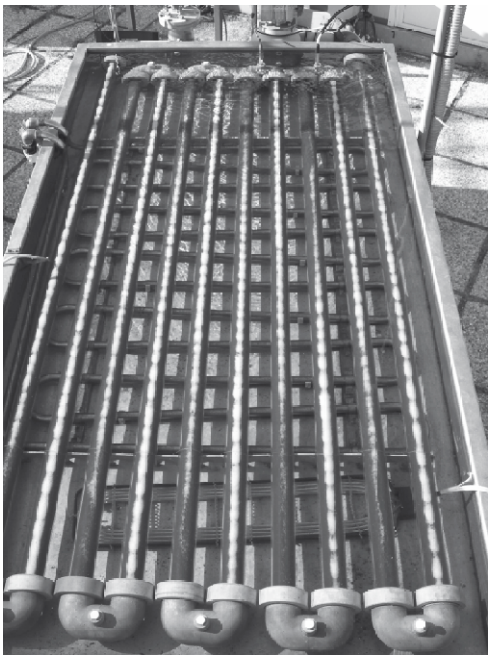

Figure 34. General view of the 50 L horizontal tubular photobioreactor used for outdoor hydrogen experiments with *Rhodopseudomonas palustris* 42OL.

The photobioreactor (Figure 34) consisted of 10 parallel Pyrex glass tubes (length 2 m, internal diameter 4.85 cm) connected by PVC U-bends with watertight flanges. For a more detailed description of the PBR see [30]. The illuminated area of the reactor, calculated on the basis of the tube semi-circumference, was 1.523 m^2, and the corresponding surface-to-volume ratio was 30.46 m^{-1}. The tubes were placed horizontally in a stainless-steel basin containing thermostated (28±0.5°C) demineralised water. The culture was recycled by means of a centrifuge PVC pump. The speed of the culture was adjusted to a value within 0.2 m s^{-1} yielding Reynolds number (*Re*) of 12.000. At the end of the circuit (total length 23 m), the culture flowed

into a 2-liter transparent PVC cylindrical degasser (internal diameter 10 cm, height, 28 cm). The degasser contained several hose-fittings for fresh medium additions, for culture sampling and biogas recovering. During the hydrogen production experiments, the head space of the PBR, i.e., the volume above the culture level, was about 0.2 L (0.4% of the total volume). The reactor was oriented North/South, and the latitude and longitude at the site where it was operated were 43° 50' North and 11° 11' East, respectively. The system was covered with a net which cut the 50% of the global radiation impinging on the reactor.

Before being used, the photobioreactor was cleaned by circulating 50 L of a 1% solution of sodium hypochlorite for 12 hours; the reactor was then washed twice with sterilized deionised water.

3.2.3 Analytical methods

The gas produced was measured by the use of a system similar to the one described in [31] composed of: a gas-to-liquid conversion bottle; a liquid accumulating bottle; a digital balance; a lap-top computer for continuous data recording. The digital balance, monitoring the changes in the weight of the liquid accumulating bottle (Acculab, ALC models, Sartorius Group, Goettingen, Germany), was connected to a lap-top computer. The pH was measured by a pHmeter and the oxygen concentration in the culture was polarographically determined with a Clark-type electrode. A software for automatic culture control and data acquisition (gas accumulation, pH and O_2 concentration) was developed by Chemitec (Florence, Italy).The dissolved H_2 was measured by the use of an amperometic micro-sensor (AMT Analysenmeßtechnik GmbH, Germany), dipped in the culture, measuring H_2 concentration in the range 0.0 to 1.5 mg L^{-1} of dissolved H_2.

The amount of hydrogen in the gas phase of the photobioreactor was determined by gas chromatography as described in [29] with Ar as a carrier gas.

The concentration of malate in the culture medium was measured with an HPLC chromatograph (1100 series, Agilent Technologies, USA) equipped with an Aminex 87H column (Bio-Rad, USA). An UV detector (λ = 210 nm) and a refractive index (RI) detector were used for the determination and quantification of the organic acids. The column was maintained at 65°C and the eluent was 0.01 N H_2SO_4, at a flow rate of 0.6 mL min^{-1}.

The cellular protein concentration was determined by Lowry method [32].

3.2.4 Fluorescence measurements

Bacteriochlorophyll fluorescence was recorded using a portable pulse-amplitude-modulation fluorometer (PAM-2100, H. Walz, Effeltrich, Germany) operated by an external PC using PamWin software. For F_v/F_m determination, 0.5-ml samples were taken from the outdoor photobioreactor (PBR), and dark-adapted for 15 minutes. Minimum Bchl a fluorescence, F_o, was measured by modulated light (< 0.5 μmol photons m^{-2} s^{-1}) coming from a light-emitting diode (peak wavelength at 655 nm, 600 Hz). A single high-intensity flash (6000 μmol photons m^{-2} s^{-1}, 0.8 s in duration) provided by a miniature 8V/20W halogen lamp (Bellphot, Osram) was

used to raise the fluorescence yield to the maximum, F_m. The effective quantum yield of the photosystem (PS) was measured as $(F'_m-F_s)/F'_m$, where F'_m and F_s are the maximum and the steady-state fluorescence under light exposure, respectively. The fluorescence nomenclature follows Van Kooten and Snel [33]. The fraction of absorbed light utilized in electron transport was given by the effective PS quantum yield, $\Delta F/F_m' = (F_m'-F_s)/F_m'$ [34]. The relative electron transfer rate (rETR) of the cultures during the day was calculated as (30):

$$rETR = [(F_m'-F_s)/F_m'] \times PFD \quad (30)$$

where PFD is the photon flux density (μmol photons m^{-2} s^{-1}).

We used the non-linear least-squares regression model by Eilers and Peeters [35] to fit the ETR/PFD curves performed on culture samples taken from the outdoor cultures, and to estimate the maximum electron transport rate (ETR_{max}), α (i.e. the initial slope of the curve), which is proportional to the maximum light utilization efficiency; the I_k (i.e. the saturation irradiance) was given as an intercept between α and ETR_{max}.

Calculation of substrate conversion and photosynthetic efficiency (PE)

The conversion yield of the carbon substrate (malate) to hydrogen was calculated as the percentage of the actual amount of hydrogen produced on the maximum amount of hydrogen theoretically obtainable from the complete conversion of the substrate to hydrogen, according to the following reaction (31):

$$C_4H_6O_5 + 3H_2O \rightarrow 6H_2 + 4CO_2 \quad (31)$$

The photosynthetic efficiency (PE), i.e. the efficiency of conversion of light energy to hydrogen energy, was calculated assuming that all the incident light was absorbed by the culture; the following equation (32), based on [5] was applied:

$$PE = \frac{Combustion\ enthalpy\ of\ H_2 \times H_2\ production\ rate}{Absorbed\ light\ energy \times 100} \quad (32)$$

Being solar light the only light source, the "Absorbed light energy" factor was calculated as in (33):

Absorbed light energy = global radiation x 0.5 x illuminated surface x 0.64 (33)

where 0.5 is due to the light screen applied on the reactor and 0.64 is the correction factor to determine the photosynthetic available radiation of *Rp. palustris* [23].

3.3 Results

3.3.1 Growth characteristics and culture parameters in outdoor culture of *Rp. palustris*

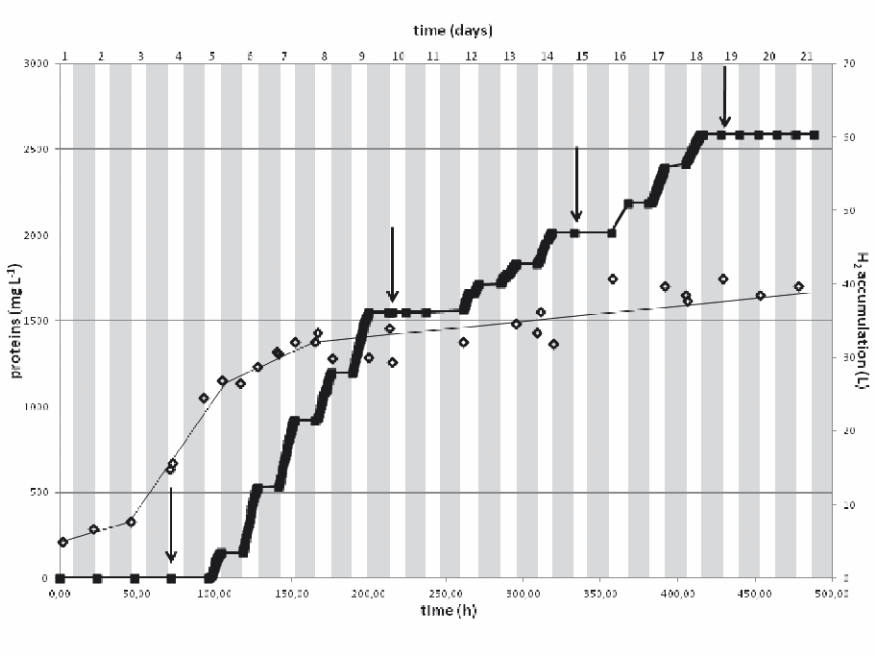

Figure 35. Time course of cumulative hydrogen production (■) and cell protein concentration (◊) of *Rp. palustris* 42OL cells grown in an outdoors 50 L photobioreactor. The black arrows indicate nutrient additions. The grey bands indicate nighttime periods.

The experiment was carried on for twenty one days under natural solar light as the only light source. A H_2 producing preculture of *Rp. palustris* cells was used for inoculating, at 20% v/v dilution, the 50 L photobioreactor containing RPP medium. Glutamate was added at the beginning of the experimental time in order to stimulate cell growth until the proper cell density was achieved. After the beginning of H_2 production, glutamate was not added anymore. After the inoculation, the culture showed a lag phase of 46 hours, followed by a linear phase of growth characterized by a specific growth rate of 0.021 h^{-1}. The growth mostly stopped after150 hours, and the culture entered the stationary phase, as shown by the plateau observed in the curve of protein concentration (Figure 35).

The pH of the culture was constantly monitored with an electrode in the medium and its value was found to be stable around 6.93± 0.14 for the whole duration of the experiment (data not shown); in order to minimize the risk of contamination, slight corrections of pH were done in concomitance with the addition of nutrients. The concentration of dissolved O_2 was also measured, as a control of the anaerobic condition inside the reactor. At the beginning of the experiment, the dissolved O_2 inside

the reactor was 6.3 ppm, but within 20 hours it went down to 0.0 ppm and constantly remained at this value for the whole period. Slight variations from 0.0 to 0.3 ppm were occasionally observed right after nutrient additions, but in the following few hours the concentration of dissolved O_2 went down again to 0.0 ppm (data not shown).

3.3.2 Outdoor hydrogen production with *Rp. palustris* in a 50 L photobioreactor

Eleven days, out of twenty one, were of H_2 production for a total of 60.31 L of H_2 collected, with a mean production rate (calculated on the total hours of production) of 10.69 ± 4.36 ml L^{-1} h^{-1}. At day 4, malate concentration decreased down to 1.46 g L^{-1}, thus a concentrated solution of malate was added to restore the initial 4 g l^{-1} concentration. As shown in Figure 35, gas accumulation started after 97.5 hours (i.e. at day 5) and lasted for five subsequent days, stopping every night. At day 10, malate concentration was found to be 0.65 g l^{-1}, so the organic acid was added to restore 4 g l^{-1} concentration (Figure 35, second black arrow). This addition was followed by a lag phase in H_2 production of two days and then, at day 12, H_2 production started again and continued for the three following days. At day 15, the concentration of malate was 0.55 g L^{-1}, but at this point 10% of the culture was replaced with fresh RPP medium, in order to supply the cells with both sufficient amount of malate and other nutrients. After one day of lag phase, H_2 production was reactivated, at day 16, and lasted for three other days. At day 19, H_2 production stopped again but, after further addition of malate (Figure 35, fourth black arrow), it was not restored in the following days.

The concentrated solutions of malate were slightly acidic (pH 5.5), with the purpose of lowering the pH of the whole culture, since no pH correction was included in the system.

The highest daily hydrogen production rate, obtained at day 6, was 18.38 ml L^{-1} h^{-1} (Figure 36); during the same day the maximum hour rate of 27.24 ml L^{-1} h^{-1} was registered two hours after the highest light intensity at noon and was sustained for one hour. 80% of the gas produced by the culture was composed by H_2, while the remaining 20% was estimated to be CO_2.

During the experimental period (June and July 2011), the weather conditions were generally sunny, with the exception of the days 10-13 and 15-17, when the presence of clouds resulted in a decrease of the global radiation, as indicated by the striped bars in Figure 36.

A light conversion efficiency of 0.63 ± 0.19% was calculated for the days of production taking into account the value of the global radiation recorded for each day; the maximum daily light conversion efficiency of 0.92 % was obtained at day 9 (Table 14).

The maximum molar conversion of malate to hydrogen obtained was 49.7 %, recorded at day 8, while a 10.3% conversion was calculated for the whole process; this value was calculated for the period of H_2 production (i.e. between day 5 and 18), thus also including the amount of malate consumed during the periods of no H_2 production.

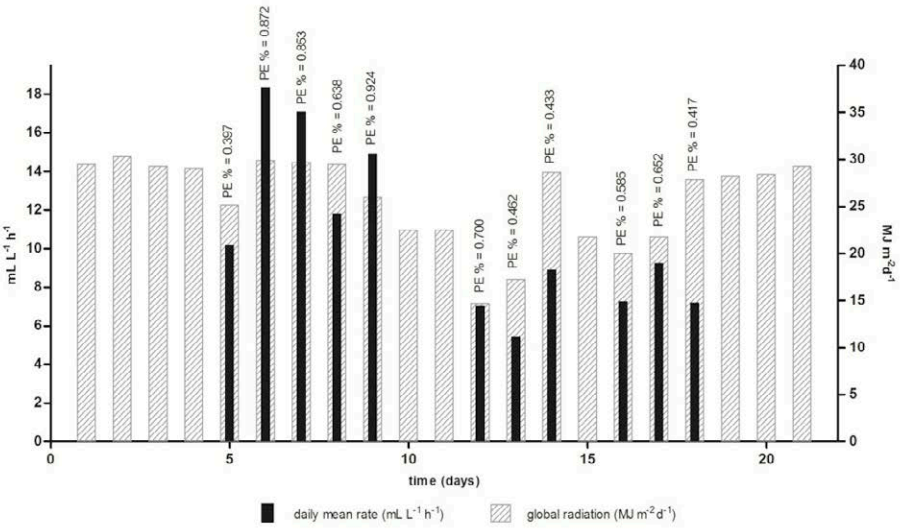

Figure 36. Daily global radiation (striped histograms) and hydrogen production daily mean rates (full dark histograms) for an outdoor culture of *Rp. palustris* 42OL; Day1 = 20 June 2010; PE (photosynthetic efficiency) is reported for each day of production.

Table 14. Mean and maximum values for H_2 productivity.

	Hour Rate[a]	Daily Rate[b]	Daily Rate per illuminated surface[c]	Substrate conversion	PE[d]
units	ml L^{-1} h^{-1}	ml L^{-1} d^{-1}	L m^{-2} d^{-1}	%	%
Mean	10.7 ± 4.4	107.8 ± 47.0	3.54 ± 1.53	10.3	0.63 ± 0.19
Max	27.2[e]	180	5.89	49.7	0.92

Notes: [a] the hour rate is calculated dividing the total amount of hydrogen produced per liter of culture by the total amount of hours of production; [b] the daily rate was calculated dividing the total daily amount of hydrogen produced per liter of culture; [c] the daily rate per illuminated surface was calculated dividing the total daily amount of hydrogen produced per square meter of illuminated surface; [d] PE = photosynthetic efficiency; [e] this rate was maintained for one hour.

The gas began to be accumulated in the gas-collecting system 4.31 ± 0.86 h after the sunrise, a mean value calculated for the eleven days of production. However, using the amperometric micro-sensor, it was possible to precisely determine when the re-activation of H_2 production occurred after sunrise. Figure 37 shows a detail of the data registered with the micro-sensor for measuring the dissolved H_2 concentration inserted in the culture: taking as an example day 7 (in the inset in Figure 37, dissolved H_2 was detected in the culture 2.40 h before the gas reached the collecting

system. Once the culture medium was H_2-saturated, gas started to be accumulated in the collecting system: in correspondence to the moment the gas was released from the culture, a small drop in voltage was observed, followed by a new increase due to attainment of the saturating concentration. Using the data, the mean daily lag time (calculated on the eleven days of production) was estimated to be of 1.26 ± 0.17 h after sunrise, taking as a reference the moment the electrode started to register an increase in the voltage due to the presence of H_2 in the culture medium.
The microsensor for measuring the dissolved H_2 concentration also revealed a decrease in the voltage during the night. As shown in Figure 37, the voltage decreased from a value of about 270-280 mV, corresponding to the saturating concentration of H_2, to a value of about 10 mV, the base-line of the electrode. This behavior was repeated for each day of production.

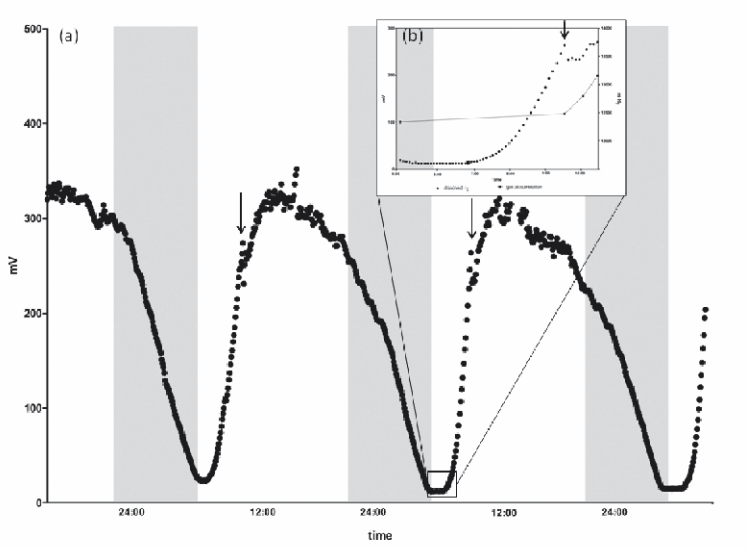

Figure 37. Time course of voltage variations responding to the concentration of dissolved H_2 (●) in an outdoor culture of *Rp. palustris* 42OL; (a) during an operation period of 3 days (6-8); (b) detail for an operation period of 5 hours (during day 7). The grey bands indicate nighttime periods. The black arrows indicate the moment the gas started to be accumulated in the collecting system.

3.3.4 Photosynthesis performances of the strain during outdoor growth and hydrogen production

In order to obtain preliminary information on the photosynthetic performance of *Rp. palustris* to different light intensities, diluted suspensions [5 mg (Bchl *a*) L^{-1}] were exposed to increasing light irradiance and the relative electron transfer rates were recorded (Figure 38). The saturation irradiance was found to be close to 300 µmol photons m^{-2} s^{-1}; the light-saturated rate (I_s) was achieved at about 800 µmol photons m^{-2} s^{-1}; and the initial slope (α), which is proportional to the maximum photosynthesis efficiency, was 0.48 (relative value). Further information on the perfor-

mance of the culture was extracted from rETR vs PFD curves measured at time intervals during the hydrogen production. As it can be seen, all the photosynthesis parameters (I_k, α, $rETR_{max}$) were scarcely affected during the production process. The quantum yield of PS monitored as changes in F_v/F_m ratio was lower at the beginning of the experiments, most likely as a result of the acclimation of the culture to the outdoor high light conditions (Figure 39).

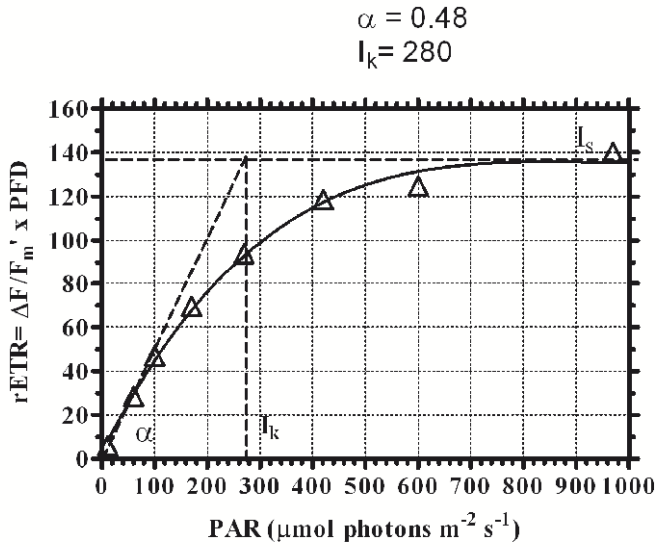

Figure 38. Relative electron transfer rate response curve (rETR) recorded in the *Rp. palustris* 42OL strain. The rETR were recorded with stepwise increase in irradiance (red-light), supplied by the PAM-2100 LEDs.

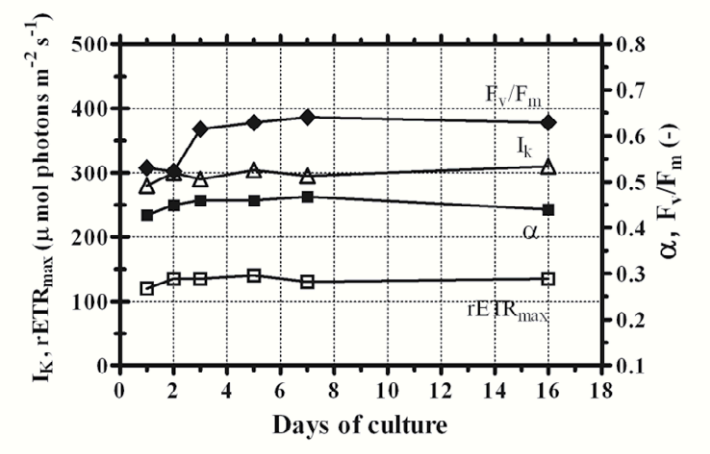

Figure 39. Time course of the main fluorescence (F_v/F_m, and rETR) and photosynthesis (α, Ik) parameters measured during the hydrogen production outdoors in a 50 L tubular photobioreactor.

3.4 Discussion

3.4.1 Outdoor hydrogen production with *Rp. palustris* in a 50 L photobioreactor

Over 60 L of hydrogen were produced by a 50 L outdoor culture of a wild type strain of *Rp. palustris*, with no need of using artificial illumination in any phase; to the best of our knowledge, it is the first time this organism was used for hydrogen production in an outdoor system of this dimension.

The whole volumetric ratio of 1.2 $L_{H2}\,L_c^{-1}$ (where L_c indicates the liters of culture) is larger than the values so far obtained with other solar photobioreactors of comparable dimensions: Gebicki et al. [26] obtained a production of 0.68 $L_{H2}\,L_c^{-1}$ for a 65 L tubular photobioreactor, while for a 25x4 L flat plate photobioreactor the ratio was 0.96 $L_{H2}\,L_c^{-1}$; recently Boran et al. [26] reported a production of almost 80 L for an 80 L tubular photobioreactor, giving a ratio of about 1 $L_{H2}\,L_c^{-1}$.

The culture acclimated to outdoor conditions during the first 50 hours, than linear growth phase started at a rate of 0.021 h^{-1}, rather comparable with the growth rate of 0.025 h^{-1} obtained with outdoor *Rhodobacter capsulatus* cultures [26]. However, *Rp. palustris* 42OLshowed a faster acclimation to the outdoor conditions than *Rb. capsulatus*, taking into account the lag phase in their growth curves. Indeed, *Rb. capsulatus* showed a lag phase of 70-90 h under artificial light, a condition used for inducing cell growth [26], while *Rp. palustris* showed a lag phase of only 46 h, with no need of using artificial illumination for starting the growth as it was done by [26]. This can be considered as a further evidence of the already suggested wide versatility of *Rp. palustris* [2, 3, 22], that allows it to easily acclimate to different environmental conditions and, particularly in this case, to different light conditions.

H_2 production started after a lag period of about 100 hours, in correspondence to the late linear growth phase obtained after the acclimation of the culture to the outdoor conditions and after a first replenishment of the carbon source. The daily rate of hydrogen per illuminated surface (3.54 ± 1.53 L m^{-2} d^{-1}) obtained is the highest so far, for comparable outdoor processes carried out in tubular photobioreactors. This result strongly supports the suggested relevancy of using reactors characterized by an elevated illuminated surface to volume ratio [36] (30.46 m^{-1} for the present study, 25.00 m^{-1} for Boran et al. [25, 26] and 13.85 m^{-1} for the tubular reactor in Gebicki et al. [27, 28]).

Moreover, the maximum hour rate attained (27.2 ml L^{-1} h^{-1}) is noticeably higher than the maximum rates obtained outdoors using reactors of comparable dimensions. It is also worth stressing that this value is even higher that the rates showed by *Rp. palustris* 42OL under laboratory conditions (i.e. 18-20 ml L^{-1} h^{-1}, data not shown), thus demonstrating a remarkable capability of the strain to acclimate to the outdoors conditions.

The initial mean rate (day 5- 9) of 14.5 ml L^{-1} h^{-1}, calculated on the hours of production, was very similar to the rates recorded for the same strain in the same medium under continuous artificial illumination in indoor conditions (13.8 ± 2.4 ml L^{-1} h^{-1}, data not shown). The rates of the two following production periods, i.e. day 12-14 and day 16-18, were significantly lower (respectively 7.1 and 7.9 ml L^{-1} h^{-1}); this reduction was most likely due to the lower light intensity caused by weather in-

stability, as the rates were described to follow the variations of light intensity in outdoor cultures [28]. In addition to the unstable weather conditions, the culture age and the lack in fresh medium components may have played a significant role as well: indeed, it has been described that hydrogen production rates during active cell growth are higher than those production rates occurring in stationary phase [37]. In particular, the reduction in the H_2 production rates might be due to the high metabolic cost of the synthesis of nitrogenase (almost 200 genes are involved in nitrogenase assembly, regulation and functioning [38]), so the lack of macro and microelements might have negatively affected the renewing of this crucial enzyme. Indeed, the substitution of a 10% of culture with fresh medium at day 15 was able to induce the restart of H_2 production. A fed-batch mode with a daily medium replenishment most probably would be a good solution for preventing the stop in H_2 production and for avoiding lag phases during the production period, according to previous findings in indoor cultures carried out with immobilized *Rp. palustris* cells [39].

The total substrate conversion was lower than for other comparable processes, due to the consumption of substrate also during the non H_2 producing periods. During these periods, the culture most probably utilized the energy coming from the sunlight and the carbon deriving from substrates for synthesizing products other than proteins or H_2. Indeed, it has been previously reported that purple bacteria, and particularly *Rp. palustris*, synthesize glycogen or poly-β-hydroxybutyrate during H_2 production processes as alternative ways for dissipating the excess of reducing equivalents [40, 41]. Moreover, genes encoding enzymes that synthesize and degrade those carbon storage polymers have been identified in *Rp. palustris* [2].

The data registered by the probe for dissolved H_2 open a question never reported to this detail: the H_2 dissolved in the culture medium decreased during the night, and thus the corresponding amount of H_2 needs to be produced at the beginning of the following day in order to saturate again the solution before it can be recovered and accumulated in the gas collecting system in the morning. System gas leakage can be excluded as a possible cause of H_2 night decrease since both the reactor and the collecting system were tightly closed every night and also because any other change, for example in O_2 concentration, was detected in the meanwhile. Whether the removal of dissolved H_2 is a *Rp. palustris* metabolic process was not investigated in this study, but it is worth of further investigations; indeed, as far as it is known, H_2 is re-assimilated by uptake-hydrogenase only in: (i) nitrogen fixing conditions (light), (ii) non nitrogen fixing conditions (either in light or darkness) in presence of small amounts of oxygen, (iii) in autotrophic conditions [42, 43]. However, it has also to be taken in consideration that, given the dimensions of the photobioreactor, it is not possible to exclude the presence of contaminants that could have had a role in H_2 consumption, even if we could not detect any evidence of their presence.

In any case, the process-related issue that came to light from this result is that, considering as an approximation the solubility of H_2 in pure water [44], every night 858 ml of H_2 were lost, and thus every morning the culture had to re-saturate the solution (process that, as described earlier, took a mean of 3.05 h). Considering the mean hour rate of the process, the re-saturation roughly corresponds to a daily loss of 1632 ml of H_2 which might have been accumulated in the gas collecting system

without this process, in addition to the once-for-all 858 ml of H_2 that has to be dissolved in the medium at the beginning of the H_2 production process.

This strongly suggests the need of integrating solar light with artificial illumination systems during dark periods, as already reported by [45, 11, 12], to achieve a continuous H_2 production process but still exploiting free solar radiation as long as possible.

3.4.2 Photosynthesis performance of the *Rp. palustris* cultures monitored via bacteriochlorophyll a fluorescence

In this study it was for the first time demonstrated the applicability of variable Bchl *a* fluorescence as a tool for readily monitoring the physiological status of outdoor mass cultures of *Rp. palustris* during the process of hydrogen production. The recorded variable fluorescence signal reflects the redox state of the reaction center, where low fluorescence yield indicated an open reaction center (no charge, reaction center is ready to perform photochemistry) while high fluorescence yield indicated a closed reaction center (charged reaction center, transiently non-functional photochemistry) [17]. The difference between the minimum fluorescence F_o and the maximum F_m makes possible to get an estimate of the quantum yield of primary charge separation (estimated from the F_v/F_m ratio). In this study the F_v/F_m ratios in *Rp. palustris* were found in the range of 0.55-0.65, which roughly agree with values (~ 0.65- 0.7) found by Law et al. [46] and more recently by Koblizek et al. [17] for lab cultures of PNS bacteria.

It was interesting to note that cultures of *Rp. palustris* exposed to increasing light displayed a light curve pattern similar to that usually observed in microalgae and higher plants. These findings thus point out the possibility of using bacteriochlorophyll fluorescence as a tool for strain selection and for monitoring the culture performance for both growth and hydrogen production outdoors in a similar way as it is already done for mass culture of microalgae and cyanobacteria [20, 30].

In the above described experiments, the F_v/F_m ratio of *Rp. palustris* cultures did not significantly change over the hydrogen production period, contrary to what it was observed with the microalga *Chlamydomonas reinhardtii* during hydrogen production process [47, 30]. However, it must be stressed that hydrogen production with *C. reinhardtii* is carried out under sulfur-deprivation in order to down-regulate the PSII oxygen release, which is toxic to hydrogenase, at the same level of respiration. This fact strongly reduces the performance of this microalga compared to *Rp. palustris*. However, in spite of the much higher hydrogen output achieved with *Rp. palustris* the photosynthetic efficiency was rather low, being lower than 1% (see table 14), which is still one order of magnitude lower than the theoretical one [48]. One of the factors that may have affected the hydrogen performance is the design of the photobioreactor. Indeed, Scoma et al. [30] recently showed that the hydrogen output of *C. reinhardtii* cultures grown in a PBR similar to the one used in this study, was affected by the pattern of illumination and the mixing time of the PBR. Cultures of *C. reinhardtii* illuminated by both sides of the PBR proved to be more hydrogen productive than when illuminated from one side. Moreover, a long mixing time of the culture, such as the one measured in the 50 L tubular reactor (1 hour)

used in this study, can represent a serious obstacle for the stripping of hydrogen from the cultures [30] and thus the improvement of the design of the PBR and of the mixing of the culture is one of the key issues for improving their performances.

3.5 Conclusions

From the above reported results it is possible to draw four main conclusions:

- *Rp. palustris* 42OL possesses a very good potential for being used in outdoor H_2 production. Indeed, the maximum H_2 production rates showed by the strain and the mean rate per illuminated surface were the highest so far reported for outdoor experiments carried out with PNS bacteria in tubular photobioreactors of comparable dimensions.
- During the dark periods, the dissolved hydrogen was lost, and a number of hours in the morning were needed to re-saturate the solution, thus negatively affecting negatively the total amount of gas collectable. Therefore, a light supply to the culture during the night is highly recommendable to achieve a continuous H_2 production process without losses.
- The direct measurement of the fluorescence of Bchl *a*, a technique used for the first time with PNS bacteria growing in outdoor large cultures, showed to be an effective tool for monitoring the performance of the bacterial photosystem and, consequently, for checking the physiological status of the cultures.
- The measurements of the fluorescence of Bchl *a* showed that the photosystem was active without any reduction in its activity, all along the period of hydrogen production, suggesting that the decrease in the rates of hydrogen production observed during the stationary phase was induced by other factors. Indeed, the reduction in the H_2 production rates might have been caused by factors such as a reduced light availability, due to the weather conditions, and/or a reduced metabolic activity of the cells due to the lack of an efficient turn-over of some key metabolic process of the cells.

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General Conclusions

This thesis had the aim of advancing the knowledge on photofermentative hydrogen production towards an up-scaling of the process, in particular with regard to the use of substrates deriving from other fermentations and the use of solar irradiation.

These two aspects are of major interest as they are crucial for an up-scale of a process that at the present moment is carried out mostly at a lab scale.

The preliminary activity of this thesis confirmed the validity of searching, in natural environments having characteristics suitable for harbouring PNS bacteria, new strains to be exploited for the biological production of hydrogen. In particular, the good performances obtained with *Rp. palustris* AV33, even under not optimized growth conditions, suggest carrying out further studies aimed at improving the H_2-production performances of the strain. Moreover, the capability of *Rp. palustris* AV33 to produce H_2 in the presence of lactate is rather promising for the utilization of this strain in integrated H_2- producing systems combining dark- and photo-fermentation.

The use of substrates deriving from other fermentations allows the recovering of energy from waste material, or from substrates not completely oxidized in other production processes. With this work it was demonstrated that *Rp. palustris* is able to use a wide variety of substrates to produce hydrogen for long time periods.

From the activity carried out about photo-fermentation of vegetable residues and of effluents derived from previous dark fermentations it emerged the possibility to recover energy from organic wastes by coupling the production of H_2 with waste disposal or to integrate microbial processes to get a higher total hydrogen yield than with the single independent processes.

The addition of nutrients, like Fe (III), resulted to be in some cases necessary for hydrogen production, in other cases it improved the rates and/or the total amount of gas produced.

However, the utilization of this kind of substrates needs optimization, in particular for minimizing the shift of the microbial metabolism from H_2 production to cell growth. Possible solutions stand in the removal of fixed N sources from the medium or in the use of strains insensitive to fixed nitrogen.

The feasibility of this last solution was investigated by the use of a *nif*A mutant strain, insensitive to ammonium. From the comparison with the wild type strain it was possible to draw the following conclusions:

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- The *nif*A* mutation actually made the *Rp. palustris* strain insensitive to ammonium in a concentration range between 2.5 and 10 mM.
- Both the wild type and the mutant strain were able to produce H_2 on the undiluted VWD medium, but the mutation allowed a faster and higher H_2 production.
- The mutant strain started earlier and produced more H_2 than the wild type.
- The wild type strain was able to produce H_2 only after the ammonium was consumed for cellular growth.
- Diluting the VWD medium sped up the process, shortened the H_2 production lag time and increased the substrate-to-hydrogen conversion.

The major conclusion of this study was that the mutant strain of *Rp. palustris* was able to produce H_2 independently from NH_4^+ concentration, both in a synthetic medium and in an actual waste derived medium, and thus it can be considered suitable for using it in the production of hydrogen using substrates deriving from previous fermentation of vegetable wastes.

The use of solar irradiation would appear as the best solution to irradiate the cultures in a cost effective system. However, the investigation on this field is spreading only in the last few years.

The results obtained indoors with artificial irradiation demonstrated the ability of the *Rp. palustris* strain 42OL to operate in light/dark cycles conditions, ensuring hydrogen production at every new light period, as long as the substrate was available. However, a light/dark cycle of 8h light/16h dark showed not to be suitable for an efficient hydrogen producing process.

A lag time in hydrogen production was observed, but the method chosen for the determination of the starting point was not enough sensitive. An electrode for measuring dissolved H_2 in the culture was used for the subsequent outdoor experimentation.

A method for the direct measurement of the fluorescence of bacteriochlorophyll *a* (Bchl *a)* was developed in the present thesis (PAM fluorometry) in order to use this technique on whole cell of PNS bacteria; the method, with the measuring conditions set up in this thesis, resulted to be applicable to cultures of PNS bacteria both in growing and in hydrogen producing conditions. It was therefore suitable for outdoor massive cultures as well.

Given the results attained in the indoor experimentations it was possible to conduct an outdoor experimentation. It emerged that:

- *Rp. palustris* 42OL possesses a very good potential for being used in outdoor H_2 production. Indeed, the maximum H_2 production rates showed by the strain and the mean rate per illuminated surface were the highest so far reported for outdoor experiments carried out with PNS bacteria in tubular photobioreactors of comparable dimensions.
- During the dark periods, the dissolved hydrogen was lost, and a number of hours in the morning were needed to re-saturate the solution, thus negatively affecting the total amount of gas collectable. Therefore, a light supply to the culture during the night is highly recommendable to achieve a continuous H_2 production process without losses.

- The direct measurement of the fluorescence of Bchl *a*, a technique used for the first time with PNS bacteria growing in outdoor large cultures, showed to be an effective tool for monitoring the performance of the bacterial photosystem and, consequently, for checking the physiological status of the cultures.
- The measurements of the fluorescence of Bchl *a* showed that the photosystem was active without any reduction in its activity, all along the period of hydrogen production, suggesting that the decrease in the rates of hydrogen production observed during the stationary phase was induced by other factors.

As a general conclusion, it is possible to say that the process for hydrogen production is ready for a gradual up-scaling from lab conditions; a first step was made in the present thesis towards a semi pilot scale H_2 production process with cultures irradiated by natural sunlight. A further step would be the use of low cost substrates (such as substrates deriving from vegetable wastes fermentation) in larger scale processes, possibly using PNS bacterial strains (such as ammonium insensitive strains) that allow to obtain larger H_2 amounts.

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