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Hydrogen Production by Immobilized *Rhodopseudomonas* sp. Cells in Calcium Alginate Beads

Eleftherios Touloupakis [1,*], Angeliki Chatziathanasiou [2], Demetrios F. Ghanotakis [2], Pietro Carlozzi [1] and Isabella Pecorini [3]

[1] Research Institute on Terrestrial Ecosystems, National Research Council, Via Madonna del Piano 10, 50019 Sesto Fiorentino, Italy
[2] Department of Chemistry, University of Crete, Voutes Campus, 70013 Heraklion, Greece
[3] DESTEC—Department of Energy, Systems, Territory and Construction Engineering, University of Pisa, 56122 Pisa, Italy
* Correspondence: eleftherios.touloupakis@cnr.it

Abstract: The present investigation concerns the potentiality of *Rhodopseudomonas* sp. cells to produce clean energy such as molecular hydrogen (H_2). The abovementioned goal could be reached by improving the capability of purple non-sulfur bacteria to produce H_2 via a photofermentative process through the enzyme nitrogenase. *Rhodopseudomonas* sp. cells were immobilized in calcium alginate gel beads and cultured in a cylindrical photobioreactor at a working volume of 0.22 L. The semi-continuous process, which lasted for 11 days, was interspersed with the washing of the beads with the aim of increasing the H_2 production rate. The maximum H_2 production rate reached 5.25 ± 0.93 mL/h with a total output of 505 mL. The productivity was 40.9 μL (of H_2)/mg (of cells)/h or 10.2 mL (of H_2)/L (of culture)/h with a light conversion efficiency of 1.20%.

Keywords: *Rhodopseudomonas* sp.; photobioreactor; calcium alginate; photofermentation; hydrogen production

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1. Introduction

Most of the fuels, chemicals, and raw materials that we use every day come from refineries that use petroleum as fuel, thus, leading to higher levels of pollution and greenhouse gas emissions. The transition from a wasteful fossil fuel-based economy to a more sustainable circular economy is currently a global issue. Global and in particular European policies are also promoting renewable hydrogen (H_2) production for decarbonization purposes [1,2].

Molecular H_2 is a potential alternative energy source that is receiving a lot of attention [3,4]. H_2 production by biological means can be achieved by: (i) the biophotolysis of water using oxygenic photosynthetic microorganisms (cyanobacteria and microalgae), (ii) the photodegradation of low molecular weight organic compounds using anoxygenic photosynthetic bacteria, or (iii) the fermentation of organic substrates using anaerobic chemoheterotrophic bacteria. There are also integrated systems in which a first fermentation phase is carried out by anaerobic chemoheterotrophic bacteria and a second phase is carried out by anoxygenic photosynthetic bacteria using the acids that had been produced in the previous phase [5–7]. Scientists have recently developed techniques that greatly enhance the ability of photosynthetic microorganisms to produce H_2 as a result of the energy-related H_2 demand [8,9].

Photosynthetic microorganisms such as microalgae, cyanobacteria, and purple bacteria are interesting candidates for the use as feedstock for H_2 production [8–10]. They can be used to produce a wide range of high value-added products, biomass, and bioenergy [11–14]. Purple non-sulfur bacteria (PNSB) are being explored as a potential renewable

energy source such as H_2 [15]. PNSB can grow as photoautotrophs, photoheterotrophs, or chemoheterotrophs depending on the availability of light, carbon, and oxygen (O_2).

PNSB use the photofermentation process to produce H_2 through the anaerobic conversion of organic molecules to H_2, and the enzyme nitrogenase is responsible for this process [16]. The photofermentative process often involves several phases. First, the tricarboxylic acid cycle uses substrates, such as carbohydrates or organic acids, in an oxidation reaction that produces electrons. Carbon dioxide is also released in the process. The subsequent oxidation and reduction of the electron carriers transfers electrons from the tricarboxylic acid to nitrogenase. Nitrogenase uses electrons and protons that are produced by the oxidation of organic substances, as well as solar energy, to produce H_2 in the presence of nitrogen deficiency. The ability to use either organic acids, sugars, or waste materials, as well as the lack of an O_2-evolving activity, are just some of the advantages of photofermentation [17]. Under certain limitations, single organic acids or mixtures of organic acids can serve as carbon sources for H_2 synthesis [17]. The H_2 production rate should increase when the growth conditions such as light, temperature, pH, and composition of the culture media are optimized. Numerous reports have been published on photofermentative H_2 production both in the laboratory and in the field [15,18–20].

PNSB have been explored for H_2 synthesis using a variety of carbon sources, including wastewater-derived volatile fatty acids [21]. Under nitrogen-limited conditions, single or mixed organic acids (such as acetate, malate, and succinate) can also be used as carbon sources for H_2 synthesis [15]. The rate of H_2 production increases with the improvement of the growth conditions, including the physical (light, temperature, and pH) and chemical conditions (composition of the growth medium).

Cell immobilization is a technique for immobilizing cells on a matrix. The main goal of this strategy is to increase the H_2 production by immobilizing the bacteria. In several studies, the bacteria were found in large amounts and the immobilized cells were able to produce large amounts of H_2 [22]. Compared to suspension cell culture systems, immobilization offers several advantages that reduce the overall cost of the system. These advantages include the ability to use a high cell biomass, add nutrients without harvesting the cells, a reduced risk of cell contamination, and an improved cell stability [22]. The other advantages of immobilization are that the cells are not washed out, the cell is stable, and it is protected from mechanical stress [23,24]. In addition, immobilization provides a much more stable microenvironment for cells when it is compared to a suspension culture. In biohydrogen research, immobilization technology is specifically used to increase H_2 synthesis, yield, and rate [25].

Photosynthetic microorganisms can be immobilized by a variety of techniques, however, gel entrapment seems to be one of the most commonly used techniques. Gel entrapment can be performed by using synthetic polymers, proteins, and natural polysaccharides [26]. Among the natural polysaccharides, alginate is most commonly used one for photosynthetic cell encapsulation since this hydrogel is transparent, permeable, and nontoxic [24,27,28].

The reuse of immobilized cells over a long period of time is a major advantage over suspension cultures. Immobilized systems are more desirable because they have comparatively better H_2 yields and rates. In addition, the immobilization technique is easier to scale up and requires less space for photobioreactor (PBR) construction. To date, there is very little information on H_2 production using immobilized PNSB in PBRs. The objective of this work is to investigate H_2 production in a PBR using immobilized *Rhodopseudomonas* sp. cells.

2. Materials and Methods

2.1. Algal Strain and Growth Conditions

Rhodopseudomonas sp. was cultured in a sterilized van Niel's medium according to previous reports [29]. The medium contained glutamate (1.0 g/L), acetate (4.0 g/L), and 100 mg/L of KH_2PO_4 (instead of 1.0 g/L). The cultures were illuminated using a halogen

lamp (150 W OSRAM power-star HQI-TS; 80 W/m^2). This lamp type shows four peaks the main peak is around 590 nm. The temperature of the culture was maintained at 30.0 ± 0.1 °C using a Plexiglas water bath with a heat exchanger. The sterilization was performed by using a Vapor Matic 770 autoclave (Vacuum Service srl, Civezzano, Italy).

2.2. Immobilization

The cell culture was centrifuged in a Sorvall Super T21 centrifuge at 5000× *g* for 10 min, and the pellet was rinsed twice with sterile saline solution. Equal parts of 25 mL of the cells (1.6 g/L of biomass cell dry weight (DW)) in sterile saline solution and a 6% *w*/*v* of sodium alginate (in saline solution) were mixed. The mixture was added dropwise to a sterile $CaCl_2$ solution (2% *w*/*v* in saline solution), and then, it was allowed to set for 30 min (Figure 1). After curing them in the $CaCl_2$ solution, the beads were washed, harvested, and placed in a cylindrical glass PBR. Calcium alginate beads were mounted on slides and observed in bright field using a Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan).

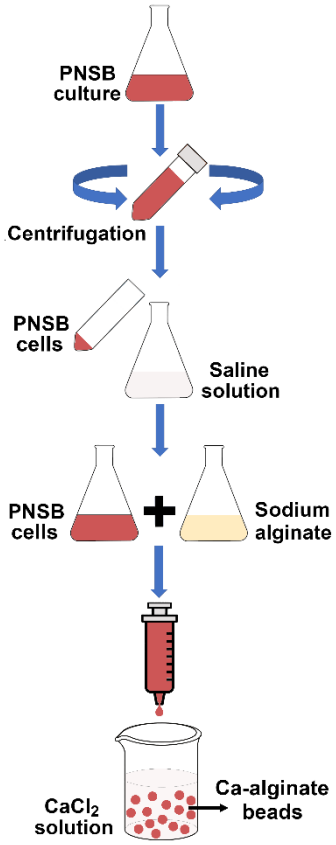

Figure 1. Flow chart showing the method used to immobilize the PNSB cells.

2.3. Hydrogen Production

The H_2 production by the immobilized *Rhodopseudomonas* cells in the PBR, which was filled with the sterile van Niel's medium, was performed in anaerobic mode. The PBR (internal diameter of 4.0 cm) with a working volume of 220 mL was operated in batch mode, illuminated using a halogen lamp (80 W/m^2), and maintained at 30.0 ± 0.1 °C. The calcium alginate beads were stirred using a magnetic stirrer. The pH was maintained at 7.2 by adding a sterile HCl solution. The pH values and oxidation-reduction potential (ORP) were monitored using two electrodes connected to a control unit (Chemitec srl, Florence, Italy). A silicone plug on the PBR was pierced with a needle to introduce the sterile HCl solution. Before the experiment, anaerobiosis was achieved by dark incubation for 16 h. The H_2 produced by the immobilized cells was collected on a calibrated column submerged in a CO_2-absorber solution (1.0 M NaOH, 3.4 M NaCl). The samples were collected from

the calibrated column and analyzed using a gas chromatograph (Clarus 500, Perkin Elmer, Waltham, MA, USA) with a packed column (Carbosieve SII Spherical Carbon, Supelco Inc., Bellefonte, PA, USA). The gas chromatography operated with an isothermal program at 35 °C for 2.25 min, a nitrogen carrier gas flow of 30 mL/min, and a 150 °C injection and detector temperature. The reported peak areas were converted to H_2 moles using a standard H_2 curve. The beads were washed twice during the experiment with sterile saline solution.

2.4. Analytical Procedures

Before the immobilization procedure, the biomass DW was determined according to the method of Touloupakis et al. [30]. After the immobilization procedure, the biomass DW was also checked after the beads were dissolved and the cells were released. For the dissolution procedure, the beads were incubated in 50 mM sodium phosphate (pH = 6.0) for 24 h. The bacteriochlorophyll (Bchl) concentration was determined according to Carlozzi et al. [31]. The light conversion efficiency (LCE) was calculated as the following ratio (energy output)/(energy input) × 100 [32], where energy output = (energy of the H_2 produced + energy of the biomass produced). The energy input is the energy of the organic molecules that were consumed and the irradiance that was impinged on the reactor surface.

3. Results

The *Rhodopseudomonas* cells were first cultured in a modified van Niel's medium until they reached a DW of 1.6 g/L. The cells were washed, mixed with sodium alginate, and dropped into the calcium chloride solution. Once the alginate droplets reached the calcium chloride solution, they immediately formed gel spheres that entrapped the *Rhodopseudomonas* cells. The formed calcium alginate spheres containing *Rhodopseudomonas* cells which were transparent and reddish in color (Figure 2). They had a diameter of 4.15 ± 0.18 mm, a volume of 37.6 ± 1.6 μL, and contained 30.7 ± 1.3 μg of cells (DW/bead).

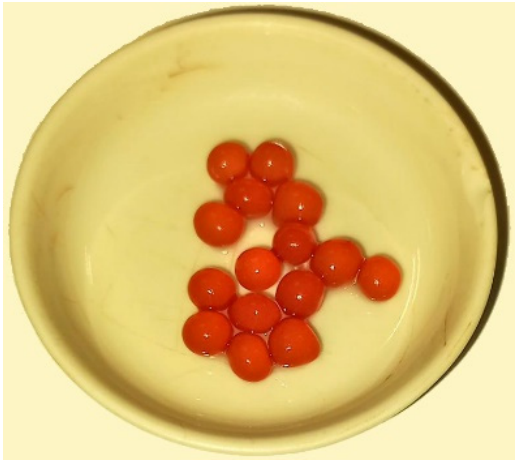

Figure 2. Calcium alginate beads with entrapped *Rhodopseudomonas* sp. cells.

One thousand six hundred and thirty-seven beads, corresponding to 61.5 mL of total volume and 50.2 mg of the immobilized cells, were transferred to the cylindrical PBR at a 0.22 L working volume (Figure 3). The PBR was then filled with van Niel's medium for H_2 production, sealed, and left in the dark for 16 h to support the anaerobiosis process. Subsequently, the PBR was transferred under light conditions to start the H_2 photofermentation process. The ORP showed a typical pattern, with a sharp decrease from the positive to the negative values, which was followed by a stabilization between −430 mV and −500 mV, which is attributed to the anaerobic conditions that were obtained (Figure 4).

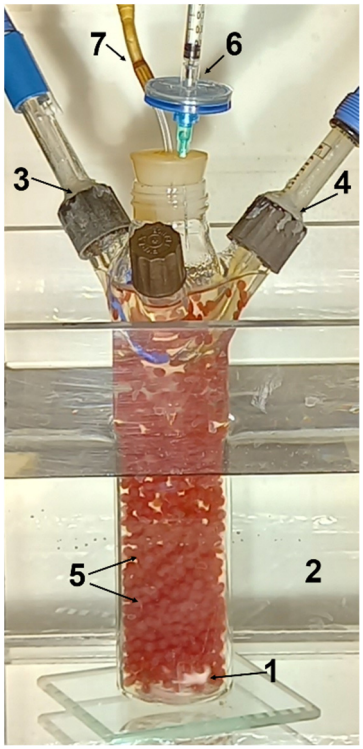

Figure 3. Picture of the cylindrical photobioreactor (0.22 L working volume). (1) Magnet; (2) water bath; (3) pH sensor; (4) redox sensor; (5) calcium alginate beads; (6) HCl sterile solution inlet; (7) H_2 exit.

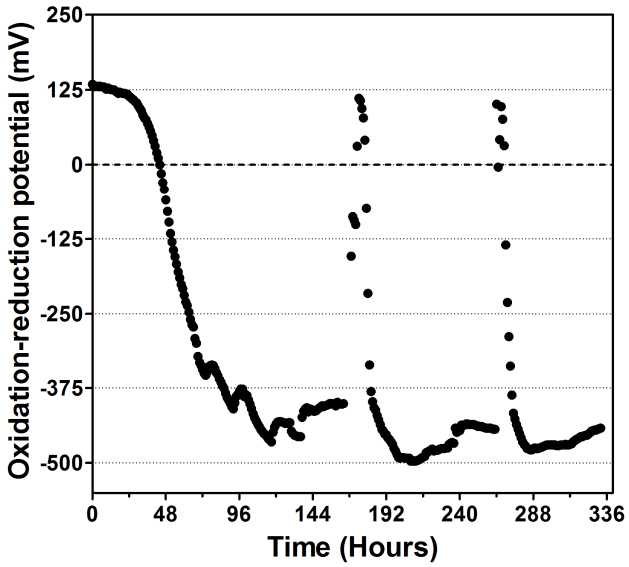

Figure 4. Time course of oxidation–redox potential of the immobilized *Rhodopseudomonas* sp. cells in calcium alginate beads, which were immersed in the cylindrical photobioreactor. The redox potential increased at 165 and 264 h which correspond to the presence of O_2 that entered in the system during the beads washing procedure.

Successively, the redox potential increased rapidly again, and then, it decreased in concomitance with the two washes that were carried out (Figure 4). The H_2 production started immediately after the redox potential stabilized in the negative range. The cumulative H_2 over the entire period of the bacterial cultivation was achieved in three steps: at the beginning of the *Rhodopseudomonas* sp. cultivation (86.2 mL of H_2), after the first

wash (163.5 mL of H_2), and after the second wash (255.5 mL of H_2) (Figure 5). The final cumulative output was 505 mL of H_2 which corresponds to 2295 mL (H_2)/L. After calculating the H_2 production rate for each period (considering washing of the beads), we obtained the following three values: 0.80, 1.97, and 4.40 mL of H_2/h, respectively. The average production rate was 2.05 mL of H_2/h with a maximum H_2 production rate of 5.25 ± 0.93 mL/h.

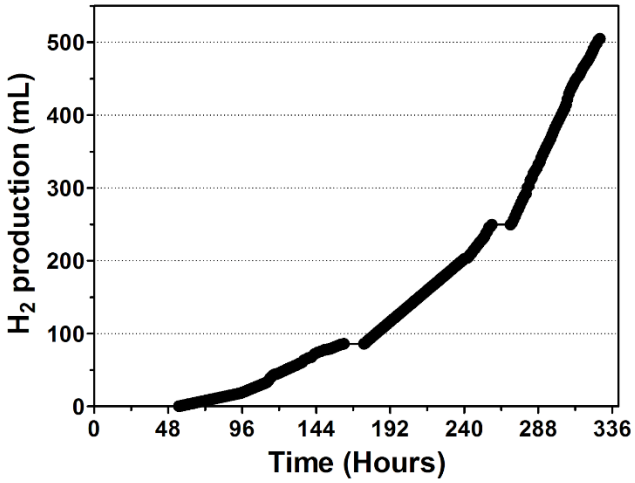

Figure 5. H_2 production by immobilized *Rhodopseudomonas* sp. cells in calcium alginate beads immersed in the cylindrical photobioreactor. Immobilized cells were washed and retested for their capacity to produce H_2 at 165 and 264 h.

The average productivity was 40.9 µL (of H_2)/mg (of cells)/h or 10.2 mL (of H_2)/L (of culture)/h with a maximum value of 22.2 mL (of H_2)/L (of culture)/h which was obtained after the second wash. At the end of the H_2 production, the immobilized *Rhodopseudomonas* cells were viable and showed minimal leakage.

For the calculation of the LCE of the process, only the production of H_2 was considered (for the stored energy) since the amount of biomass that was produced in the calcium alginate beads was insignificant. For the total energy input, we considered both the irradiance at the reactor surface and the consumption of the organic compounds [32]. We considered: (a) 12.94 J/mL as the energy content of H_2 at 25 °C; (b) 0.67 J/s the light irradiation impinged on the PBR surface, which was calculated as 80 J/m^2/s (light intensity) × 0.00942 m^2 (reactor surface) × 0.89 (glass transparency of the PBR); (c) 2569 kJ/mol the heat combustion of glutamate; (d) 708.8 kJ/mol the heat combustion of acetate.

The LCE that was calculated for the entire experiment was 1.20%. After calculating the LCE for each period (considering bead washing), three values were obtained: 0.41%, 1.00%, and 2.19% for the three periods from 55 to 162 h, from 176 to 258 h, and from 271 to 328 h, respectively.

4. Discussion

In an anaerobic environment, photosynthetic bacteria produce H_2. The most popular and promising photosynthetic group for biological H_2 production is PNSB [22]. PNSB use organic substrates as electron donors to produce H_2 in anaerobic environments; this process is called photofermentation. Photofermentation in PNSB is influenced by many culture parameters such as the type of carbon source, the N/C ratio, and the pH [15,33]. In contrast to the low C/N ratios, which promote higher cell growth, high C/N ratios lead to higher H_2 production [6]. At low C/N ratios, the organic acids are consumed for cell development instead of producing H_2, thus leading to a decrease in conversion efficiency.

The ability to use solar energy, a wide range of light wavelengths, a variety of carbon sources, and their operation at low pressures and temperatures make photofermenta-

tion a viable technique. The great bulk of photofermentation research relies on PBRs in combination with suspension cultures for H_2 production [22,34].

Whole cell immobilization in a calcium alginate matrix is a common strategy since calcium alginate is a cheap, biocompatible transparent material that is well suited for the immobilization of PNSB cells. It has strong mechanical properties, allows for gas diffusion to occur, and is stable in most growth media. The H_2 production by immobilized systems is influenced by the materials that are used for the immobilization, the type of substrate, the light source and its intensity, the pH, the temperature, the mode of operation, and the bacterial strains [35]. Cell immobilization in calcium alginate offers several advantages when it is compared to the suspension cell culture techniques, such as: the protection against mechanical stress, the protection against contamination, the ability to use a high cell biomass, and a nutrient supply without having to perform cell harvesting. Cell immobilization has been proposed to avoid the harvesting problem and to preserve the high-quality cell biomass for further processing. It is frequently employed in H_2 production systems to increase the H_2 production yield and rate [25,36]. In the immobilized systems, the choice of substrate is crucial because different substrates have different utilization and conversion efficiencies. Acetate, succinate, and sucrose are just some examples of substrates that have been used to generate H_2 in these systems [37–39].

Rhodopseudomonas sp. cells that were immobilized in calcium alginate beads were able to produce H_2 in PBR. In this work, acetate was used because in our previous research, it proved to be the best carbon source for H_2 production by *Rhodopseudomonas* sp. [15]. Four g/L of acetate (67.8 mM) was used in our system because higher acetate concentrations may cause the inhibition of H_2 production in PNSB [40]. The pH in the PBR was maintained at 7.2 during our experiment because the nitrogenase activity is optimal at a neutral pH in most PNSB [41]. The phosphate concentration in the van Niel's medium was reduced to 100 mg/L instead of 1.0 g/L to protect the integrity of the beads. The integrity of the calcium alginate beads was not affected by the low levels of EDTA and phosphate in the culture medium.

Anaerobiosis is a fundamental condition under which the photofermentative H_2 production process can be carried out. The immobilized *Rhodopseudomonas* cells began to produce H_2 after two days when they reached the anaerobic conditions in the presence of acetate and glutamate (C/N~21). The culture showed the highest rate of H_2 production in the last two days of the experiment, after which, the H_2 production stopped after eleven days. The ORP remained stable at these values (<−375 mV) during the experiment as the excess energy was dissipated by the photofermentative H_2 production process (Figure 3). A higher H_2 production rate was observed when we were washing the immobilized cells (Figure 5), which is contrary to the results of Zagrodnik et al. [42] who indicated that the H_2 production decreased with increasing number of operating cycles. The H_2 production rate increased by 2.5 and 5.5 times after the first and second washing steps, respectively. The cumulative H_2 that was obtained from the immobilized *Rhodopseudomonas* sp. cells was 505 mL, which corresponds to 2295 mL/L. This value is the same of that which was recently reported (2286 mL/L) when the researchers were culturing non-immobilized cells of the same bacterium [15]. The maximum LCE value that was obtained in our system (2.19%) remained moderate when it was compared with those of other works. Adessi et al. obtained a maximum LCE value of 0.92% using malic acid as a substrate for *Rhodopseudomonas palustris* 42OL in an outdoor PBR [35]. Chen et al. reported an LCE of 1.93% using *Rhodopseudomonas palustris* WP3-5, a light intensity of 95 W/m^2, and they used acetate as an organic substrate [43]. Liao et al. reported an LCE of 8.9% when they were using LEDs to illuminate *Rhodopseudomonas palustris* CQK 01, and glucose as a substrate [44]. Carlozzi reported an LCE of 0.78% when they were using malic acid as a substrate and a halogen lamp (480 W/m^2) to illuminate *Rhodopseudomonas palustris* 42OL [45]. Cui et al. reported an LCE of 1.6% when they were using acetate to feed *Rhodopseudomonas faecalis* RLD-53 in a 1.5 L PBR [46]. Zhang et al. reported an LCE of 3.8% when they were using glucose to feed *Rhodopseudomonas palustris* GCA009 in a groove-type

PBR that was illuminated by an LED (6.75 W/m^2) [47]. Wang et al. reported an LCE of 5.34% when glucose was used as substrate for feeding *Rhodopseudomonas palustris* GCA009 in a grid columnar flat panel PBR that was illuminated using lamps (210 W/m^2) [48]. We retain that the LCE of the immobilized cells was negatively affected by the lack of homogeneous light penetration and distribution [49]. Table 1 compares the H_2 production rates from several studies using different carbon sources and PBR types.

Table 1. Comparison of H_2 production studies of immobilized photosynthetic bacteria cultures.

Organism	Immobilization Material	PBR (mL)	Light Intensity (W/m^2)	Substrate (g/L)	H_2 Production Rate (mL/L/h)	Reference
Rhodobacter capsulatus YO3	Agar	1400	200	Acetic acid (3.6)	31.2	[50]
Rhodopseudomonas faecalis RLD-53	Agar	100	150	Acetic acid (4.1)	32.8	[51]
Rhodobacter sphaeroides O.U.001	Porous glass	235	102	Malic acid (2.0)	12.7	[52]
Rhodobacter sphaeroides	Porous glass	200	64	Malic acid (2.0)	59	[42]
Rhodopseudomonas sp. *nov. strain A7*	Biofilm	25	150	Acetate (4.1)	25	[53]
Rhodopseudomonas palustris CQK 01	Biofilm	1200	39.5	Glucose (21.6)	38.9	[54]
Rhodopseudomonas palustris CQK 01	Biofilm	125	12	Glucose (9.0)	39.2	[55]
Rhodobacter capsulatus YO3	Agar	1400	200	Sucrose (1.7)	17.8	[38]
Rhodopseudomonas sp.	Alginate	200	80	Acetate (4.0)	22.2	This work

The separation of the cells from the medium is necessary for the H_2 production process when a change of culture medium is required. This procedure would be facilitated by immobilizing the cells in calcium alginate, and the culture media can be recycled. The reuse of culture media can lead to massive savings in water and chemical consumption. The strategy of reusing the medium can be considered from the point of view of low-cost and highly efficient production of value-added materials such as poly(3-hydroxybutyrate) and/or proteins [56,57]. After the product's extraction, the remaining part of the biomass could be used as fertilizer.

5. Conclusions

The purpose of this study concerns the role, and above all, the benefits that PNSB can bring to the supply of green energy that can gradually replace fossil fuels. The study demonstrates that the immobilized cells of *Rhodopseudomonas* sp. could avoid the harvesting problem by reducing the high cost of biomass harvesting, thus preserving the high quality of bacterial cell biomass for further processing to produce the cleanest energy such as bio-H_2. The calcium alginate matrix, in a similar way to agar and porous glass, shows that it can be used for a long period of time without being damaged by the repeated washing of the beads. In conclusion, the present study shows some results that lead to the production of a renewable energy source by biological means from the consumption of acetate (a volatile fatty acid), in which many wastewater effluents are rich, so that volatile fatty acids could be recovered by the use of biological means in view of a circular economy.

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