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Full Review Article

Photofermentative hydrogen production by immobilized *Rhodopseudomonas* sp. S16-VOGS3 cells in photobioreactors

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ABSTRACT

One of the most important solutions to overcome energy and environmental problems and to replace the fossil fuel-based economy could be the use of photosynthetic microorganisms. The use of photosynthetic microorganisms is a potential alternative to energy generation from fossil fuels because they efficiently produce hydrogen (H_2). Immobilization of photosynthetic microorganisms is used for many biotechnological applications such as H_2 production. This method appears attractive because it restricts cell movement in an entrapped matrix. Immobilization of *Rhodopseudomonas* sp. S16-VOGS3 cells is a promising way to improve H_2 production. In this work, the ability of immobilized *Rhodopseudomonas* sp. S16-VOGS3 cells to produce H_2 was investigated in two types of PBRs. The PBRs used in this work were a cylindrical one with 0.2 L working volume (C-PBR) and a flat Roux type with 0.6 L working volume (FRT-PBR). The calcium alginate beads prepared were resistant to culture mixing and showed little leakage of cells, and the immobilized cells continued the photofermentation process in both PBRs. The immobilized cells in the C-PBR produced 936.8 mL of H_2 with an average H_2 production rate of 2.99 mL/h. The average productivity was 126.4 μL (H_2)/mg (cells)/h or 14.96 mL (H_2)/L (culture)/h, and the light conversion efficiency was 2.37 %. The immobilized cells in the FRT-PBR produced a total of 662.2 mL of H_2 with an average H_2 production rate of 1.55 mL/h. The average productivity was 31.1 μL (H_2)/mg (cells)/h or 2.58 mL (H_2)/L (culture)/h, and the light conversion efficiency was 0.52 %. The more uniform and therefore more efficient degree of bacterial cell mixing achieved in the C-PBR with cylindrical configuration played an important role compared to the FRT-PBR. In the FRT-PBR, the beads were aggregated at the bottom, which limited light penetration and resulted in low H_2 production efficiency.

1. Introduction

Worldwide primary energy consumption has increased because of global economic growth, population growth, and technological improvements. The research community is under pressure to find alternatives to a fossil fuel-based economy because fossil energy resources are limited. As an alternative to our existing economic system, the idea of a green circular economy has emerged, using mainly renewable resources. One of the most important approaches to overcoming energy and environmental problems and replacing the fossil fuel-based economy is the application of microbes in bioprocessing and exploring the possibility of using waste streams as substrates or feed streams.

Coffee silverskin is also a potential carbon source to replace glucose or other valuable substrates and improve cost efficiency in the production of value-added products [1]. Bioenergy is usually produced through biological processes (fermentation or anaerobic digestion) using an effective and appropriate microbial system and sustainable raw substrates [2]. Hydrogen (H_2) is an effective, environmentally friendly, and renewable fuel source that can be produced during dark-fermentation and photo-fermentation by various facultative and obligate anaerobic and purple bacteria and microalgae [3]. Molecular H_2 offers the greatest potential due to its high conversion efficiency, recyclability, and environmentally friendly nature [4]. H_2 production from cheap and readily available substrates such as crude glycerol or various industrial, agricultural, and other carbon-based wastes by bacteria is a sustainable technology [3]. Regarding light-dependent microbes, photosynthetic microorganisms such as microalgae, cyanobacteria, and purple bacteria are promising candidates as potential producers of H_2 [5–7].

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Green energy produced by cultivating photosynthetic microorganisms (cyanobacteria, microalgae and photosynthetic bacteria) will become increasingly important in the world economy, to limit damages such as global changes caused by fossil fuel consumption. Photosynthetic microorganisms, which grow extensively on land and aquatic environments (freshwater and seawater), contribute significantly to the production of bioproducts (proteins, nucleic acids, carbohydrates, lipids, and H_2), by fixing CO_2 through the photosynthetic process. Through the intensive cultivation of photosynthetic microorganisms, it is also possible to produce commercial green energy such as biodiesel and/or molecular hydrogen. Biodiesel, one of the most widely used biofuels, is considered an ideal recyclable energy source and thus a potential primary energy source [8]. Biodiesel is a green and sustainable energy source; molecular hydrogen is also green and an emission free energy carrier, as its combustion produces energy and water. When burned, H_2 releases a significant amount of energy per unit weight and can be easily converted to electricity in fuel cells.

Gaffron first discovered H_2 synthesis in microalgae in 1942, when he noticed that in addition to fermentative H_2 synthesis in the dark, the cells of *Scenedesmus obliquus* can also produce H_2 in the light under anaerobic conditions [9,10]. Since oxygen inhibits the hydrogenase enzymes anaerobic conditions are required for H_2 formation. Some cyanobacteria possess a hydrogenase that is less susceptible to long-term inactivation by oxygen and can catalyze both the generation and uptake of H_2 [11,12]. Photosynthetic H_2 formation can be distinguished into direct and indirect pathways of biophotolysis. While the indirect pathway uses electrons from carbohydrates as a substrate for H_2 generation, the direct pathway generates H_2 from the electrons and protons produced by the light reactions [13,14]. Temporal separation of photosynthetic oxygen evolution and H_2 synthesis is required because hydrogenase is oxygen sensitive. Melis et al. have presented a two-step mechanism for H_2 production in microalgae in which the process of oxygen and H_2 production are separated in time [15]. In step I, cells grow with normal photosynthesis, while in step II a nutrient (sulphate) is withdrawn, resulting in reduced photosynthetic activity, followed by the production of H_2. When the process of H_2 production is scaled up, the two-step approach of Melis et al. has a disadvantage. Repeated washing of cells in sulphate-depleted media takes time and increases the risk of contamination of the culture [16].

Purple non-sulfur bacteria are the most commonly used photosynthetic bacteria for H_2 production [17]. They absorb solar energy in the visible and near-infrared spectrum and have no oxygen evolving activity. One of the biological processes mainly driven by purple non-sulfur bacteria is photofermentation, in which organic substrates are used as electron donors to produce molecular H_2 [18]. Nitrogenase catalyzes proton reduction to produce H_2 in the absence of molecular nitrogen, anaerobic, and light conditions at the expense of 4 mol of adenosine triphosphate (ATP): $8H^+ + 8e^- + 16ATP \rightarrow 4H_2 + 16ADP + 16Pi$ [19]. The process of photofermentation begins with the utilization of the carbon source by the tricarboxylic acid cycle to produce electrons and carbon dioxide. Then the electrons are transferred to nitrogenase by successive oxidation and reduction of electron carriers such as NAD/-NADH and (Fd)ox/(Fd)red. The electrons and protons are transferred to nitrogenase along with ATP generated by the photosynthetic process to synthesize H_2 [20]. Various purple non-sulfur bacteria such as *Rhodobacter sphaeroides, Rhodobacter capsulatus* and *Rhodopseudomonas palustris* as well as organic acids such as acetate, lactate and malate were used for photofermentation [21–24]. The use of effective photobioreactors (PBRs), the low light conversion efficiency, the low H_2 production rate (HPR), and the limitation of the process by the day-night cycle are some disadvantages of photofermentation.

PBRs play an essential role in the cultivation of photosynthetic bacteria (purple non-sulfur bacteria) and microalgae. They can be placed indoors or outdoors. In both cases, the light source and light intensity are critical factors that can affect the performance of photosynthetic microorganism growth. Although many efforts have been made to develop

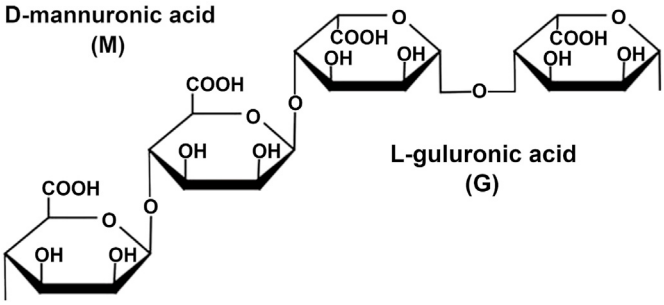

Fig. 1. The main structure of alginate consists of two monomeric units: β-(1,4)-linked D-mannuronic acid (M) and α-(1,4)-linked L-guluronic acid (G).

efficient and cost-effective PBRs, the high cost of installing and operating artificial light sources in conventional PBRs remains a major problem [25]. The scaled-up production of molecular hydrogen faces a number of technical hurdles that make the development of H_2 production economically unfeasible, currently. Therefore, it is necessary to develop the cultivation of photosynthetic microorganisms, the design of PBRs, and low-cost technologies for H_2 production.

The techno-economic analysis shows that the process of photofermentative H_2 production is close to commercialization. However, much experience is still needed to overcome some challenges, such as low H_2 production rate, low photochemical efficiency, increased cost of bioreactors, suitability of low-cost substrates, sensitivity of hydrogenase to oxygen, cost of H_2 storage material to ensure economic feasibility [7]. In practice, we need to make the whole system of molecular H_2 production, its storage and distribution more efficient.

Waste products such as olive mill effluent, cheese whey and molasses, can be profitably used to feed photosynthetic bacteria instead of synthetic sugars and/or organic acids [23]. Single organic acids or combinations of organic acids can be used as carbon sources for H_2 production under certain conditions [26]. H_2 production depends on growth parameters such as light, temperature, pH and the composition of the culture medium. The production of photofermentative H_2, both in the laboratory and in the field, has been the subject of several publications [27–29]. Purple bacteria have been studied for H_2 production using various carbon sources, including volatile fatty acids from wastewater [30,31]. Recently, *Rhodopseudomonas* sp. was reported to produce H_2 when cultured with acetate [32]. This organic acid proved to be the best carbon source for this microorganism and showed the highest cumulative H_2 production [31]. However, to increase the HPR, the physical and chemical parameters should still be improved.

The improvement and stabilization of photofermentative H_2 production could be achieved by immobilization. This method has been widely used for purple bacterial cells [33–38]. Purple bacterial cells can be immobilized by many different methods, but the gel entrapment method seems to be one of the most popular. Cell immobilization has a number of advantages over traditional cell culture (suspension) that can reduce the cost of the system. These advantages include better cell stability and resistance to mechanical stress; increased cell biomass; nutritional supplementation without the need to harvest cells; reduced risk of cell contamination. In addition, immobilization provides cells with a much more stable microenvironment compared to planktonic culture. This technology aims to improve H_2 synthesis, yield and speed in biohydrogen research [38,39].

This method seems to be attractive because it restricts the movement of the cells in a confined matrix and because it is easier to scale [40–43]. It also requires less area to build a photobioreactor (PBR) and increases cell density. The immobilization approach has the advantages of not washing out the cells, stability of the cell system, protection from mechanical stress, and reduced contamination [41,44].

Common immobilization techniques in photo and dark fermentation include entrapment, biofilms, adsorption, and encapsulation, resulting in

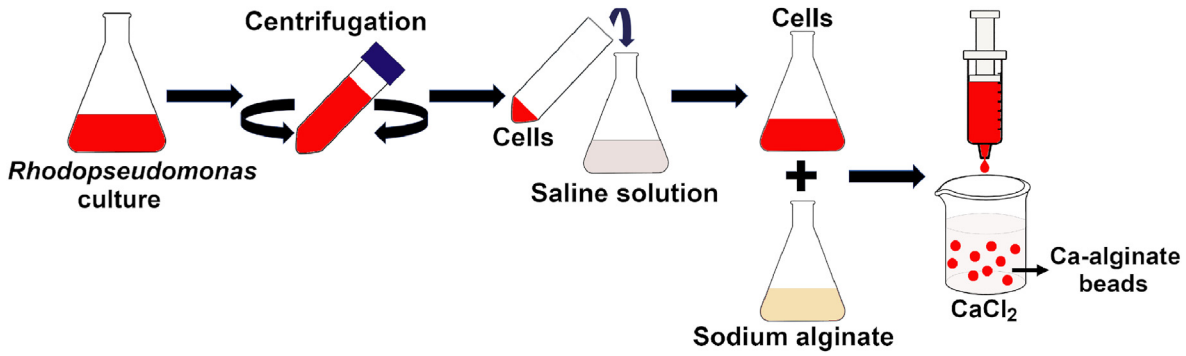

Fig. 2. Procedure for *Rhodopseudomonas* cells immobilization in calcium alginate beads.

high yields and reusability compared to suspended systems [45,46]. Materials such as glass beads, alginate, agar, chitosan hydrogels and polyvinyl alcohol cryogels have been used to immobilise purple bacteria cells [47–51].

Alginates, which are unbranched polysaccharides are commonly used for encapsulation of purple bacteria [33,38,44]. They are mainly obtained from brown algae and consist of (1–4)-linked a-L-guluronic acid and b-D-mannuronic acid residues in varying proportions (Fig. 1) [52,53]. They are frequently employed for immobilization and microencapsulation technologies because they are abundant, nontoxic, inexpensive, and compatible with biological systems [54]. Gelation of alginate occurs by ion exchange between Na^{+} from the salts of guluronic acid and divalent cations such as Ca^{2+}. The gelling ability of alginates is based on ionic bonding. The metal affinity of alginates increases in the following order: $Mg^{2+} < Ca^{2+} < Sr^{2+} < Ba^{2+}$. Alginates have several special properties that have allowed them to be used as a matrix for the entrapment of various photosynthetic microorganisms [41,42,44]. These properties include: i) transparency; ii) a relatively inert aqueous environment within the gel; iii) a mild encapsulation process; iv) the high porosity of the gel, which allows gas and nutrient exchange between the gel and its environment; v) the reversibility of the gelation process. Alginate gels have some disadvantages, including limitations in mass transfer, low mechanical strength, gel degradation, and large pores [55]. Alginate gel is sensitive to chelating agents such as ethylenediaminetetraacetic acid (EDTA) and phosphate and to antigelling cations such as magnesium. Other disadvantages include its susceptibility to pH and ionic strength [56], and gelation and mechanical properties are strongly influenced by its composition and concentration [57,58].

In this work, we studied the photofermentation process in two types of laboratory PBRs (cylindrical and flat) using immobilized *Rhodopseudomonas* sp. S16-VOGS3 cells (hereafter *Rhodopseudomonas*). The two PBRs used, the first with a working volume of 0.2 L and the second with 0.6 L, are compared in terms of light conversion efficiency (LCE), H_2 productivity and HPR.

2. Materials and methods

2.1. Algal strain and growth conditions

The bacterium *Rhodopseudomonas* sp. S16-VOGS3 from the culture collection of the National Research Council - Research Institute on Terrestrial Ecosystems, Florence, Italy, was used in this study. The 16S sequence of *Rhodopseudomonas* sp. S16-VOGS3 has been deposited in GenBank under the following accession numbers: KU899101 - KU899105. The bacterium was pre-cultured using a modified van Niel growth medium [59]. The medium contained 2 g/L acetate, 0.5 g/L NH_4Cl, 1 g/L KH_2PO_4, 0.4 g/L NaCl, 0.4 g/L $MgSO_4$ $7H_2O$, 0.05 g/L $CaCl_2$ $2H_2O$, 0.1 mg/L *p*-aminobenzoic acid, 0.005 g/L ferric citrate and 10 mL/L mineral solution for micronutrients. The mineral solution (1 L) contained 1 mg $CuCl_2$ $2H_2O$, 2 mg $NiCl_2$ $6H_2O$, 3 mg $MnCl_2$ $4H_2O$, 10 mg

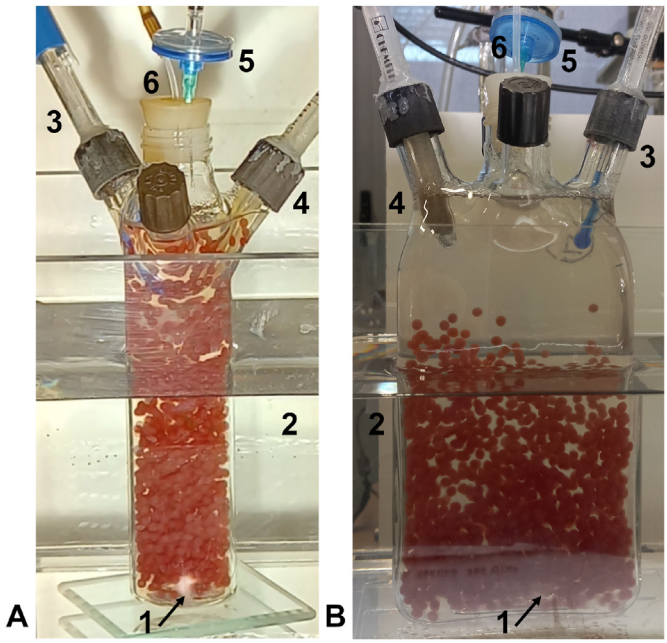

Fig. 3. Image of the two photobioreactors used in this study containing the Ca-alginate beads with the immobilized *Rhodopseudomonas* cells. A) The C-PBR. B) The FRT-PBR. (1) Magnetic stirring bar; (2) water bath; (3) pH probe; (4) ORP probe; (5) HCl solution inlet; (6) H_2 exit.

$ZnSO_4$ $7H_2O$, 20 mg $CoCl_2$ $6H_2O$, 30 mg H_3BO_3, 200 mg $FeSO_4$ $7H_2O$, and 500 mg Na_2MoO_4 $7H_2O$. The pH of the medium was adjusted (once daily) to 6.8 with sterile NaOH (1.0 mM). The medium was sterilized using a Vapor Matic autoclave (Vacuum Service srl, Civezzano, Italy) model 770/A for 20 min at a pressure of 1 bar and a temperature of 121 °C. Cultures were continuously illuminated from one side with a power-star HQI-TS OSRAM halogen lamp (80 W/m^2, main peak at 590 nm) and maintained at 30 °C. A LICOR radiometer model LI-185B (LICOR, Lincoln, USA) was used to measure irradiance.

2.2. Immobilization

To remove the growth medium, the *Rhodopseudomonas* culture was first centrifuged (3000 rpm for 10 min) in a Sorvall Super T21 centrifuge (Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA) and the pellet was washed twice with sterile saline solution (NaCl 0.9 %). 50 mL of the resuspended cells (46 mg cell dry weight (CDW)) in saline solution were mixed with 50 mL of a 6 % sodium alginate (SPALGS100, Gioia Group srl, Torino, Italy) in saline solution (Fig. 2). The mixture was then added dropwise to a 2 % $CaCl_2$ in saline solution stirred with a magnetic stirrer and allowed to set at 25 °C for 30 min. Under sterile conditions

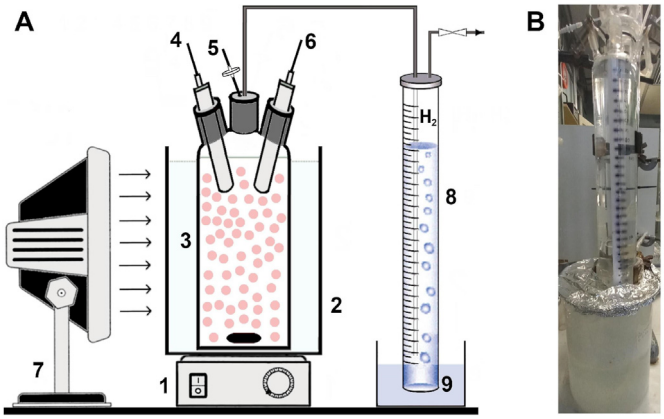

Fig. 4. (A) Schematic diagram of the H_2 production set-up; 1) magnetic stirrer; 2) water bath; 3) photobioreactor with calcium alginate beads containing *Rhodopseudomonas* cells; 4) pH sensor; 5) inlet for sterile HCl solution; 6) ORP probe; 7) lamp; 8) calibrated glass column; 9) CO_2-absorber solution. (B) Image of the calibrated glass column.

using a laminar flow hood Asalair 1200 FLO (Asal, Milan, Italy), the calcium alginate beads with the immobilized cells were then washed twice with sterile saline solution, harvested, and placed in the PBR. The beads were observed with a Nikon Eclipse E600 microscope (Nikon Corporation, Tokyo, Japan).

2.3. Photobioreactors

Two different PBR shapes were used in the present study: a cylindrical 0.2 L photobioreactor (C-PBR) and a flat 0.6 L Roux type photobioreactor (FRT-PBR); both PBRs with 4.0 cm light path (Fig. 3). Two OSRAM power-star HQI-TS lamps, (Osram GmbH Munich Germany, 150 W) continuously illuminated the PBR surfaces. The average irradiance measured on a flat surface (IF_{avg}), perpendicular to the PBR, was 80 W/m^2. The irradiated area of the C-PBR was calculated as ½ of the cylindrical reactor surface (2πrih), where ri and h indicate the inner radius and height of the cylindrical reactor, respectively [60]. Due to the geometry of the cylindrical PBR shape, a dilution factor of 1.57 was applied to determine the average effective irradiance that impinged on the semi-circumference of the PBR [61]. The C-PBR consisted of a cylindrical Pyrex bottle (15 cm height × 4 cm inner diameter, 200 mL working volume) with a flat bottom and ports for the pH and oxidation-reduction potential (ORP) electrodes (Fig. 3A). The FRT-PBR consisted of a Pyrex-Roux culture bottle (600 mL working volume, 4 cm light path) with a flat cross-section (15 × 10 × 4 cm, H × W × D), a flat bottom, and ports for the pH and ORP electrodes (Fig. 3B). The main port at the top (2.5 cm i.d.) of each PBR was closed with a silicone stopper equipped with a Tygon tube for the outflow of culture gases. A needle was inserted into the silicone stopper for the addition of sterile HCl solution. The PBRs were placed in a thermostatic water bath (type M900-TI Basic MPM Instruments srl, Bernareggio, Italy) at a constant temperature (30.0 ± 0.1 °C) and the immobilized *Rhodopseudomonas* cells were stirred using a Falc F30 magnetic stirrer (Falc Instruments srl, Treviglio, Italy).

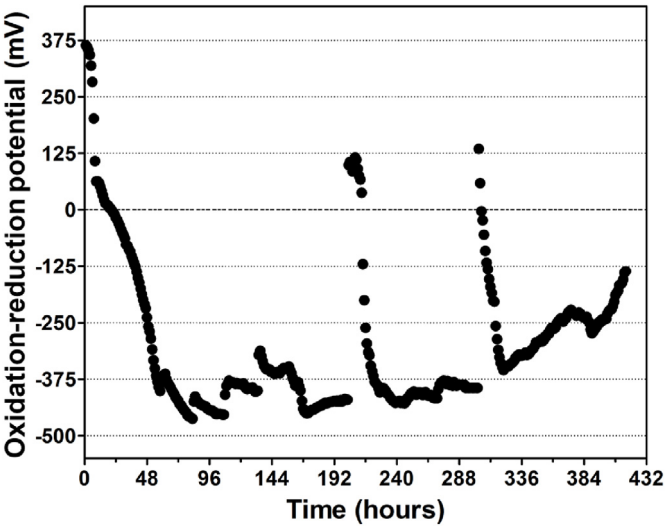

Fig. 5. Oxidation-reduction potential values of the immobilized *Rhodopseudomonas* cells in the C-PBR during the photofermentation process. The increase in values at 204 and 304 h corresponds to the entry of atmospheric O_2 into the system during the bead-washing process.

2.4. H_2 production

Photofermentative H_2 production was performed in an anaerobic mode using the immobilized *Rhodopseudomonas* cells in the PBRs filled with a modified Van Niel growth medium. The modified Van Niel growth medium contained 4.0 g/L acetate instead of 2.0 g/L, 1.0 g/L glutamate instead of NH_4Cl, and 0.1 g/L potassium dihydrogen phosphate instead of 1.0 g/L (C/N = 21). The pH of the bacterial culture was maintained at 7.2 by the addition of a sterile HCl solution (10 mM). All experiments were performed in a thermostatic room at atmospheric pressure. Two probes coupled to a controller (Chemitec srl, Florence, Italy) were used to measure ORP and pH (Figs. 3 and 4). A calibrated column immersed in a CO_2-absorber solution was used to collect the H_2 produced by the immobilized cells (Fig. 4B). During the experiment, the beads were washed twice with a sterile saline solution. The experiment was stopped when the calcium alginate beads containing the immobilized bacteria started to lose their structural stability.

2.5. Analytical procedures

Biomass cell density (as CDW) was determined by dry weight measurements performed in triplicate according to the following protocol [44]. Samples of 10 mL were taken from the culture and filtered through pre-weighed Whatman GF/F filters with 0.7 mm pore size. They were then dried in an MPM Instruments type M60-VN oven (MPM Instruments srl, Bernareggio, Italy) at 70 °C for 16 h and weighed in a PBI model bc analytical balance (VWR International (PBI) Srl, Milan, Italy).

Organic acid content was determined using an HPLC (Thermo Finnigan-Spectra System 6000 LP) (Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA) with an analytical C18 column (250 × 4.6 mm) and H_3PO_4 (0.1 % w/v) as the mobile phase at a flow rate of 1.0 mL/min and 25 °C [59].

The light conversion efficiency (LCE) was calculated as the following ratio (energy output)/(energy input) × 100 [60]. The energy output is equal to the energy of the H_2 produced plus the energy of the biomass produced. The energy input consists of the irradiance impinging on the surface of the PBRs and the energy of the organic molecules consumed. We considered (i) 12.94 J/mL as the energy content of H_2 at 25 °C; (ii) the light irradiance on the PBR surface, calculated as: light intensity (J/m^2/s) × reactor surface area (m^2) × 0.89 (glass transparency of PBR); (iii) 2569 kJ/mol the heat of combustion of glutamate; (iv) 708.8 kJ/mol the heat of combustion of acetate.

The gas produced by the culture was analyzed using a Clarus 500 model gas chromatograph (PerkinElmer, Waltham, Massachusetts USA) with a Carbosieve SII Spherical Carbon packed column (Supelco. Inc., Bellefonte, Pennsylvania, USA) and a thermal conductivity detector. Gas chromatography was performed under the following operating conditions. An isothermal program at 35 °C for 2.25 min, the nitrogen carrier gas was set to a flow of 30 mL/min, the injection temperature was set to 150 °C, and the detector temperature was set to 150 °C. Known doses of pure gas were injected to develop a calibration curve of the H_2.

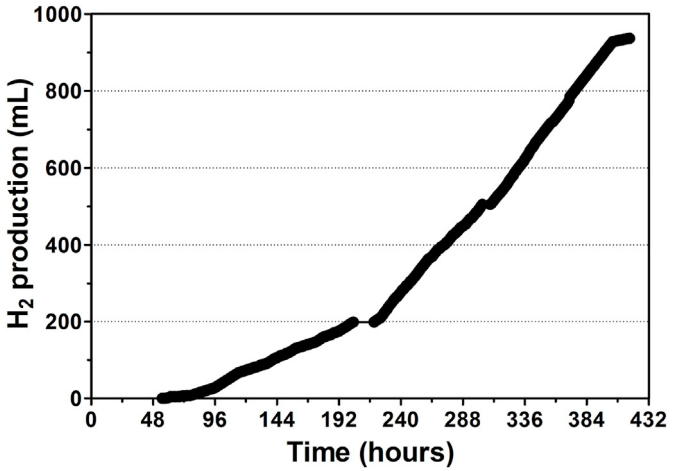

Fig. 6. H_2 production by immobilized *Rhodopseudomonas* cells in the C-PBR. The immobilized cells were washed and retested for their ability to produce H_2 twice at 204 and 304 h.

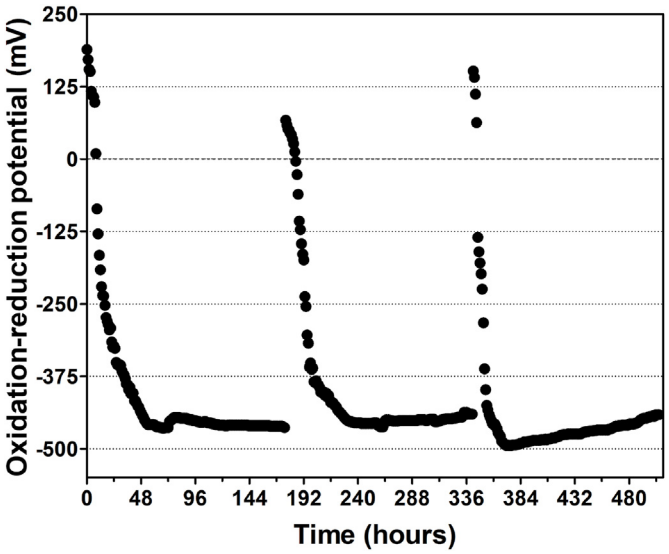

Fig. 7. Oxidation-reduction potential values of the immobilized *Rhodopseudomonas* cells in the FRT-PBR during the photofermentation process. The increase in values at 176 and 342 h corresponds to the entry of atmospheric O_2 into the system during the bead-washing process.

3. Results

3.1. H_2 production by immobilized Rhodopseudomonas in the C-PBR

3.1.1. Immobilization of Rhodopseudomonas cells

A total of 46.0 ± 1.5 mg *Rhodopseudomonas* cells were washed with saline solution, mixed with sodium alginate, and dropped into a $CaCl_2$ solution. Once the alginate droplets reached the $CaCl_2$ solution, spherical beads with entrapped cells were formed. The beads formed were 4.34 ± 0.18 mm in diameter, 42.9 ± 1.8 μL in volume, and contained 19.7 ± 1.0 μg of cells (CDW/bead).

3.1.2. H_2 production

1200 beads, corresponding to 51.5 mL total volume and 23.68 mg immobilized cells, were added to the C-PBR (Fig. 3A). The PBR was filled with the medium for H_2 production, sealed, and left in the dark overnight to support anaerobiosis. Then, the PBR was placed in the light under anoxic conditions that triggered photofermentation. Photofermentative H_2 production was carried out in this immobilized system in a sequential batch mode. The process took 18 days and consisted of three sequential steps.

The ORP of the culture showed a sharp initial drop followed by stabilization between −400 mV and −460 mV, mainly due to the anaerobic conditions achieved (Fig. 5). It then increased rapidly and then decreased in line with the two washes performed (Fig. 5). H_2 production by immobilized *Rhodopseudomonas* cells began 55 h after anaerobic conditions were reached (Fig. 6).

Three steps were taken to determine the cumulative amount of H_2 during the experiment: at the start (198.6 mL), after the first wash at 204 h (306.5 mL), and after the second wash at 304 h (431.7 mL) (Fig. 6). A total of 936.8 mL of H_2 was produced, corresponding to 4258 mL (H_2) per liter of culture (Fig. 6). The following results were obtained by calculating the HPR for each time period considering bead washing: 1.33, 3.65, and 3.99 mL H_2/h for the three time periods of 55–203 h, 220–303 h, and 310–417 h, respectively. The maximum HPR was 5.39 mL/h, with an average HPR of 2.99 mL/h. Productivity ranged from 6.66 to 19.98 mL (H_2)/L (culture)/h with an average of 14.96 mL (H_2)/L (culture)/h or 126.4 μL (H_2)/mg (cells)/h.

An LCE of 2.37 % was calculated for the entire experiment. Since the amount of biomass produced in the calcium alginate beads was negligible, only H_2 production was considered for the calculation of LCE. Both the irradiance at the surface of the PBR and the consumption of the organic compounds were considered in the calculation of the total energy input [60]. We also considered the following factors: (i) the energy content of the H_2 produced; (ii) the heat of combustion values of glutamate and acetate. By calculating the LCE for each time period (considering bead washing), we obtained three values: 1.05 %, 2.89 %, and 3.17 % for the three periods of 55–203 h, 220–303 h, and 310–417 h, respectively.

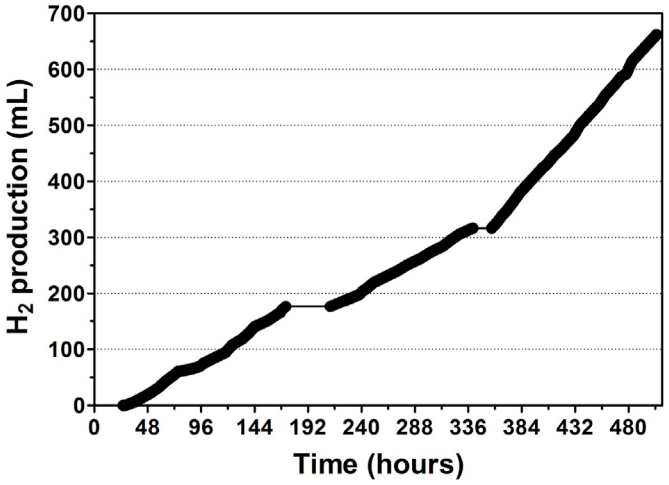

Fig. 8. H_2 production by immobilized *Rhodopseudomonas* cells in the FRT-PBR. The immobilized cells were washed and retested for their ability to produce H_2 twice at 176 and 342 h.

3.2. H_2 production by immobilized Rhodopseudomonas cells in the FRT-PBR

3.2.1. Immobilization of Rhodopseudomonas cells

In this experiment, 65.5 mg *Rhodopseudomonas* cells were washed with saline solution, mixed with sodium alginate, and dropped into the sterile $CaCl_2$ solution. The formed spherical beads with entrapped cells were 4.34 ± 0.18 mm in diameter, 53.0 ± 2.6 μL in volume, and contained 34.2 ± 1.6 μg cells (CDW/bead).

3.2.2. H_2 production

A total of 1433 beads, corresponding to 75.9 mL total volume and

Table 1
H_2 production results obtained with the two PBRs with immobilized *Rhodopseudomonas*. The average light intensity received at each step in each PBR was 51 W/m^2 and 80 W/m^2 for the C-PBR and the FRT-PBR, respectively.

Variable	C-PBR (0.2 L)				FRT-PBR (0.6 L)			
	Step-1	Step-2	Step-3		Step-1	Step-2	Step-3	
HPR (mL (H_2)/h)	1.33	3.65	3.99		1.20	1.10	2.33	
HPR (mL (H_2)/L (culture)/h)	6.66	18.24	19.98		2.01	1.84	3.88	
HPR (μL (H_2)/mg (cells)/h)	56.3	154.0	168.8		24.3	22.2	46.9	
LCE (%)	1.05	2.89	3.17		0.40	0.37	0.78	
				Total				**Total**
Cumulative H_2 (mL)	198.6	306.5	431.7	936.8	176.5	140.3	345.4	662.2
Duration (h)	149	84	108	341	146	127	148	421

49.76 mg immobilized cells, were added to the FRT-PBR (Fig. 3B). The FRT-PBR filled with the medium was transferred under light conditions after overnight dark incubation. The ORP of the culture in this experiment also showed a sharp initial drop followed by stabilization between −400 mV and −465 mV (Fig. 7). As in the previous experiment, ORP values increased again and then decreased in line with the two washes performed. H_2 production by immobilized *Rhodopseudomonas* cells began 30 h after anaerobic conditions were reached (Fig. 8).

Also in this experiment, two washing steps were performed, at 172 and at 341 h. During these three periods in the beginning, after the first wash, and after the second wash, the following amounts of H_2 were produced: 176.5 mL (HPR = 1.20 mL/h), 140.3 mL (HPR = 1.10 mL/h) and 345.4 mL (HPR = 2.33 mL/h) (Fig. 8). The total amount of H_2 produced was 662.2 mL, this value corresponds to 1103.5 mL of H_2 per liter of culture. The maximum HPR was 4.31 mL/h, with an average HPR of 1.55 mL/h. Productivity ranged from 1.84 to 3.88 mL (H_2)/L (culture)/h with an average value of 2.58 mL (H_2)/L (culture)/h or 31.1 μL (H_2)/mg (cells)/h.

The LCE of the process was 0.52 % for the entire experiment. By calculating the LCE for each period (considering the washing of the bead), the following values were obtained: 0.40 %, 0.37 %, and 0.78 % for the three periods of 26–172 h, 212–341 h, and 357–506 h, respectively.

4. Discussion

Biological H_2 production systems currently face several difficulties, including relatively low H_2 yield and HPR, which makes biological H_2 production more expensive than alternative fuels. To effectively use H_2 as a fuel, issues of production, transportation, storage, and application efficiency must be resolved. Cell immobilization could play a critical role in the development of large-scale H_2 production. This technique is useful for H_2 production because it offers several advantages, such as higher cell concentration, improved operational stability, higher substrate conversion efficiency, reduced contamination, reuse of immobilized microorganisms, and protection from mechanical stress. In addition, the immobilization process is easier to scale up and requires less area for PBR production. However, low light transmission, cell shading, low substrate diffusion, and carrier disruption are some of the main obstacles for immobilized systems [38]. Immobilization of purple bacteria has been shown to increase the rate of H_2 production [62]. Compared with suspension cultures, H_2 production by immobilized purple bacteria is more stable [63]. There are many recent studies on the production of photofermentative H_2 by immobilized purple bacteria [33,35,38,44,48,64]. The major technological obstacle to the economic viability of H_2 photoproduction is the low LCE [65]. Therefore, the benefits of various ways to increase LCE are constantly being investigated [66].

To achieve the best possible product quality, all physical and chemical parameters of the culture must be strictly controlled. Many different bioreactor designs have been developed to meet different criteria and a range of bioprocesses [67–71]. Closed bioreactors are preferred for cultures of photosynthetic microorganisms because they protect the culture from contaminants, allow better control of growth conditions, greatly minimize evaporation, and allow higher volumetric productivities and cell concentrations. The difficult link between microalgal growth and culture conditions best explains the difficulties in developing customized reactors that allow adequate yields. Therefore, a preliminary investigation using a fully specified method in which all relevant variables can be controlled with extreme accuracy is critical. The efficiency of the reactor is based on the combination of light collection, transmission and distribution [72,73].

There are a variety of PBRs with different designs and configurations (tubular, flat plate, etc.) for photofermentation [74]. Tubular PBRs consist of one or more transparent tubes made of glass or plastic with different diameters. The tubes can be arranged in a variety of shapes and orientations (loop, coil, manifold, horizontal, vertical, etc.). Pumps or airlift systems are used to aerate, mix, and circulate the culture. Flat plate PBRs consist of one or more plastic or glass light-harvesting units with a reduced light path containing the culture, which is circulated between the units by a pump.

Most published research on H_2 production is at the laboratory scale, and there is little work on scaling up to the level required for commercial production. It can be difficult to determine the variables that affect the scaling process during cultivation, which makes scaling microorganisms from a laboratory-scale facility to a commercial facility challenging. For this reason, the yield of most commercial cultures is lower than predicted in the laboratory [75]. To design a suitable PBR system, the following parameters should be considered: the cultured species, the operating conditions, and the geometry of the PBR [76]. To ensure the scalability of PBRs, the proper balance between light intensity, hydrodynamics, and environment must be carefully considered. Since light distribution within the PBR is one of the most important factors, the two PBRs in our study were selected to have the same light path (4.0 cm).

The fermentative conversion of organic substrate to H_2 has recently become the focus of extensive research worldwide [77]. Compared to other microbial systems, photofermentation has the advantages of high substrate conversion rate, absence of oxygen, wider use of wavelength range, and the ability to use organic waste substrates.

In our experiments the immobilized *Rhodopseudomonas* cells were able to produce H_2 by photofermentation in both types of PBRs. H_2 production started when the cultures reached anaerobic conditions, i.e., after 55 h and after 30 h, and lasted 17.5 and 21 days for the C-PBR and FRT-PBR, respectively. In this study, two washing steps were performed when H_2 production approached zero due to the depletion of organic compounds.

The initial system adaptation may have contributed to the lowest performance in the first step. In the C-PBR, volumetric H_2 production was 936.8 mL with an average HPR of 2.99 mL/h, whereas in the FRT-PBR, it was 662.2 mL with an average HPR of 1.55 mL/h. The productivity in the C-PBR was 14.96 mL (H_2)/L (culture)/h or 126.4 μL (H_2)/mg (cells)/h, whereas the productivity in the FRT-PBR was 2.58 mL (H_2)/L (culture)/h or 31.1 μL (H_2)/mg (cells)/h. When we compare the productivity and LCE values of the two PBR geometries, we find that the C-PBR has better performance than the FRT-PBR. The average productivity (14.96 mL

Table 2
Comparison of H_2 production studies of suspension and immobilized photosynthetic bacterial cultures.

Bacterial strain (biomass used)	PBR type (volume)	Substrate (concentration)	Matrix	Irradiance (W/m^2)	Culture type	HPR (mL/g/h)	Reference
Rhodobacter sphaeroides O.U. 001 (4820 mg)	Flat plate reactor (200 mL)	Malic acid (2.0 g/L)	Filter porous glasses	64	Immobilized	1.1	[48]
Rhodobacter capsulatus DSM 1710 (500 mg)	Roux bottle (1400 mL)	Acetate (3.6 g/L)	Agar	200	Immobilized	50.4	[36]
Rhodopseudomonas palustris WP3-5 (2800 mg)	Glass vessel (800 mL)	Acetate (1.95 g/L)	–	95	Suspension	10.9	[83]
Rhodobacter capsulatus YO3 (1000 mg)	Flat panel (1400 mL)	Acetic acid (3.6 g/L)	Agar	200	Immobilized	43.7	[36]
Rhodopseudomonas faecalis RLD-53 (87.2 mg)	Glass vessel (80 mL)	Acetate (4.1 g/L)	–	31.6	Suspension	25.3	[84]
Rhodopseudomonas sp. S16-VOGS3 (23.7 mg)	Cylindrical glass bottle (200 mL)	Acetate (2.0 g/L)	Ca-alginate	51	Immobilized	168.6	This work
Rhodopseudomonas sp. S16-VOGS3 (49.7 mg)	Flat Roux type glass bottle (600 mL)	Acetate (2.0 g/L)	Ca-alginate	80	Immobilized	46.8	This work
Rhodopseudomonas (277 mg)	Cylindrical glass bottle (220 mL)	Acetate (4.0 g/L)	–	80	Suspension	15.6	[31]

(H_2)/L(culture)/h) obtained in the C-PBR of the present study is very similar to the results obtained when *Rhodopseudomonas palustris* sp. was cultured in suspension cells (15.21 mL(H_2)/L h) at the same irradiance [78]. Table 1 summarizes the results obtained with the two types of PBR.

The differences in product yield and LCE between the two culture systems can be attributed to the cylindrical geometry of the C-PBR, which allows for better mixing efficiency and a higher surface area-to-volume ratio. In addition, the aggregation of beads at the bottom of the FRT-PBR limited light penetration and thus the efficiency of H_2 production. It is important to note that H_2 production in the FRT-PBR started 39 h after the first washing step, whereas in the C-PBR it started only after 16 h. We believe that the culture required more time to create an anaerobic environment due to the combination of the higher volume with the lower mixing efficiency.

The productivity value of the C-PBR (126.7 μL (H_2)/mg (cells)/h) is much higher compared to our previously published results with the same PBR and strain (40.9 μL (H_2)/mg (cells)/h) [44]. Compared to our previous work, the use of less immobilized material resulted in higher LCE and HPR. The use of less material likely resulted in better mixing and light utilization. The PBR exhibited a higher HPR after the second wash step (~20 mL/L/h). This value is comparable to the HPR of previous publications on immobilized purple bacteria in PBRs. Zagrodnik et al. reported an HPR of 59 mL/L/h using *Rhodobacter sphaeroides* immobilized on porous glass, a light intensity of 64 W/m^2 and malic acid as organic substrate [48]. Elkahlout et al. reported an HPR of 31.2 mL/L/h using *Rhodobacter capsulatus* YO3 immobilized in agar, a light intensity of 200 W/m^2 and acetic acid as substrate [36]. Xie et al. reported an HPR of 32.8 mL/L/h using *Rhodopseudomonas faecalis* RLD-53 immobilized in agar, a light intensity of 150 W/m^2 and acetic acid as substrate [79]. Wen et al. reported an HPR of 25 mL/L/h using *Rhodopseudomonas* sp. nov. strain A7 immobilized in biofilm, a light intensity of 150 W/m^2 and acetate as substrate [64]. The HPR obtained (~20 mL/L/h) is similar to the HRP (19.6 mL/L/h) previously published by our group, where we used the same experimental conditions and the same strain suspended in the growth medium [31]. Ross and Pott reported a maximum specific H_2 production rate of 15.74 ± 2.2 mL/g/h using *Rhodopseudomonas palustris* NCIMB 11774 immobilized in polyvinyl alcohol cryogel and glycerol as substrate [35]. In Table 2, the H_2 production studies with immobilized photosynthetic bacteria were compared with photosynthetic bacteria in suspension. The total H_2 volume obtained in C-PBR was 936.8 mL, which corresponds to 4258 mL (H_2) per Liter of culture. This value is higher than the reported value (2286 mL/L) using the same bacterium in suspension culture [31]. The LCE value obtained in our system (2.37 %) is higher than the values reported by Cui et al. [80] using *Rhodopseudomonas faecalis* RLD-53 in a 1.5 L PBR (1.6 %), by Adessi et al. [81] outdoors (0.92 %), and by Carlozzi [82] indoors (0.78 %) at a very high irradiance of 480 W/m^2. The above two research groups cultured *Rhodopseudomonas palustris* 42OL.

The lifetime and reusability of the immobilized cells were checked by adding fresh growth medium. Alginate beads are sensitive to chelating agents such as EDTA and phosphate, thus by adding fresh growth medium (more phosphate), the calcium ions in the alginate gel are replaced by sodium ions. As a result, the alginate beads lost their gel strength and became more susceptible to mechanical stress (from the stir bar) and began to lose their integrity. Many strategies have been explored to increase the mechanical stability of gels. These include the use of alginate in combination with other substances such as nanofibers, carbon nanomaterials, cellulose nanocrystal(s), and nanoparticles [85–88]. The mechanical properties of alginate can be improved by the addition of substances such as dextran sulphate, cellulose acetate phthalate, sodium carboxymethyl cellulose, polyphosphate, and cellulose sulphate [89–91].

Commercial production of microbial H_2 is progressing. Photosynthesis is the fundamental driving force that supports all synthetic biofuel processes by converting solar energy into storage products (e.g., carbohydrates and lipids) and/or H_2 [91]. Limiting the discussion to H_2 production by photofermentation, some species of purple non-sulfur bacteria (e.g., *Rhodopseudomonas faecalis*, *Rhodobacter sphaeroides*, *Rhodopseudomonas palustris* and *Rhodobacter capsulatus*) are suitable candidates for commercial production of clean energy such as H_2. Nevertheless, a comprehensive understanding of the biosynthesis and degradation of precursors, intermediates, and metabolic end products is essential to develop advanced engineering strategies to optimize H_2 production in photosynthetic microorganisms. Integration of metabolic pathways is coordinated by complex mechanisms that regulate photosynthetic performance to distribute reducing power for bioproduct and H_2 synthesis. Currently, we believe that cost-effective technologies have not yet been developed. Many efforts are still needed to produce microbial H_2 by photosynthesis.

5. Conclusions

In this study, the calcium alginate immobilization technique was used to evaluate the responses of operating conditions of immobilized *Rhodopseudomonas* cells in relation to photofermentative H_2 production. This work was an attempt to compare the photofermentation process, using immobilized purple bacterial cells, in two types of PBR. The ability of immobilized *Rhodopseudomonas* to produce H_2 in two PBRs with different volumes was effectively verified. The results suggest that although immobilization seems to be promising, further research is needed in the field of PBRs.

H_2 production technologies are constantly improving to minimize dependence on limited fossil fuels, mitigate climate change, and keep the

planet healthier. The design of PBRs and their efficiency in capturing solar radiation are very important aspects that could play a role in producing clean energy like H_2 in the near future. However, more research is needed to improve their industrial sustainability and commercialization. For example, improving the light conversion efficiency and reducing the costs associated with nutrient supply can make the process more profitable.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

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