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Immobilization Techniques of *Rhodobacter Sphaeroides* O.U. 001 in Hydrogen Generation

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1 Introduction

Among many alternative routes of hydrogen generation, the microbiological ways belong to the most promising ones. Microbiological methods of hydrogen generation consist of biophotolysis of water, indirect biophotolysis, “dark” fermentation, and photofermentaion [1]. Literature data and our earlier results showed that the application of photoheterotrophic bacteria in hydrogen generation processes can lead towards designing installations on a larger scale and, as a consequence, hydrogen generation on a small industrial scale.

The present paper describes an application of purple non-sulfur *Rhodobacter sphaeroides* O.U. 001 bacteria (PNS), in photofermentative hydrogen generation. These photohetrotrophic bacteria require for growth, not only light, but also simple organic substances [2]. Appropriate growth is assured under anaerobic conditions with a pH close to 7, temperature range of 27-32 ^{o}C and in the absence of ammonium ions. The presence of these ions as well as molecular nitrogen inhibits hydrogen evolution by transformation of ammonium ions and reduction of N_2 to NH_3 by nitrogenase [3]. In contrast, in the absence of nitrogen in this system, nitrogenase catalyzes the reduction of protons to molecular hydrogen [3].

It is important to increase the laboratory scale of the described process into the industrial one. Therefore, a cheap method of separation of liquid medium from bacteria is required. This can be achieved by immobilization of the bacterial culture. The immobilized microorganisms show higher catalytic stability, higher tolerance towards toxic substances or inhibitors and shorter lag phase [4]. Application of the appropriate photobioreactor operating under the continuous and sterile conditions during hydrogen generation is the key point in the efficient generation of hydrogen using the microbiological way. Therefore, the main goal of the presented paper was focused on the design of a new photobioreactor operating with an immobilized culture. Here, different materials were applied for immobilization: Ca-alginate, PVA and large pore silica aerogel. Both systems were deposited onto glass plates or entrapped between polymeric nets. The present paper presents, however, the results obtained for calcium alginate and PVA only. The stability of the system was tested.

2 Materials and Methods

2.1 Inoculum and medium

Photohetrotrophic bacteria *Rhodobacter sphaeroides* O.U.001 (ATCC 4919) were cultivated on Van Niel’s medium containing: K_2HPO_4 (1.0 g/l), $MgSO_4$ (0.5 g/l), yeast extract (10 g/l) and tap water filled up to 1l and then activated according to the procedure already described [5]. For hydrogen generation a modified Biebl and Pfennig medium [6] was applied as reference. Medium was sterilized at 120°C by autoclaving for 20 minutes. In all experiments

temperature was 28 ± 2°C and pH after sterilization varied between 7.0 and 7.2. The mercury-tungsten lamp (300W Ultra-Vitalux from Osram) was applied in all experiments. The intensity of illuminance was 9 klx (116 W/m^2).

2.2 Immobilization techniques

2.2.1 Ca-alginate

The *Rhodobacter sphaeroides* O.U.001 inoculum after centrifugation (12000 rpm for 7 min) was introduced into sterilized solution of sodium alginate. The initial concentration of sodium alginate solution was 5 wt. %, whereas sterilization was performed at 120 ^{o}C for 20 minutes. After introduction of inoculum the final concentration of sodium alginate reached 4 wt. % value. This solution was next introduced into calcium chloride (2 vol. %) solution . After 30 minutes of the crosslinking process, the formation of insoluble gel of calcium alginate was observed [7,8]. Finally the obtained materials were washed with sterile water. Three methods of gel formation were applied:

- injection of bacteria containing alginate solution into dissolved $CaCl_2$ with simultaneous formation of round spheres,
- deposition of bacteria containing alginate solution onto glass plates (sodium glass) followed by immersion of these plates in solution of Ca^{2+} ions,
- similarly, as above but deposition of alginate occurred between two polymeric nets.

2.2.2 PVA crosslinked with boric acid

Medium containing bacteria was centrifuged in similar manner as above and introduced into sterilized polyvinyl alcohol (PVA) solution (13.5 vol.%, av. M_w - 130,000, 99+% hydrolyzed, Aldrich) until concentration of PVA of 9 vol. % was achieved. The obtained suspension was stirred mechanically and transferred into saturated solution of boric acid. The obtained gel [9] after crosslinking and sedimentation (30 minutes) was transferred into 0.5 M solution of NaH_2PO_4. Here the phosphorylation of PVA occurred [10]. This method was applied only for polymeric nets due to difficulties of round spheres formation and lack of adhesion properties on glass plates. Distances between particular fibers in the net were 2mm whereas thickness of these fibers was close to 0.5mm.

2.3 Photobioreactors

2.3.1 Preliminary experiments

The preliminary experiments were performed in small vials made of sodium glass with capacity of 60 cm^3. The working volume was 30 cm^3 whereas remaining volume served for gas collection. The constant concentration of bacterial cells equal 0.576 g biomass/l medium was applied in all experiments but with different volume ratio of alginate to medium (1:14, 1:5, 1:2 and 1:1). Experiments were performed in periodical system: medium was exchanged in each set of experiments whereas bacteria deposited inside calcium alginate spheres represented constant value. In all studied systems the medium was initially degassed with argon after replacement.

2.3.2 Flat plate photobioreactor with immobilized culture (FP PBR with IC)

The main body of modified flat plate photobioreactor was made from stainless steel, (Figure 1) and side walls were made from sodium glass. Seal integrity of the reactor was protected with Viton™ o-rings. Occasionally the system was cooled with water flowing through stainless steel walls. The working capacity of this reactor was 200 ml. The immobilized bacteria were placed on glass plates or between two parallel polymeric nets (dimensions 10 x 10.5 cm). Glass plates were the support for 10 cm^3 of calcium alginate containing different concentrations of bacteria (0.029, 0.087, 0,116 g/l medium). In the case of the polymeric nets the concentration of bacteria cells was constant but the volume of alginate deposited was 10 cm^3 and 20 cm^3 in the case of PVA. System was deoxygenated in stream of argon after each exchange of medium.

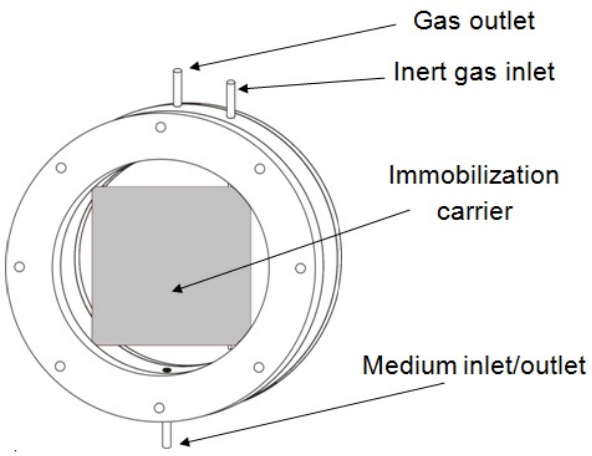

Figure 1: Flat Plate Photobioreactor with Immobilized Culture scheme.

2.4 Analytical methods

Gas metabolites were measured with gas chromatography (Varian GC-3800 equipped with Carboplot P7 capillary column and TCD) [5]. The amount of gaseous products was measured by a volumetric method applying constant detection of the gas volume evolved with a computerized camera. Each cycle of the exchange of liquid medium was completed with pH value measurements. The amount of the evolved cells from immobilizing medium was determined spectrophotometrically, measuring absorption at 660 nm (UV-Vis- Beckman DU 640).

3 Results and Discussion

The experiments performed with immobilized bacteria proved that this technique allows for multiple applications of the same bacterial cells. In all cases the observed, relatively long, lag phase at the beginning of the experiment (directly after immobilization) is significantly shorter in the subsequent replacements of the medium. This indicates a good acclimatization of microorganisms in the porous structure of the immobilizing material. However, in all studied cases the immobilized materials showed decomposition with time, and simultaneous

evolution of cells into the medium (see Figure 2). The preliminary experiments with alginate spheres showed that the best stability of the immobilized bacteria can be reached with samples containing the highest concentration of microorganisms (1:1 – up to seven cycles). Lower concentrations of bacteria in alginate resulted in higher hydrogen productivity due to a better diffusivity of hydrogen but shorter lifetime. Faster hydrogen generation causes faster formation of cracks and finally easier decomposition of the immobilized material.

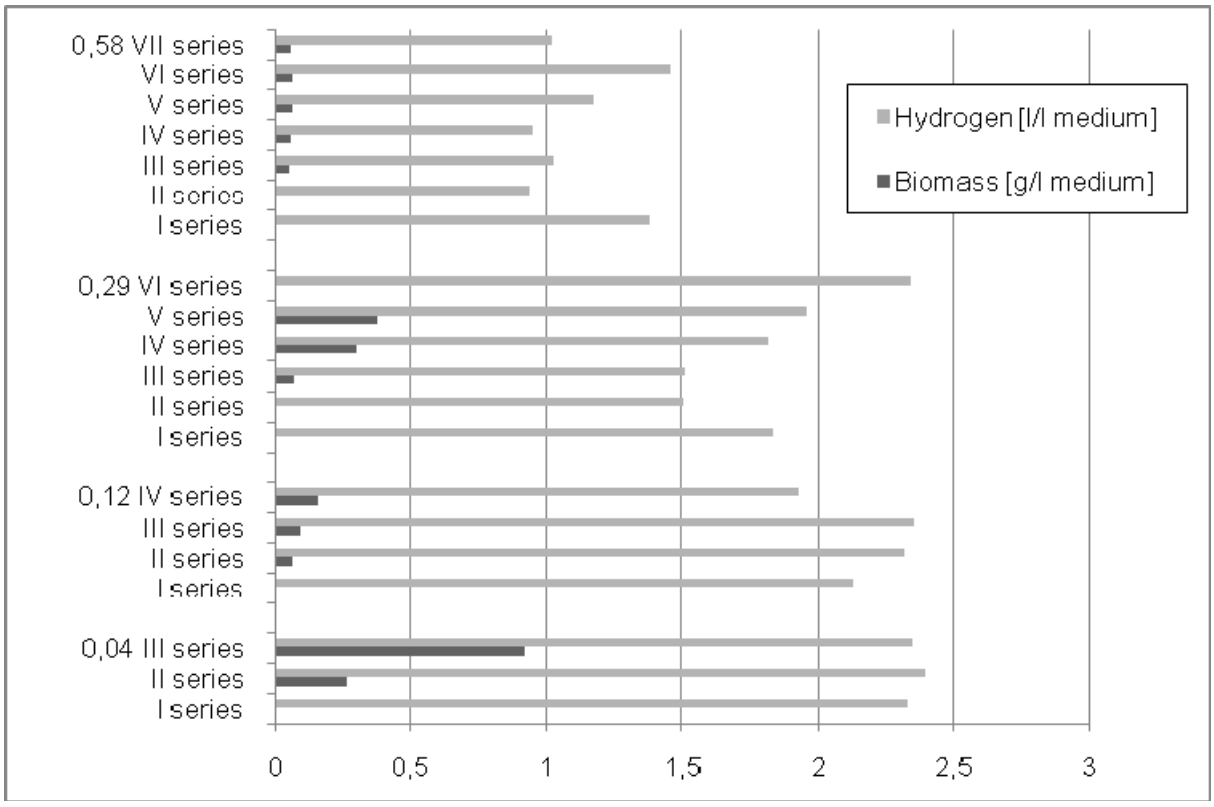

Figure 2: Calcium alginate spheres – maxima of generated hydrogen and concentration of the released cell into the medium. Cell concentrations 0.58, 0.29, 0.12, 0,04 g biomass/l of medium.

Higher activity of the released cells can be explained by better access to substrates and illumination. The access of light for immobilized bacteria is always limited in deeper parts of the alginate spheres. The observed effect leads to the conclusion that the rate of hydrogen evolution in the case of alginate can significantly influence the stability of the immobilized systems. An application of the alginate system deposited on glass plates and FP PBR reactor the best results were obtained with cell concentrations 0.058 and 0.087 g biomass /l of medium (Figure 3).

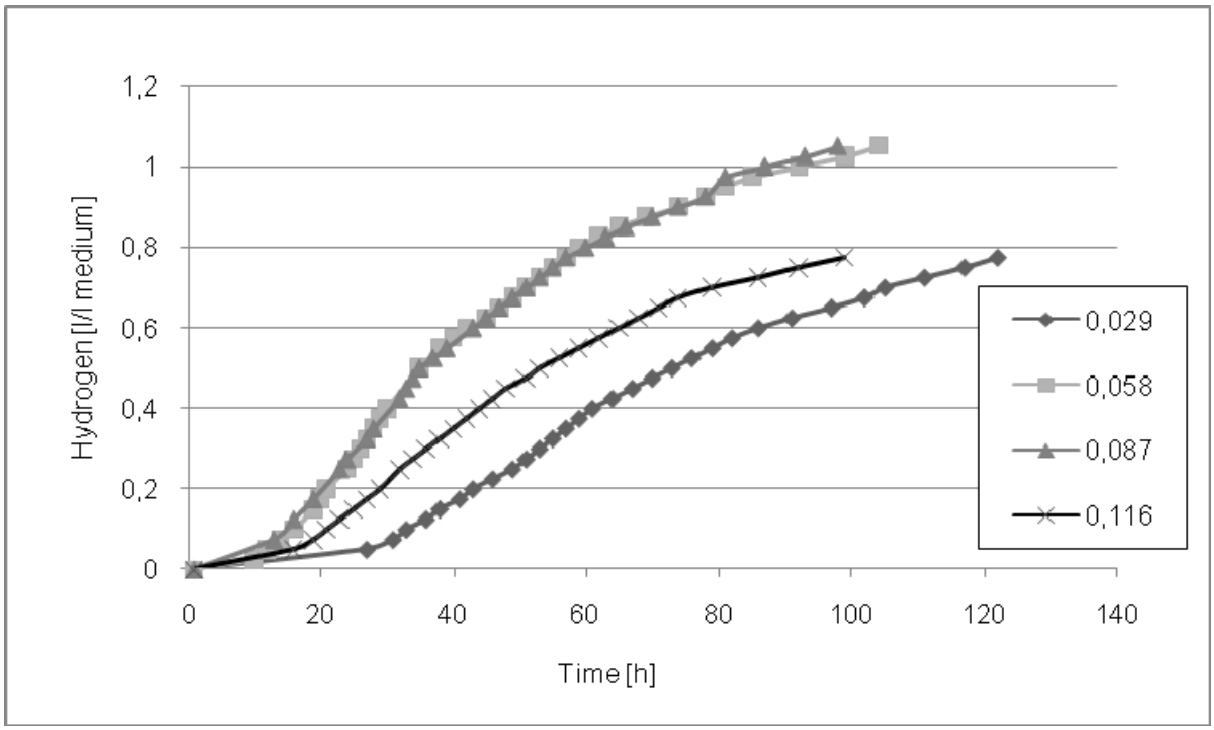

Figure 3: Calcium alginate deposited on glass plate. Different concentrations of immobilized cells.

Low productivity of hydrogen was found at the highest concentration of bacteria in alginate (0.116 g biomass/l medium). This can be related to the autoinhibition process [11]. An application of alginate immobilized on glass plates resulted in much lower stability than with the round spheres. The immobilized material could “survive” only two exchanges of medium. Therefore, new method of alginate method was applied. Here, alginate, with optimized concentration of bacteria (0.058 g biomass/l medium), was located between two parallel polymeric nets. This construction provided much better stability, and relatively high hydrogen generation. After the first run the amount of hydrogen was 0.8 lH_2/l medium and in subsequent runs this amount was close to 1.2 lH_2/l medium (see Figure 4). Much better productivity (about 30 %) can be explained by acclimatization of the bacterial strain as well as partial elution of bacterial cells into the medium. Separation of liquid medium was possible up to 4 runs. After 4 runs the stability of the system was very poor.

The search for new, more stable materials for immobilization showed that PVA is the more stable one. First run with high concentration of entrapped bacteria (0.116g biomass/l medium) indicate low productivity of hydrogen and long lag time phase (see Figure 5).

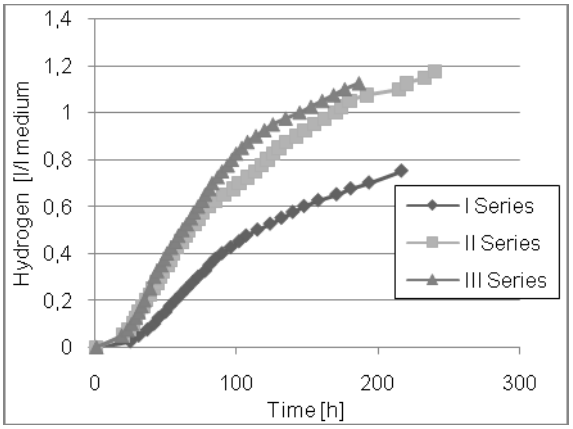

Figure 4: Bacteria immobilized in Ca-alginate located between polymeric nets.

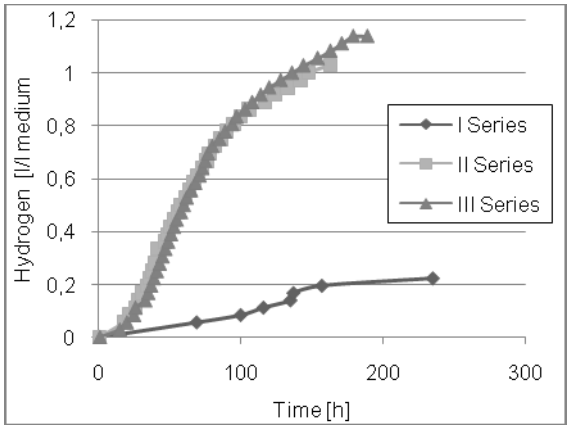

Figure 5: Bacteria immobilized in PVA located between polymeric nets.

This can result from adaptation of bacteria in this new material as well as from transfer of H_3BO_3 into the medium. The amount of photobiogenerated hydrogen was almost the same but stability of the system with PVA appeared to be much better.

The obtained results showed that application of polymeric nets significantly prolong the lifetime of the immobilized bacterial systems. Moreover, it appeared that crosslinked with boric acid and subsequently phosphorylated PVA is much better material for immobilization of bacteria. The amounts of photogenerated hydrogen is identical when alginate gels were applied.

Acknowledgements

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