BIOHYDROGEN PRODUCTION BY PHOTOFERMENTATION OF LACTIC ACID USING *THIOCAPSA ROSEOPERSICINA*

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Hidrogenul obținut prin procese biologice este denumit biohidrogen. Procesele biologice de producere a hidrogenului prezintă anumite avantaje, cum ar fi operare în condiții blânde, selectivitate ridicată şi posibilitatea utilizării surselor regenerabile, dar au şi dezavantaje, precum eficiența scăzută a conversiei şi costurile ridicate ale operării fotobioreactorului. Scopul nostru este studierea fenomenului de obținere a biohidrogenului din acidul lactic, specific subproduselor industriei laptelui, prin fotofermentație cu bacterii fotosintetice. În această lucrare sunt prezentate caracteristicile definitorii ale bacteriilor Thiocapsa roseopersicina, precum şi acele puncte critice, pe care trebuie să se pună accentul în proiectarea şi construirea unui fotoreactor inovativ.

The biologically produced hydrogen is defined as biohydrogen. Biological hydrogen production methods offer distinct advantages, such as operation under mild conditions, high selectivity and the possibility of using renewable sources, but have also some disadvantages, such as low conversion efficiency and high operating cost of photobioreactors. Our goal is to study photofermentative biohydrogen production with photosynthetic bacteria from lactic acid, also found in dairy wastewater. The focus of this paper is on the essential characteristics of Thiocapsa roseopersicina and the critical features which have to be taken into consideration when designing and constructing a novel, improved photoreactor.

Keywords: biohydrogen, photofermentation, photoreactor

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1. Introduction

Hydrogen is considered to be the „energy of future", since it is a clean source with high energy content (122 MJ/kg) [1], its combustion produces water, which is not detrimental to the environment. It is a secondary source of energy (an energy carrier) which is used to move, store and deliver energy in an easily usable form. Therefore, future energy technology will use hydrogen with an increasing trend in steady as well as unsteady combustion processes [2]. As part of this increasing trend, new processes need to be developed for cost efficient hydrogen production.

The best known industrial methods for hydrogen production include steam reformation of natural gas, coal gasification and splitting water with electricity typically generated from carbonaceous fuels. These energy-intensive industrial processes release carbon dioxide and other greenhouse gases and pollutants as byproducts [2].

Biological hydrogen production technologies are still under development, they provide a wide range of approaches, including direct biophotolysis, indirect biophotolysis, photofermentation, dark fermentation or a combination of these processes [2, 3]. Biological methods offer distinct advantages for hydrogen production, such as operation under mild conditions (at ambient temperature and pressure), high selectivity and the possibility of using renewable sources, like biomass and/or some food industry wastewaters which contain high amount of carbohydrates and organic acids, no unwanted and hazardous byproducts are formed, no special equipment is needed, in brief they are "ecofriendly" processes.

The hydrogen produced by bacteria (biologically) is called biohydrogen, a renewable biofuel produced from biorenewable feedstock through chemical, thermochemical, biological, biochemical and biophotolytical methods [2].

Microbial hydrogen production is made possible by fermentative (*Clostridia* sp., methanogens, archaebacteria etc.) or photosynthetic (purple sulphur bacteria, non-sulphur bacteria, green algae) bacteria. Anaerobic bacteria use organic substances as the sole source of electrons and energy, converting them into hydrogen. The cyanobacteria directly decompose water to hydrogen and oxygen in the presence of light energy through photosynthesis. Photosynthetic bacteria use organic substrates, like organic acids, in the presence of light, as well. In all cases, the hydrogen production is intimately linked with the respective energy metabolism [2, 4, 5, 6].

Hydrogen-intensive research work has already been carried out on the advancement of these processes, such as the development of genetically modified microorganisms, metabolic engineering, improvement of the reactor designs, use of different solid matrices for the immobilization of whole cells, biochemical

assisted bioreactor, development of two-stage processes etc., for higher H_2 production rates [2, 7].

The combination of dark fermentation and photofermentation seems to be an ideal biohydrogen producing model leading to the highest theoretical and practical H_2 yield [1, 8]. Through the process of dark fermentation, acidogenic or thermophile bacteria produce from organic substrates hydrogen, volatile fatty acids and alcohols, which can be consumed by photosynthetic bacteria in the photofermentative phase [7, 9, 10]. In such systems, the photofermentation constitutes the limiting step for the overall hydrogen producing process, because it has poor hydrogen production rate due primarily to slow growth of photosynthetic bacteria and low light conversion efficiency of photobioreactors [1]. Therefore, pilot plant experiments of the photofermentation processes require more attention. The use of cheaper raw materials and efficient biological hydrogen production processes will surely make them more competitive with the conventional H_2 generation processes in the near future [2].

The aim of our study is the examination of the photofermentation process at laboratory scale, in order to elaborate a whole technology for biohydrogen production from cheap raw material, namely from acid whey.

2. Biohydrogen production using *Thiocapsa roseopersicina*

Photofermentation is the fermentative conversion of organic substrate to biohydrogen manifested by a diverse group of photosynthetic bacteria through a series of biochemical reactions involving three steps similar to anaerobic conversion. Photofermentation differs from dark fermentation because it only proceeds in the presence of light.

The purple sulphur bacteria are a group of *Proteobacteria* capable of photosynthesis because they have photosynthetic pigments. They are anaerobic or microaerophilic, and are often found in hot springs or stagnant water. Unlike plants, algae and cyanobacteria, they do not use water as their reducing agent, and consequently, do not produce oxygen. Instead, they use hydrogen sulphide or other reduced sulphur compounds as electron donor, which is oxidized to produce granules of elemental sulphur, which become visible in cells.

One of the purple sulphur photosynthetic bacteria is the *Thiocapsa roseopersicina*, which is an anaerobic photoheterotroph microorganism. It employs light as primary energy source, the growth of bacteria in the media containing reduced sulphur compounds increases in the presence of some organic substrates, especially lactate, pyruvate, glycerol and glucose [11]. The bacteria are able to produce hydrogen through several reversible hydrogenases [12], or rather, it possesses nitrogenase activity, too, and atmospheric N_2-fixing is accompanied by H_2 production [13]. We have chosen this bacterium for biohydrogen

production due its unique characteristic, namely that in a single cell there are four distinct NiFe hydrogenases [14].

The studied *Thiocapsa roseopersicina* BBS strain (obtained from the University of Szeged, Department of Biotechnology, Hungary) has been isolated from the cold water of the North Sea, accordingly the cultivation of the strain is possible only below 30ºC, preferably at 25 – 28ºC [13, 14]. For growth in culture medium they demand mineral salts (NaCl, KH_2PO_4, $MgCl_2$, KCl, NH_4Cl, $NaHCO_3$, $Na_2S_2O_3$), trace elements (mostly Fe and Ni) and vitamin B_{12}. The initial pH has to be 7.0 – 7.5.

For hydrogen production we intend to use as substrate sweet or acid whey, the byproduct of cheese industry, which beside other fermentative compounds, contains from 1g/L to 5g/L of lactic acid with pH values around 6.0 and 4.5, respectively. Therefore, we have to complete the substrate with compounds listed above and we have to adjust the pH to 7.0 – 7.5.

Kondratieva et al. (1976) described that *Thiocapsa roseopersicina* BBS strains are able to change from phototrophy to aerobic chemolitoautotrophy in the dark under aerobic conditions. In this case, they require only vitamin B_{12} as organic compound. The cells oxidize sulphide and thiosulphate to molecular sulphur and then to sulphate, using O_2, and assimilate carbon dioxide. The growth of strains under such conditions is slower than in light, from 7 up to 12 days are needed to obtain a considerable yield of cells. In illuminated anaerobic conditions the cultures reach the stationary phase after 3 – 4 days [11].

Although dark aerobic conditions are less favorable for growth than illuminated anaerobic conditions, the capacity for such a change of metabolism may be of importance for the survival of these phototrophic bacteria in the absence of light and usable organic compounds [11].

Thiocapsa roseopersicina belongs to the *Chromatiaceae* family; these bacteria harbor as the main light-harvesting pigment bacteriochlorophyll *a*. The main absorption of bacteriochlorophyll *a* occurs beyond the limit of visible light, in the near-infrared (NIR) range, hence the colour of these bacteria is given predominantly by the optical activity of carotenoids, of which spirilloxanthin is the most abundant in *Thiocapsa roseopersicina,* so the culture has a rose colour [15].

Specific absorption coefficients of *Thiocapsa roseopersicina* at wavelengths of 480, 520, 550, 580, 860 and 880 nm were determined (Fig. 1-a). In case of growth with low aeration in the dark, the cultures had a rose colour; with high aeration they were colourless. Figure 1-b shows the absorption spectra of cells grown in the dark with low aeration; in this case the cells synthesize carotenoids and bacteriochlorophyll, although the pigment content is considerably smaller than in cultures grown in the light. In cultures grown in dark under high aeration only traces of bacteriochlorophyll could be found (Fig. 1-c) [11, 15].

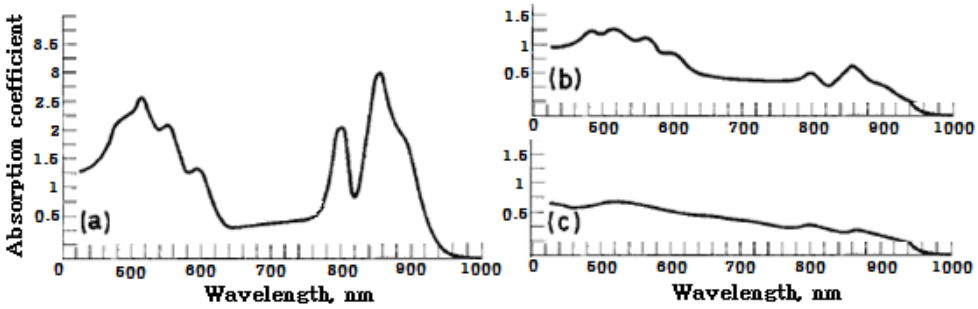

Fig. 1. The absoption spectra of *Thiocapsa roseopersicina* cells grown under different conditions: (a) anaerobic, light; (b) low aeration, dark; (c) high aeration, dark

It has been described that bacteriochlorophyll *a* and carotenoid synthesis is exclusively restricted to anoxic-dark periods, do not occur during oxic-light periods. When anoxic conditions were maintained throughout, bacteriochlorophyll synthesis occurred both during light and dark periods. The continuous presence of oxygen results in colourless cells and very much reduced yields due to chemotrophy [16].

The practical application of these strains for hydrogen production is limited by the difficulty of supplying light efficiency to/in photobioreactors.

3. Engineering a novel, improved photobioreactor for the cultivation of *Thiocapsa roseopersicina* and biohydrogen production

For hydrogen production purposes the following types of bioreactors have been used: vertical column reactors, tubular ones and flat panel photobioreactors. These reactors must comply with the following requirements: should be enclosed systems so that the produced hydrogen gas may be collected without any loss, the reactor design must allow sterilization with convenience and ease, and because light is needed for growing the strains, the illumination must be provided from inside or outside the reactor. Because light can not be stored in the reactor, it must be supplied continuously. Accordingly, the photobioreactor design should provide large surface to volume ratio in order to maximize the area of incident light [17, 18].

Among all the light sources available, solar light energy is the most abundant natural light source on earth, and has the following advantages: it is cost free and contains the full spectrum of light energy [1]. But, the cultivation of photosynthetic bacteria using sunlight as illumination source is usually carried out in open ponds, in which is difficult to control the other culture conditions

(temperature, light intensity), the eventual contamination, and it requires large areas. In conclusion, these outdoor systems lead usually to poor performances.

As a solution for this problem, the photosynthetic bacteria can be cultivated in closed systems, in indoor photobioreactors, which are usually more efficient and easier to control. But these indoor photobioreactors have disadvantages too, namely high power consumption and high operation costs due to the need of artificial light sources [1].

Various types of photobioreactor with high illumination to volume ratios have been proposed, but most are limited by cost, mass transfer, contamination, scale-up or a combination of these [17].

In order the cultivated bacterial strains to use the most energy emitted by the artificial light source (to decrease the energy loss), we have proposed to design an internally illuminated, cylindric photoreactor for cultivation of *Thiocapsa roseopersicina* strains (Fig. 2).

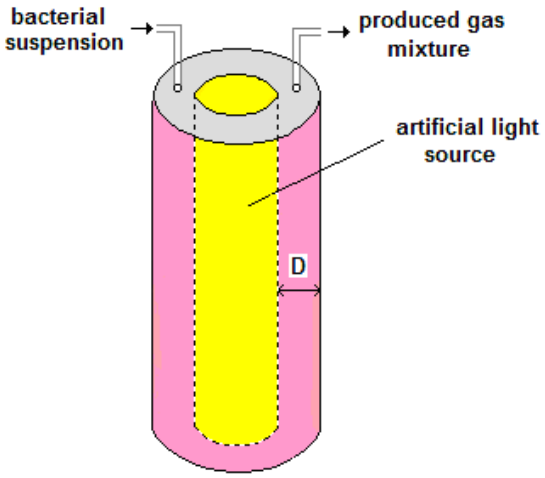

Fig. 2. Internally illuminated photoreactor (draft)

At first, we tried to solve the illumination of the culture with a fluorescent tube (T8 15W 438MM WHITE, luminous flux 900 lumen), which is a gas-discharge lamp that uses electricity to excite mercury vapor. The excited mercury atoms produce short-wave ultraviolet light that then causes phosphor to fluoresce, producing visible light. We concluded that these sources of light do not emit that quantity of light energy in the near-infrared spectrum that the photoheterotroph bacteria require.

Another possibility was the tungsten filament lamps, which emit the corresponding spectrum of light, but these lamps have an important drawback: they generate very much heat and higher temperatures (above 28 – 30ºC) destroy

the studied bacterial strains. Although it could be a viable approach, the use of such light sources to illuminate internally the bacterial culture is not recommended, because it is very difficult to cool the system, to keep it at low temperature.

As for the light source, a viable approach seems to be the LED tube (Light Emitting Diode tube), which emits the corresponding spectrum of light (containing several white LEDs) and generates almost no heat. LEDs are small and highly efficient light sources, their use in illumination systems resulted in high densities and photosynthetic activities of cells [19].

Self-shadowing raises another difficulty regarding the cultures of photosynthetic microorganisms. When microbial cell concentration is high, the light penetration is low, i.e. when light passes through a dense culture, the irradiance decays along the depth. Therefore, it is important to take into consideration a basic analysis on light distribution and light penetration [5].

Another question in photoreactor design was that of how the light intensity (which is emitted by the light source installed in the center of the reactor) decreases while passes through the bacterial suspension. To answer this question, we measured with the help of a spectrophotometer (VARIAN CARY UV-VIS Spectrophotometer) the absorbance (A) of a dense culture of *Thiocapsa roseopersicina* at 600 nm. The *Thiocapsa roseopersicina* culture was prepared by growing the strains under anaerobic conditions in 5 mL PC culture medium (containing in 1 L 20g NaCl, 1g KH_2PO_4, 1g $MgCl_2$, 1g KCl, 1g NH_4Cl, 2g $NaHCO_3$, 4g $Na_2S_2O_3$, 20µl of 1000µg/mL B_{12} vitamine, 1mL trace elements solution, 1mL Fe-EDTA) for 5 days at 25 – 28ºC. The growth medium was illuminated using a tungsten lamp (Tungsram, 60 W, 700 lumen). The average value of the measured absorbance of the 1 cm thick bacterial culture was equal to 2.4.

Using the Lambert-Beer equation (eq.1) we can calculate the variation of light intensity knowing that the entrance light intensity that passes trough the culture is 1500 W/m^2 at 600 nm:

$$A = \log_{10} \frac{I_0}{I} \tag{1}$$

where: A – absorbance; I_0, I – entrance and exit light intensity, W/m^2.

So, the light intensity after passing trough 1 cm thick bacterial suspension is (eq.2):

$$I = \frac{I_0}{10^A} = \frac{1500}{10^{2.4}} = 5.97 \text{ W/m}^2. \tag{2}$$

Similarly to equation 2, can be calculated the intensity of light which passes in the second 1 cm layer of the culture (eq.3). In this case, the entering

light intensity is considered to be equal with the one leaving the first one. The calculated light intensity after passing through the 2 cm thick culture (I_{2cm}) is:

$$I_{2cm} = \frac{I}{10^{A}} = \frac{5.97}{10^{2.4}} = 0.023 \text{ W/m}^2. \tag{3}$$

The obtained intensity value shows that the distance between the two cylinders (D, see Fig.2), where the culture of bacterial strains will be found, can not be more than 2 cm, because after this point the bacteria do not get light energy, they will overshadow each other.

By increasing the entering light intensity is possible to increase the distance between the two cylinders to more than 2 cm, but this increase is not significant. Additionally, higher light intensities either inhibit the bacterial growth, or a part of the energy will not be used, the effect of light intensity on photosynthetic bacterial growth being described by a saturation growth curve (Fig. 3). The saturation growth curve is characteristic for many species and depends on the light source used. The essential feature of this curve is that it behaves as a first-order response at low intensities and zero order at high light intensities. The first-order part, featuring a linear increase in specific growth rate (μ) with light intensity, is light-limited. At higher values of intensity the curve becomes flat, because growth is light-saturated. In Fig. 3, the values of light intensity corresponding to the maximum observed specific growth rate are those for which the culture is light saturated. As presented in Fig. 3, when the intensities are higher, the excess light is not used [17].

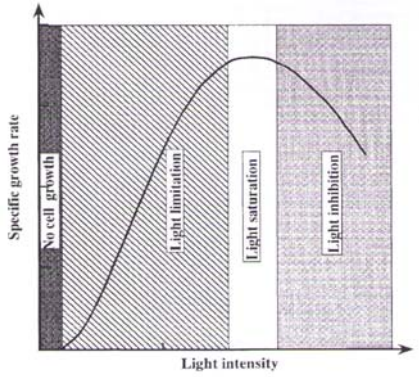

Fig. 3. Effect on light intensity on growth of photosynthetic cells

Photosynthetic H_2 production requires more sophisticated closed bioreactors to maintain anaerobic conditions and to collect the H_2 gas. Depending on their complexity, however, closed systems do have the advantage that they assist in maintaining sterility and optimal culture parameters, such as light levels, CO_2 and nutrient supply, temperature [6].

4. Conclusions

The cultivation of photosynthetic bacteria for hydrogen production purposes is only possible in photobioreactors. For the cultivation of the selected hydrogen producing, purple sulphur bacteria *Thiocapsa roseopersicina is* very important to ensure the appropriate conditions in the photoreactor, namely anaerobic conditions by introducing nitrogen gas, temperature below 30ºC, near-infrared range of light and substrate for growth. Beside the presented critical features, there are other conditions that contribute to the successful operability of a photoreactor, such as: full pH-control, uniform heating/cooling, permanent sterility. Considering the discussed starting points (to ensure adequate light source, temperature, light intensity at each point of the reactor), we conclude that a photoreactor engineered on the bases here set, should be suitable for the cultivation of *Thiocapsa roseopersicina* strains and for the biohydrogen production.

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