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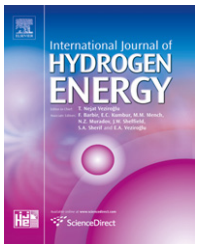

Brewery wastewaters in photobiological hydrogen generation in presence of *Rhodobacter sphaeroides* O.U. 001

*K. Seifert, M. Waligorska, M. Laniecki**

Faculty of Chemistry, A. Mickiewicz University, Grunwaldzka 6, 60-780 Poznań, Poland

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ABSTRACT

Rhodobacter sphaeroides O.U. 001 (concentration of inoculum-0.36 g dry wt/l) and brewery wastewaters were applied in photobiogeneration of hydrogen under illumination of 116 W/m^2. The best results were obtained with filtered wastewaters sterilized at 120 °C for 20 min and maximal concentration of waste in medium equal 10% v/v. The main product in generated biogas was hydrogen (90%). After sterilization the amount of generated hydrogen was tripled (from 0.76 to 2.2 l H_2/l medium), whereas waste concentration of 10% v/v resulted in the best substrate yield (0.22 l H_2/l of waste). Under these conditions the amount of generated hydrogen was 2.24 l H_2/l medium and light conversion efficiency reached value of 1.7%. The modified Gompertz equations served in modeling of the kinetics of the studied process.

1. Introduction

The decomposition of organic compounds in presence heterotrophic bacteria under illumination of visible light with simultaneous evolution of hydrogen and carbon dioxide is known as photofermentation process. The ideal substrates for this process can be organic wastes with low concentration of total nitrogen (both organic and inorganic). It is well known from literature that purple non-sulfur bacteria of *Rhodobacter sphaeroides* O.U. 001 are very efficient biocatalyst in hydrogen generation process from wastes originating from food, dairy, sugar or alcohol-distilling industry [1–5]. A very high concentration of organic substances (average Chemical Oxygen Demand-COD: 0.8–2.5 kg/hl) in wastes from breweries suggests an application of these wastes in hydrogen generation. The amount of waste during beer production is enormous and equals the amount of water applied for production diminished with water present in beer (usually 3–4 hl of waste per 1 hl of beer). A chemical composition of waste strongly depends on the kind of beer produced and fermentation degree. Such waste can contain aminoacids, proteins, organic acids, sugars, alcohols, as well as vitamins of the B group [6]. All these substrates can be efficiently used in photobiological hydrogen production [7,8].

Constantly increasing demand for energy requires search for new sources and methods of generation. Utilization of waste organic material makes biological hydrogen production a novel promising approach to meet the increasing needs for energy. The US Department of Energy Hydrogen Program in United States estimates that contribution of hydrogen to total energy market will be 8–10% by 2025 [9]. The production of hydrogen from renewable sources, known as ''green technology'' has received considerable attention in recent years. It is predicted that hydrogen will become the main carrier of energy in the near future due to environmental and universal applications reasons. It is clean, highly energetic energy carrier (142.35 kJ/g), with almost tripled gravimetric energy density compared to

* *Corresponding author*. Tel.: +48 61 8291339; fax: +48 61 8291505.
E-mail address: laniecki@amu.edu.pl (M. Laniecki).

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ordinary hydrocarbons. Today, besides well-known industrial methods of hydrogen generation, the biological generation of hydrogen represents the most intensively researched and developed area. In the photofermentation the yield of biologically generated hydrogen depends on nitrogenase activity and its final amount is the compromise between hydrogen generated and consumed by hydrogenase in oxidation process [10]. Although the described method is relatively simple and cheap it still requires optimization due to the obtained unsatisfied yields.

This paper presents the research performed with *R. sphaeroides* O.U. 001 and brewery wastes in photobiological hydrogen generation. It was found the influence of the amount of as waste in medium, a method of waste pretreatment, concentration of sodium glutamate, biomass increase and changes of COD and pH on the amount of generated hydrogen.

2. Materials and methods

2.1. Inoculum, medium and procedures

Phototrophic bacteria *R. sphaeroides* O.U. 001 (ATCC 4919) was 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 1 l and then activated according to the procedure already described [9]. For hydrogen generation a modified Biebl and Pfennig medium [12] was applied as the reference. This standard medium contained following compounds (g/l): KH_2PO_4 0.5; $MgSO_4 \cdot 7H_2O$ 0.2; NaCl 0.4; $CaCl_2 \cdot 2H_2O$ 0.05, L-malic acid 2.0; sodium glutamate 0.36, iron citrate 0.005; yeast extract 0.17 and microelements: $ZnCl_2$ 0.07; $MnCl_2 \cdot 4H_2O$ 0.1; H_3BO_3 0.06; $CoCl_2 \cdot 6H_2O$ 0.2; $CuCl_2 \cdot 2H_2O$ 0.02; $NiCl_2 \cdot 6H_2O$ 0.02; $NaMoO_4 \cdot 2H_2O$ 0.04; HCl 25% (1 ml/1).

The untreated brewery waste from local brewery was filtered through cotton wool, next sterilized at 120 °C by autoclaving for 20 min and re-filtered applying paper filter. In all experiments, except the reference experiments with standard medium, the pretreated liquid waste in concentration varying between 1 and 20% v/v was added into Biebl and Pfennig medium replacing malic acid. The medium was inoculated with bacteria 30% v/v (0.36 g dry wt/l) and next was cultivated for 12 h. The process was performed in small vials (25 ml) made from sodium glass and filled with 12.5 ml of inoculated medium. Tightly closed vials were carefully deaerated with argon before starting the illumination. In all experiments the temperature was 28 ± 2 °C and pH after sterilization and inoculation varied between 7.0 and 7.2. Certain experiments were performed in the absence of sodium glutamate (raw waste).

The mercury–tungsten lamp (300 W Ultra-Vitalux from Osram) was applied in all experiments. The intensity of illuminance was 9 klx (116 W/m^2). In "day–night" experiments samples in cycles were illuminated 12 h, followed by 12 h in the light absence, till the process was completed. All experiments were performed in two series with three samples.

In order to establish the influence of organic nitrogen concentration on the effectiveness of hydrogen generation the amount of sodium glutamate was changed twice.

2.2. Analytical methods

The content of H_2 and CO_2 were measured with gas chromatography (Varian GC-3800 equipped with Carboplot P7 capillary column and TCD) [11].

The loss of organic substances was monitored with COD using the dichromate method after centrifugation of biomass [13]. The biomass content was established spectrophotometrically measuring optical density at 660 nm (DU640 UV–vis spectrophotometer from Beckmann). The samples collected after specified time intervals (10 ml) were centrifugated at 12 000 g for 12 min. The obtained pellets were washed twice with deionized water and dried at 80 °C for 4 h. The cell dry weight was determined using gravimetric method. Total suspended solids (TSS) of the brewery waste were measured with elemental analysis (C,H,N,O) in triplicate using an elemental analyser (Vario EL III Elementary), whereas concentration of Fe, Ca, Mg was measured by ICP OES spectroscopy. The intensity of illuminance was measured at the external wall of the bottles with luxometer Lx204 made by Slandi, Poland and a pyranometer CMP3 by Kipp & Zonen [11].

The modified Gompertz Eq. (1) was applied for calculations of cumulative amounts of hydrogen and carbon dioxide [14–20]:

$$H = H_{max}\exp\left\{-\exp\left[\frac{R_{max,H_2}e}{H_{max}}(\lambda - t) + 1\right]\right\} \quad (1)$$

where: H – cumulative hydrogen (l/l$_{medium}$), H_{max} – maximum cumulative hydrogen (l/l$_{medium}$), $R_{max,H2}$ – maximum rate of hydrogen production (l/l/h), t – photofermentation time (h), λ – lag time (h), e – exp. In the case of CO_2 appropriate values and subscripts for carbon dioxide in the same equation were applied.

The kinetic program Micro Math SCIENTIST from Software Co was applied in modeling of this process. The R^2 coefficient was chosen as an indicator of goodness-of-fit.

The light conversion efficiency (η) is calculated applying following formula [9,21]

$$\eta(\%) = \frac{33.61 \cdot \rho \cdot V}{I \cdot A \cdot t} \quad (2)$$

where "V" is the volume of produced H_2 in liters, "ρ" represents density of the produced hydrogen gas in g/l, "I" is the light intensity in W/m^2, "A" – irradiated area in m^2 and "t" duration of hydrogen production in hours.

3. Results and discussion

3.1. Pretreatment of brewery waste

The characteristic of applied waste is given in Table 1. The COD value of 202 g O_2/l as well as carbon content, indicate that such waste can serve as good medium for applied bacteria. The concentration of the nitrogen compounds is high and it can negatively influence the yield of produced hydrogen [22].

In order to establish the influence of the waste pretreatment conditions on the final production of hydrogen a series of experiments with non-treated and sterilized waste were performed. These measurements were performed with solution containing waste at concentration of 10% v/v inoculated

Table 1 – Characteristic of the brewery wastewater.

Parameters	Value
Ca [mg/l]	37.2
Fe [mg/l]	1.04
Mg [mg/l]	95.8
N [%]	0.669
C [%]	36.74
H [%]	6.98
S [%]	0.045
pH	4.71
COD [g O_2/l]	202
N-NH_4^+ [mg/l]	95.8

with 10% and 30% v/v of inoculum. The results of these experiments shown on Fig. 1 indicate that thermal pretreatment can significantly improve the yield of generated hydrogen. Application of the sterilized waste with concentration of inoculum 10% v/v resulted in doubled amount of produced hydrogen. Triplication was observed at higher concentration of inoculum (30% v/v). Many laboratories apply similar pretreatment conditions. Thermal treatment at 95 °C for 45 min [3], filtration or sedimentation [23] as well as dilution leads towards removal of fermentation bacteria and solid sediments from medium. This type of thermal treatment appears to be the best. It provides better access of light inside photobioreactor and in consequence better hydrogen production.

Considering literature data and results obtained in our preliminary experiments (Fig. 1) it was decided that in further research only sterilized waste containing 30% v/v of inoculum (0.36 g dry wt/l) will be applied.

3.2. *Hydrogen in presence of nitrogen compounds*

Among different types of liquid wastes, the brewery waste contains significant amount of nitrogen compounds both of organic and inorganic origin (see Table 1). The ammonium ions are the source of nitrogen for biomass growth but simultaneously cause the decrease of nitrogenase activity responsible for the yield of generated hydrogen [22,24]. It is known that C/N ratio plays an essential role in photobiologically generated hydrogen [3,10]. Therefore, in the series of experiments it was tested the influence of nitrogen on the yield of obtained hydrogen. This was realized by applying of media containing no sodium glutamate (only sterilized waste) and those with 0.36 and 0.72 g/l of sodium glutamate (equivalent 27 mg N/l and 54 mg N/l, respectively – see Table 2). In all these experiments concentration of brewery waste was equal 5% v/v. Media were inoculated with inoculum containing 0.36 g dry wt/l and illuminated with light intensity of 116 W/m^2.

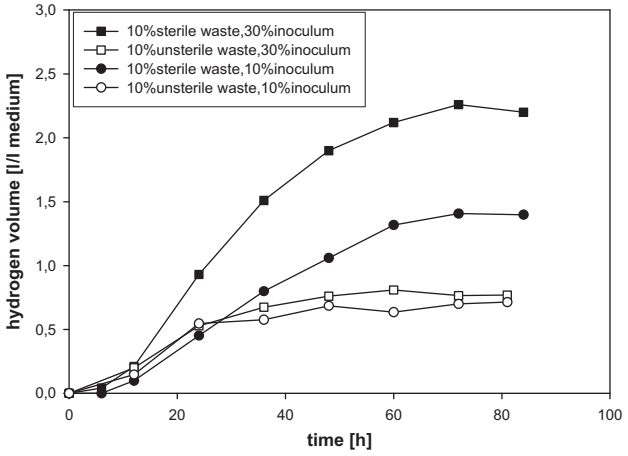

Fig. 1 – Influence of sterilization of brewery wastewaters on kinetics of hydrogen generation.

Data presented in Table 2 shows that change in sodium glutamate concentration does not influence the amount of generated hydrogen. At all studied concentration the amount of evolved hydrogen is very similar (1.5–1.7 l/l). In contrast to hydrogen, the amount of photogenerated biomass increase from 1.4 to 2.6 g dry wt/l as well as the COD values (from 2.5 to 3.3 g O_2/l). These results suggest that the amount of nitrogen compounds present in 5% v/v medium is sufficient for the growth of phototrophic bacteria and does not influence the effectiveness of evolved hydrogen. However, our earlier results [22] showed that an increase of NH_4^+ ions concentration from 1 to 5 mmol/l decreases several times the amount of photogenerated hydrogen. Therefore, an application of waste with concentration equal or higher than 20% v/v (containing >1 mmol NH_4^+/l) can reduce the amount of evolved hydrogen, as well.

3.3. *Waste concentration vs. hydrogen production*

In the previous section it was found that at certain concentration level of waste, namely 5% v/v, the amount of photogenerated hydrogen practically does not depend on concentration of nitrogen compounds in medium. In order to optimize the concentration of waste in medium a series of experiments were performed. Data presented on Fig. 2a show that among brewery waste water containing either 1, 3, 5, 10 or 20% v/v the best results were obtained for medium with 10% v/v. Up to the concentration of 10% v/v, the amount of generated hydrogen increases from 0.86 to 2.24 l per liter of medium, whereas the yield increased from 0.009 to 0.22 l H_2 per liter of waste (see also Table 3). An increase of waste concentration up to 20% resulted in significant decrease of generated hydrogen (0.52 l/l) and the drop in hydrogen yield (down to 0.1 l/l waste). In this case, the inhibition of nitrogenase by ammonium ions is well documented [1]. In medium containing waste with concentration 20% v/v (equivalent of 1.5 mmol NH_4^+/l) in presence of *R. sphaeroides* bacteria a two times lower hydrogen yield, as well as prolongation of lag phase, was observed (Table 3). The

Table 2 – Hydrogen generation in the presence of glutamate.

Concentration of sodium glutamate [g/l]	H_2 [l/l]	Biomass increase [g dry wt/l]	pH final	COD loss [g O_2/l]
0	1.71 ± 0.04	1.43	6.42	2.51
0.36	1.64 ± 0.05	1.92	6.26	2.80
0.72	1.54 ± 0.06	2.58	6.73	3.27

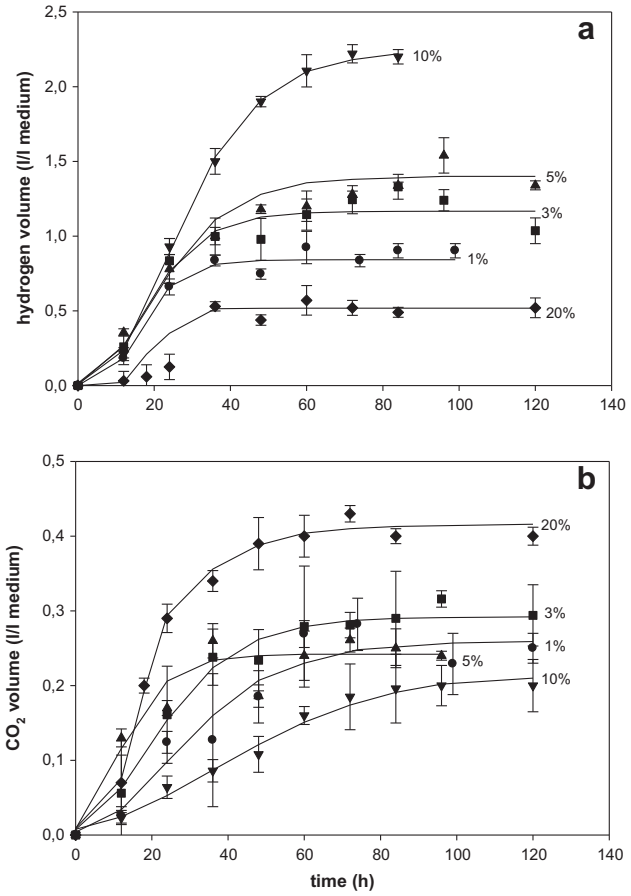

Fig. 2 – Kinetics of hydrogen (a) and carbon dioxide (b) evolution at different concentrations of brewery wastewater (inoculum 0.36 g dry wt/l, illumination 116 W/m^2).

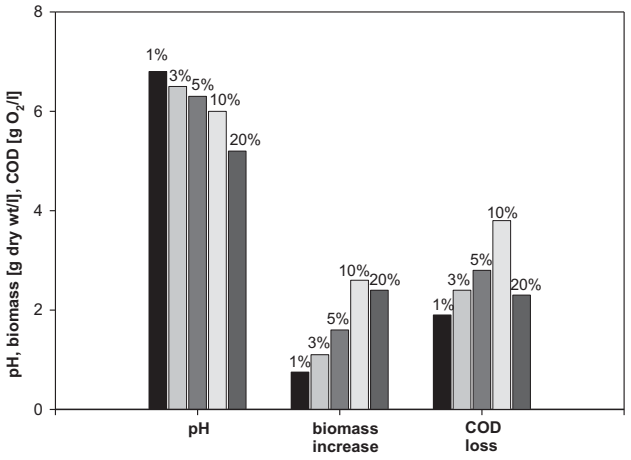

Fig. 3 – Influence of brewery wastewater concentrations on pH, biomass increase and COD number (medium containing 30% v/v of inoculum).

mechanism of interaction of ammonium ions with nitrogenase is well known [24–26]. However, it is puzzling that at such strong decrease in hydrogen yield there was no decrease in biomass content (Fig. 3). It happened that at very high concentrations of ammonium ions the amount of biomass was doubled whereas no hydrogen was observed [22]. These observations lead to the conclusion that only nitrogen from ammonium ions can be involved in biomass generation. Zhu et al. [4] using tofu wastewater containing 2 mmol/l of NH_4^+ and *R. sphaeroides* bacteria immobilized with agar gel obtained relatively high yield of hydrogen (>50%). It was established that almost 90% of ammonium ions was consumed. It can be assumed that immobilization protects bacteria against inhibition by ammonium ions. High concentration of different organic compounds (COD = 202 g O_2/l) in brewery wastewater certainly influences the inhibiting effects of NH_4^+ ions.

The final pH values presented on Fig. 3 show the drop from 7.1 to 5.2, respectively. This effect is caused mainly by formation of lactic and acetic acids [10]. The higher was the concentration of the waste the higher was the amount of detected acids and lower value of pH. This can be explained by higher ability of transfer of undissociated form of acids towards the cell, followed by dissociation inside the cell, proton release and final inhibition of the process [27]. Eroglu et al. [21] studying the range of concentrations of olive mill waste water in hydrogen bioproduction established that at high concentration of waste in medium the amount of inhibiting substances increases. The color of the medium at

Table 3 – Kinetic parameters of cumulative hydrogen and carbon dioxide production for different initial concentration of brewery waste (inoculum 0.36 g dry wt/l), calculated from Gompertz eq. (1).

Run	Concentration of brewery waste (% v/v)	H_{max} (l/l)	R_{max,H_2} (l/l/h)	λ_{H_2} (h)	R^2	Y (l H_2/l waste)	η (%)	H_{max,CO_2} (l/l)	$R_{max,CO2}$ (l/l/h)	λ_{CO_2} (h)	R^2
1	1	0.86 ± 0.02	0.046 ± 0.007	8.0 ± 1.4	0.96	0.009	0.65	0.260 ± 0.015	0.006 ± 0.001	7.1 ± 4.9	0.91
2	3	1.17 ± 0.05	0.045 ± 0.009	6.1 ± 2.7	0.89	0.035	0.88	0.292 ± 0.011	0.007 ± 0.001	4.2 ± 2.6	0.94
3	5	1.40 ± 0.05	0.042 ± 0.008	6.1 ± 2.1	0.96	0.07	1.07	0.240 ± 0.023	0.011 ± 0.005	1.7 ± 5.5	0.92
4	10	2.24 ± 0.09	0.061 ± 0.009	9.4 ± 2.6	0.95	0.22	1.7	0.223 ± 0.012	0.003 ± 0.0002	6.4 ± 2.6	0.97
5	20	0.52 ± 0.02	0.040 ± 0.015	18.7 ± 2.2	0.93	0.010	0.4	0.416 ± 0.020	0.010 ± 0.002	5.1 ± 3.6	0.95
6	10[a]	1.41 ± 0.04	0.034 ± 0.004	11.6 ± 2.9	0.89	0.14	1.06	0.173 ± 0.009	0.0029 ± 0.0004	5.3 ± 3.6	0.93
7	Standard[b]	2.3 ± 0.06	0.047 ± 0.004	2.7 ± 1.8	0.97	–	1.74	0.14 ± 0.01	0.01 ± 0.003	4 ± 2	0.94

a Concentration of inoculum 0.086 g dry wt/l.
b Standard medium with L-malic acid.

higher concentration of the waste was more dark and this is probably resulted from fermentative pathway rather than photoheterotrophic H_2 production. The possible fermentative metabolism can be proved by low concentration of CO_2 in evolved gases. It is well known from literature [10,21] that decolorization leads towards high conversion efficiency. Moreover, it was found that higher amount of generated hydrogen involve lower CO_2 content (see Table 3). In those experiments in which no hydrogen was found Eroglu et al. [21] observed low amount of CO_2.

The color of medium at higher concentrations become more dark and therefore the access of light inside the photobioreactor is much more limited. Yetis and coworkers [3], studying an application of sugar refinery waste water in hydrogen generation with *R. sphaeroides*, obtained only small amounts of hydrogen at concentration close to 20% v/v (0.13 l H_2/l) but at 30% v/v the process was stopped. An application of standard medium of Biebl and Pfenning, in which L-malic acid was replaced with 20% v/v of waste water, improved significantly the yield (2.0 l H_2/l) of studied process but still did not reached standard results with malic acid (2.4 l H_2/l). In our studies introduction of 10% v/v of waste instead of L-malic acid, resulted not only in generation of 2.2 l H_2/l (with malic acid 2.3 l H_2/l) but also improved the rate of process (compare data in Table 3).

One of the important parameter in photoactive reactions that gained widespread acceptance is the light conversion efficiency – η [10,21]. This is the ratio of energy accumulated in hydrogen to light energy input in bioreactor. In our experiments, the highest value of η (close to 1.7%) was obtained for the waste concentration in medium of 10% v/v. Koku et al. [10] calculating η for different systems and the same strain of bacteria found that light conversion efficiency can vary between 1 and 5%.

3.4. *Kinetics of hydrogen generation*

The modified Gompertz equations were applied in modeling kinetics of the hydrogen generation process. This equation is generally accepted because it represents the best fitting for all steps of hydrogen production curve. The experimental results are shown on Fig. 2. Both hydrogen evolution (Fig. 2a) and carbon dioxide evolution (Fig. 2b) show typical shape – similar in all cases; well correlated with Gompertz equation ($R^2 > 0.91$). The basic differences between all these curves are related with the rate of formation, duration of lag phase and the final concentration of H_2 or CO_2. The overall rate of the process in all studied cases is similar. The rate is slower at lower concentration of bacteria (Table 3, run 6) and with higher concentration of the waste that inhibits (Table 3, run 5) photofermentation. The best results were obtained for waste concentration of 10% v/v (Table 3, run 4). Here the rate of hydrogen evolution was 0.06 l/l/h. In runs 1–3 (Table 3), with low concentration of the waste, the rates were similar (~0.045 l/l/h). This indicates that at up to 5% v/v of the waste, the concentration does not limits hydrogen generation process. Eroglu et al. [28], studying hydrogen photoevolution (reactor capacity – 400 cm^3, illumination – 200 W/m^2) at different concentrations of malic acid as the only source of organic carbon, obtained the maximum reaction rate of 0.01 l/l/h. In experiments by Kitajima et al. [29], the rate of hydrogen generation was 0.04 l/l/h, while applying plane type photosynthethic bioreactor. Much lower values (0.007 l/l/h) were obtained by Su et al. [15] in photoreaction with cassava starch. Literature data as well as our results show that hydrogen generation rate depends on such conditions as: type and concentration of the substrate, light intensity and type of the photobioreactor [30].

Another parameter which influences the rate of reaction is lag phase. In our case, an increase of brewery waste waster concentration in medium resulted the lag phase prolongation. A comparison of the most effective system (Table 3, run 4) with standard (Table 3, run 7) show that lag phase in the case of brewery wastewater is at least three times longer. The extension of the lag phase is influenced by composition of the applied waste. High load of different organic compounds (COD = 202 g O_2/l) and presence of ammonium ions slow down the whole process. In such a case, microorganisms require more time for a propagation and acclimatization. Recently, applying cobalt ions as an example, it was shown [31] the influence of heavy metals on the growth of *R. sphaeroides*. Presence of heavy metals in certain circumstances and appropriate concentrations is essential for bacterial growth and it is required in several metabolic pathways, including those involved in phototrophic growth. Too high concentration of cobalt ions results in weakening of an impaired expression and activity of porphobilinogen deaminase (PBGD) enzyme. The PBGD specifically belongs to the porphyrine and bacteriochlorophyll biosynthetic pathways and its down-regulation may account for the reduced ability to synthesize bacteriochlorophylls required for assembling LH1 (light harvesting antenna protein).

3.5. *Hydrogen generation in simulated day/night sequence*

In order to check the possibility of hydrogen generation by *R. sphaeroides* under natural solar irradiation a series of experiments simulating natural day/night conditions were performed. The amount of generated hydrogen, changes in pH, formation of biomass and COD values were monitored under illumination

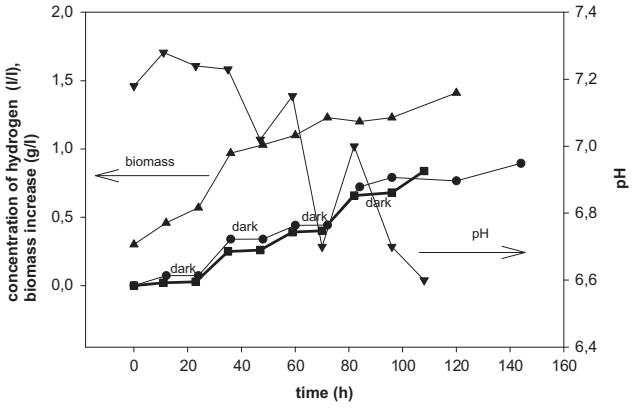

Fig. 4 – Hydrogen generation in the sequence day/night. "Day" illumination (−116 W/m^2) ● – hydrogen generation in medium containing 10% v/v of waste and 10% v/v of inoculum ■ – hydrogen generation in medium containing 10% v/v of waste and 30% v/v of inoculum ▲ – biomass increase ▼ – pH.

and in the dark (Fig. 4). There was no hydrogen evolution during dark period of the studied reaction. However, the slight increase of biomass was observed also without illumination. Similar effect was observed by Uvar et al. [32]. It was shown that cells cannot grow in bioreactor if it is not illuminated after inoculation. This indicates that *R. sphaeroides* cannot grow under dark anaerobic conditions, however, they can survive in fermentation mode while consuming organic substances. The decrease of pH value (Fig. 4) during the initial dark periods proves that bacteria without illumination shift their metabolic mode and fermentation starts to occur. Literature data [33] show that illumination is the essential factor for transportation of electrons and synthesis of ATP. These electrons with help of ferrodoxine and NAD are transferred towards nitrogenase and finally reduce protons to molecular hydrogen. In the absence of light the PNS bacteria shift towards fermentation mode and nitrogenase become inactive.

4. Conclusions

It was established that brewery wastewater can serve as an excellent substrate in hydrogen photobiogeneration in presence of *R. sphaeroides*. The amount of photogenerated hydrogen increases with the increase of wastewater concentration in medium up to 10% v/v.

It represents 97% of the amount obtained with standard medium containing malic acid. At higher concentration of the wastewater in medium a significant decrease of photogenerated hydrogen is observed. Simultaneously, the COD and the yield of the reaction drops down (to 0.1 l H_2/l waste). This effect results from too high concentration of NH_4^+ ions concentration (above 1 mM NH_4^+) in reaction medium. Under these conditions the activity of nitrogenase drops down significantly and in consequence the amount of photogenerated hydrogen, as well. Additionally, the much more intense color of the reaction medium at higher concentrations of the waste water causes the limited access of the light in photoreactor and in consequence lowers the total activity of the studied system.

Kinetic studies performed in the day/night conditions proved total inhibition of the process in the absence of light: no hydrogen evolution, very low growth of bacteria, decrease of pH and lack of COD reduction. It was established that modified Gompertz equation describes well the kinetics both of the generated hydrogen ($R^2 > 0.89$) and evolved CO_2 ($R^2 > 0.91$).

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Nomenclature

H: cumulative hydrogen, l/l_{medium}
H_{max}: maximum cumulative hydrogen, l/l_{medium}
$R_{max,H2}$: maximum rate of hydrogen production, l/l/h
t: fermentation time, h
λ: lag time, h
e: exp. = 2.718
R^2: coefficient of determination
Y: substrate yield, l hydrogen/l waste
η: light conversion efficiency, %
V: volume of produced H_2, l
ρ: density of the produced hydrogen gas, g/l
I: light intensity, W/m^2
A: irradiated area, m^2