A BACTERIAL HYDROGEN PRODUCTION TEST SYSTEM
FOR MEASURING H₂ CONCENTRATIONS IN LIQUIDS AND GASESI. Klepere¹, I. Muiznieks¹, J. Kleperis²¹University of Latvia, Faculty of Biology,
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The hydrogen production by biological processes using fermentative micro-organisms is an environment-friendly way for storage and use of energy. A laboratory-scale model of the fermentation system was developed and tested, which allows studying the kinetics of hydrogen production by a standard *Escherichia coli* MSCL (Microbial Strain Collection of Latvia) strain 332 used as indicator at the sanitary analyses of water. In this work, we compared the hydrogen concentrations in liquid (the cultivation media) and gaseous environments. During the anaerobic growth of *E. coli* MSCL332 bacteria in glucose-containing media a hydrogen over-saturation in the liquid phase was observed. The maximum of dissolved hydrogen concentration (2481 $\mu\text{mol/l}$) was reached by the fourth hour of fermentation, while for gaseous phase the mass-spectrometric analysis showed that hydrogen of the highest concentration occupied only 0.4% volume.

1. INTRODUCTION

Today the energy production is mainly based on fossil fuels, and these climate changing energy resources will soon be exhausted. Consequently, efforts of researchers worldwide are directed towards the innovations that are environment-friendly and sustainable.

Hydrogen is the most abundant element in the Universe, though scarcely available on the Earth in a molecular (dihydrogen, H₂) form. Nevertheless, dihydrogen can be produced from many different sources (e.g. biomass rich in carbohydrates), and in many different ways [1]. Every physical or chemical method of H₂ production requires a powerful electrolytic, thermal or photolytic source of energy, which is not always environment-friendly. On the contrary, biological processes of hydrogen recovery and collection from organic resources such as municipal wastewater and sludge facilitate recycling of sewage and are environmentally benign [2]. Nowadays, many institutions and universities worldwide are involved in the research of hydrogen production by microorganisms and algae [2–4].

Microorganisms are capable of producing H₂ via two main pathways: fermentation and photosynthesis. The processes of bio-hydrogen production include:

- 1) direct biophotolysis by green algae – the photosynthetic production of hydrogen by splitting water into molecular hydrogen and oxygen using sunlight under specific conditions;

- 2) indirect biophotolysis by cyanobacteria with specialized cells (heterocysts) that perform nitrogen fixation and contain enzymes (nitrogenase and hydrogenase) directly involved in hydrogen metabolism and synthesis of molecular H₂;
- 3) photo-fermentation by purple non-sulfur bacteria that evolve molecular H₂ catalyzed by nitrogenase enzyme under nitrogen-deficient conditions using the energy of light and organic acids;
- 4) dark-fermentation by anaerobic bacteria grown in the dark on carbohydrate-rich substrates [1, 5, 6].

Bacterial hydrogen production by fermentation of carbohydrate-containing substrates (glucose, cellulose, starch and organic waste materials) is frequently preferred to photolysis, because it does not rely on the availability of light sources [5]. In the fermentation of glucose by enterobacteria, e.g. *Escherichia coli*, one of the pyruvate oxidation products, alongside with acetyl-CoA, is formate, which is produced by pyruvate formate lyase and is the sole source of hydrogen in these bacteria. The formate is split into CO₂ and H₂ by formate hydrogen lyase (FHL) complex, which comprises seven proteins, six of them being encoded *hyc* operon. Five *hyc* operon encoded proteins are membrane-embedded electron transporters. The *hycE* protein is one of the three *E.coli* NiFe hydrogenases (also referred to as Hyd-3). The *hycE* and FDH-H components of FHL complex are soluble periplasmic proteins. The hydrogen evolved from FHL is consumed by *E.coli* uptake hydrogenases Hyd-1 and Hyd-2. In contrast to enterobacteria, strictly anaerobic fermenters, e.g. *Clostridia*, use a reduced ferredoxin (required to oxidize pyruvate to acetyl-CoA) for H₂ production by the hydrogenase that generates ferredoxin in the oxidized form and releases electrons as molecular hydrogen [5, 7–9].

Glucose fermentation by enteric bacteria yields the maximum of 2 mol H₂/mol glucose [10]. To enhance the hydrogen production and utilize the substrate in full measure for complete conversion, the synergy of biological processes (two-stage/hybrid ones) should be applied [11].

Gaseous hydrogen is formed in liquid media during bacterial fermentation. Hydrogen gas hardly dissolves in aquatic solutions [12], but special methods are required to discharge hydrogen into atmosphere and in so doing to escape over-saturation [13]. In the atmosphere the parameters of hydrogen gas are measured by classical volumetric, mass-spectrometric and chromatography methods, or using chemical gas sensors [14]. Wilkins [15] described a method for measuring gas production by microorganisms using a platinum electrode and a reference Calomel (Hg–Hg₂Cl₂) electrode. To measure hydrogen gas concentration in liquid a hydrogen electrode is usually used (Pt or another noble metal – gold, rhodium, palladium, etc.). The hydrogen H⁺ ions and the molecular hydrogen H₂ set the equilibrium potential in compliance with the reaction: H₂ ⇌ 2H⁺ + 2e[−] [16]. This reaction proceeds very fast, so in its course the equilibrium state remains stable; in electrochemistry this electrode is adopted as zero reference (with zero potential). In microbiology, to measure dissolved oxygen and hydrogen gases the micro-respiration Clark electrodes are used [17]. In a Clark's electrode the cathode polarized versus an internal Ag/AgCl anode is placed behind an electrically insulating silicone rubber membrane, which is extremely permeable to oxygen. The flow of electrons from the anode to the oxygen-reducing cathode reflects linearly

the partial oxygen pressure around the sensor tip and is in the pA range. The same principle holds for a hydrogen Clark-type sensor: the environmental hydrogen is driven by the external partial pressure and penetrates through the sensor tip membrane to be oxidized at the platinum anode surface [18, 19]. Flynn *et al.* [20] used chemochromic sensors for screening in order to identify positive (i.e. hydrogen-producing) algal colonies. A chemochromic sensor film, which is normally transparent, turns blue in the presence of hydrogen gas.

Hydrogen gas is produced during the bacterial fermentation process in anaerobic conditions. In practice, hydrogen is collected in the gaseous state, since dissolved hydrogen tends to become gas. To optimize the hydrogen collection methods it is necessary to study properly the hydrogen production kinetics in liquid phase during the fermentation process. The experimental test system for bacterial hydrogen production and micro-sensors (Unisense, Denmark) were used to determine the hydrogen gas concentrations in liquid; the mass-spectrometry method was employed for measurements in the hydrogen-containing head space.

2. EXPERIMENTAL

Escherichia coli strain MSCL 332 (i.e. from Microbial Strain Collection of Latvia) was grown on Luria-Bertani (LB) nutrient agar plates (5 g/l yeast extract, 10 g/l tryptone, 10 g/l sodium chloride, 15 g/l Bacto agar [21]). *E.coli* from single colonies on the agar plates were inoculated in 2 x 150 ml flasks containing LB liquid medium. The flasks were aerobically shaken at 37°C for 12 hours at 120 rpm using a multi-shaker PSU-20 (BioSan, Latvia). The bacteria cell number in the overnight culture was titrated at 10^{-6} dilution. The amount of bacterial cell protein was calculated assuming that one *E.coli* cell contains 1.54×10^{-13} g of protein [22]. The overnight culture in LB liquid medium was mixed (1:1) with phosphate buffer saline (PBS) pH 7.3 (0.8 g/l NaCl, 0.2 g/l KCl, 1.43 g/l Na_2HPO_4 , 0.2 g/l KH_2PO_4 [23]) in a vessel sterilized for measurements. The PBS contained a complex trace element medium pH 6.5 (0.039g/l $\text{Fe}(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$, 0.172 mg/l Na_2SeO_3 , 0.02 mg/l NiCl_2 , 0.4 mg/l $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ [24]). Glucose (3.3mM, final concentration, sterilized through membrane 0.2µm filter) was added at the start of experiment.

The hydrogen and oxygen concentrations were measured with Clark-type microsensors (Unisense, Denmark) in the sample liquid phase. The microsensors were connected with the signal amplifier – a pico-ammeter and an A/D current converter connected to PC using USB port.

Before the measurements, both oxygen and hydrogen microsensors were calibrated in a liquid culture medium (similar to the sample measured by 15 min bubbling Ar) for zero concentrations and hydrogen gas (99.999% supplied from AGA SIA or using a proton exchange membrane electrolyser from Fuelcellstore.com) and clean air for 100% dissolved H_2 and O_2 concentrations (730 and 760 µmol/l, accordingly). The system is able to work independently when measurements are made in one sample. If there are several samples at a time, it is necessary to move microsensors manually and to sterilize the sensor tip using 96% ethanol, 0.1 M NaOH and distilled water every time when it is taken out from the sample [19].

The gas from the headspace of liquid bacterial culture in the test vessel was taken to an RGAPro-100 mass-spectrometer to analyze its components. The gas from an argon balloon through a diffuser was let in the test vessel with bacteria

culture to sustain the anaerobic environment (see Fig.1) and put in a water bath to maintain a temperature of 37 ± 2 °C.

Fig. 1. Experimental test system for H_2 concentration measurements with mass-spectrometer.

Argon gas was bubbled through the liquid for 15 min (flow 13 l/h) and gas measurements were made with 30 min and an hour intervals. The total time of measurements was six hours. The gas volume taken for each analysis was 20 cm^3 . During the mass-spectrometric analysis, simultaneous measurements with a hydrogen microsensor were taken in order to make unbiased comparison of mass-spectrometric and hydrogen microsensor analyses. The concentration of dissolved hydrogen gas was measured with a microsensor; the mass-spectrometric analyses were made for the atmospheric composition in the headspace of the sample bacteria culture and nutrients.

The experimental results of microsensoric measurements were analyzed using *Sensor Trace Basic* and *MicOX* (A/S Unisense) programs, and processed by *Microsoft Office Excel 2007*. The mass-spectrometric data were analyzed by *RGA 3.0 Software for SR Residual Gas Analyzers* program.

3. RESULTS AND DISCUSSION

The hydrogen output was measured for seven hours after the beginning of fermentation process; increase in the hydrogen concentration was observed starting from the second hour after adding glucose. The constancy of oxygen concentration in the measurements evidences that the system had reliable anaerobic conditions (Fig. 2). As is seen from this figure, the concentration of dissolved hydrogen stopped increasing after 5–10 h as glucose exhausted (similar results are reported in [25]).

The maximum rate of hydrogen formation in the test system was $612\text{ }\mu\text{mol/l/20 min}$ or $1.4\text{ mmol [2.4 mg] /l per h}$ for 43 mg protein mass (i.e. $32.6\text{ }\mu\text{mol/mg}$ protein mass). The maximum concentration of dissolved hydrogen ($2481\text{ }\mu\text{mol/l}$ or 2.5 mmol/l) is reached in the fourth hour of fermentation as is seen in Fig. 2. This concentration at least three times exceeds the maximum thermodynamically allowed concentration of dissolved hydrogen in water ($730\text{ }\mu\text{mol/l}$ [13]).

Fig. 2. Microsensors for fermentation measurements on the sample with *E. coli*.

To demonstrate that the hydrogen production began only after glucose had been added various glucose concentrations were tested. The correlation between the glucose concentration and the hydrogen output is shown in Fig. 3.

Fig. 3. Time dependence of hydrogen output at different glucose concentrations (microsensors for fermentation measurements on the sample with *E. coli*).

To calculate the partial pressure of hydrogen in the headspace Henry's law was used. The calculations were done using the measured dissolved hydrogen concentrations in the test system (2481 $\mu\text{mol/l}$ after 4 h fermentation). At room temperature and normal atmospheric pressure the Henry constant is:

$$k_H = 1282.05 \frac{l}{\text{atm} \cdot \text{mol}},$$

therefore $p_H = 1282.05 \cdot c_H$ (atm) and in our case $p_H = 1282.05 \cdot 2481 \cdot 10^{-6} = 3.18$ atm, which obviously does not fit the experimental results obtained in the mass-spectrometric analysis. As is seen from Fig. 4, the partial pressure of hydrogen of 3.18 atm above the test system's headspace is inadequate to the concentrations determined by mass-spectrometric analysis – only $6 \cdot 10^{-3}$ atm or 0.6% vol.

Fig. 4. Mass-spectrometric analysis of the sample with bacteria *E.coli* before fermentation (light gray curve) and after 6h (black curve).

The mass-spectrometric analysis has revealed the presence of different volatile substances – the end products of bacterial formation: acetate, carbon dioxide, ethanol, acetone, and hydrogen gas. In three separate measurements (without Ar bubbling and liquid mixing, with argon bubbling only, and with argon bubbling & liquid mixing) the mass-spectrometric analysis showed a hydrogen concentration increase from 0% to 0.4% after 6 h fermentation only in one measurement when no argon bubbling and mixing was applied. Such an increase in the hydrogen concentration (only $3.6 \cdot 10^{-3}$ atm partial pressure) is not convincing as compared with the concentrations measured in liquid phase. A reason for that could be the limited hydrogen migration from liquid to gaseous phase (dissolved hydrogen oversaturation); besides, the mass-spectrometry measurement method was not perfect (it is to be optimized in the future experiments). As reported in scientific literature [26, 27], in experiments with dissolved and gaseous hydrogen a low liquid-to-gas mass transfer was observed. Despite hydrogen being a poorly-soluble gas in anaerobic environment, it can become over-saturated in liquids [27]. Concentrations of dissolved gas are higher than theoretically possible in the anaerobic processes where gases are formed in the liquid phase and tend to reach

the gas phase. Such over-saturation could be associated with biological processes: lower pH due to the formation of gases (e.g. CO₂, H₂S) in anaerobic processes; besides, a negative thermodynamic effect is caused by inhibitor gases – e.g. H₂, since the hydrogen synthesising enzymes are sensitive to H₂ concentrations and are subject to the end-product inhibition. As concentrations of hydrogen increase, its synthesis rate decreases: the evolved H₂ is consumed by *E.coli* uptake hydrogenases Hyd-1 and Hyd-2 [14, 6, 9].

As mentioned, the hydrogen synthesising enzymes are sensitive to the end product – the hydrogen gas concentration. As this concentration increases the synthesis rate decreases, with formation of mixed-acid hydrogen-containing fermentation products (ethanol, acetate, butane) [6]. To enhance the hydrogen gas output, the bacterial metabolism has to be switched from alcohol and acid formation to volatile fatty acids [14]. This can be facilitated by the system's optimization, for example, using continuous bubbling with inert gas to reduce the partial pressure of hydrogen in the liquid phase thereby increasing its formation in the gaseous phase. To enhance hydrogen formation, very delicate bubbling/mixing procedures should be applied, since in our measurements, with intense bubbling and mixing by a magnetic stirrer, the least hydrogen increase in the headspace was observed. However, Oh Y-K *et al.* [28] report that the bubbling with argon or nitrogen increases the production of hydrogen in fermentation processes as compared with bioreactors where no bubbling occurs.

4. CONCLUSIONS

The developed lab-scale test system allows estimation of the hydrogen production. As a hydrogen producing microorganism, *Escherichia coli* wild-type strain MSCL 332 from Microbial Strain Collection of Latvia was successfully used. The hydrogen concentration in the headspace was analyzed by mass spectrometry. Due to incompleteness of the test system the hydrogen concentration in the gaseous phase was not detected, which therefore remains to be a subject of future activities. Also, the gas measuring system should be improved based on the mass-spectrometry. To enhance the transfer of dissolved hydrogen into the headspace, continuous gas bubbling and mixing are required.

The dissolved hydrogen and oxygen concentrations in liquid phase during the fermentation process were measured using microsensors (Unisense, Denmark). The maximum of dissolved hydrogen concentration (2481 µmol/l) was reached by the fourth hour of fermentation, which is markedly higher than predicted by Henry's law (730 µmol/l). Also, alternative methods should be employed for the hydrogen collection directly from the nutritional broth thus making it possible to develop commercial hydrogen production.

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REFERENCES

1. Holladay, J.D., Hu J., King, D.L., & Wang, Y. (2009). An overview of hydrogen production technologies. *Catalysis Today*, 139, 244–260.
2. Das, D., & Veziroglu, N.T. (2008). Advances in biological hydrogen production processes. *Intern. J. Hydrogen Energy*, 33, 6046–6057.
3. Kotay Meher, S., & Das, D. (2008). Biohydrogen as a renewable energy resource – prospects and potentials. *Intern. J. Hydrogen Energy*, 76, 258–263.
4. Donohue, T.J., & Cogdell, R.J. (2006). Microorganisms and clean energy. *Nature Reviews Microbiology* 4, 800. | doi:10.1038/nrmicro1534.
5. Das, D., & Veziroglu, T.N. (2001). Hydrogen production by biological processes: a survey of literature. *Intern. J. Hydrogen Energy*, 26, 13–28.
6. Levin, D.B., Pitt, L., & Love, M. (2004). Biohydrogen production: prospects and limitations to practical application. *Intern. J. Hydrogen Energy*, 29, 173–185.
7. Nath, K., & Das, D. (2004). Improvement of fermentative hydrogen production: various approaches. *Appl. Microbiol. Biotechnol.*, 65, 520–529.
8. Hallenbeck, P.C., & Benemann, J.R. (2002). Biological hydrogen production; fundamentals and limiting processes. *Intern. J. Hydrogen Energy*, 27, 1185–1193.
9. Maeda, T., Sanchez-Torres, V., & Wood, T.K. (2008). Enhanced hydrogen production from glucose by metabolically engineered *Escherichia coli*. *Appl. Microbiol. Biotechnol.*, 77, 879–890.
10. Wang, J., & Wan, W. (2009). Factors influencing fermentative hydrogen production: A review. *Intern. J. Hydrogen Energy*, 34, 799–811.
11. Nath, K., Muthukumar, M., Kumar, A., & Debabrata, D. (2008). Kinetics of two-stage fermentation process for the production of hydrogen. *Intern. J. Hydrogen Energy*, 33, 1195–1203.
12. Sander, R. (1999). *Compilation of Henry's Law Constants for Inorganic and Organic Species of Potential Importance in Environmental Chemistry*. Air Chemistry Department of Max-Planck Institute of Chemistry.
<http://www.mpch-mainz.mpg.de/~sander/res/henry.html>
13. Mandelis, A., & Christofides, C. (1993). *Physics, Chemistry and Technology of Solid State Gas Sensor Devices*. Wiley, 352.
14. Paus, A., Andre, G., Perrier, M., & Guiot, R. (1990). Liquid-to-gas transfer in anaerobic processes: Inevitable transfer limitations of methane and hydrogen in the biomethanation process. *Appl. Environ. Microbiol.*, 56(6), 1636–1644.
15. Wilkins, J. R., Stoner, G.E., & Boykin, E.H. (1974). Microbial Detection Method Based on Sensing Molecular Hydrogen. *Applied Microbiology*, 5, 949–952.
16. Mills, A. (2006). Instrumental Note: Platinized Platinum, Platinum Sponge and Platinum Black. *Bulletin Sci. Instrument Soc.*, 89, 35–37.
17. Ghirardi, M., Togasaki, R.K., & Seibert, M. (1997). Oxygen sensitivity of algal H₂-production. *Applied Biochem Biotechnol.* 63-65, 141–151.
18. Revsbech, N. P., & Jørgensen, B.B. (1986). Microelectrodes: Their Use in Microbial Ecology. *Advances in Microbial Ecology*, 9. New York: Plenum, 293–352.
19. (2007). Micro-Respiration System Manual, Hydrogen Sensor Manual, Unisense A/S, (Denmark), Version 20070828, <http://www.unisense.com/Default.aspx?ID=107>.
20. Flynn, T., Ghirardi, M.L., & Seibert, M. (2002). Accumulation of O₂-tolerant phenol-types in H₂-producing strains of *Chlamydomonas reinhardtii* by sequential applications of chemical mutagenesis and selection. *Intern. J. Hydrogen Energy*, 27, 1421–1430.
21. Atlas, M.R. (2004). *Luria Bertani media: Handbook of microbiological media* (3rd edn). CRC Press, p. 942.
22. Madigan, M.T., & Martinko, J.M. (2006). *Brock Biology of Microorganisms* (11th edn). Pearson Prentice Hall (USA), 118–119.

23. Penfold, D.W., Forster, C.F., & Macaskie, L.E. (2003). Increased hydrogen production by *Escherichia coli* strain HD 701 in comparison with wild-type strain MC4100. *Enzyme and Microbial Technology*, 33, 185–189.
24. Maeda, T., Sanchez-Torres, V., & Wood, T.K. (2008). Metabolic engineering to enhance bacterial hydrogen production. *Microbial Biotechnol.*, 1, 30–39.
25. Nandi, R., Dey, S., & Sengupta, S. (2001). Thiosulphate improves yield of hydrogen production from glucose by the immobilized formate hydrogenlyase system of *Escherichia coli*. *Biotechnol Bioeng.* 75(4), 492-494.
26. Battino, R., & Clever, L.H. (1966). The Solubility of Gases in Liquids. *Chem. Rev.*, 66 (4), 395–463.
27. Kraemer, J.T., & Bagley, D.M. (2006). Supersaturation of dissolved H₂ and CO₂ during fermentative hydrogen production with N₂ sparging. *Biotechnol Lett.*, 28, 1485–1491.
28. Oh, Y-K., Seol, E-H., Yeol Lee, E., & Park, S. (2002). Fermentative hydrogen production by a new chemolithotrophic bacterium *Rhodospseudomonas palustris* P4. *Intern. J. Hydrogen Energy*, 27, 1373–1379.

ŪDEŅRAŽA KONCENTRĀCIJU MĒRĪJUMI ŠĶIDRĀ UN GĀZVEIDA VIDĒ TESTSITĒMĀ AR ŪDEŅRADI RAŽOJOŠĀM BAKTĒRIJĀM

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K o p s a v i l k u m s

Mūsdienu enerģijas ieguves metodes ir balstītas lielākoties uz neatjaunojamā fosilā kurināmā izmantošanu, kas nespēj nodrošināt ilgtermiņa attīstību. Līdz ar to nepieciešami jauni, inovatīvi risinājumi, kas būtu videi draudzīgi un ilgstpējīgi. Bioloģiska ūdeņraža producēšana, izmantojot anaerobus mikroorganismus, ir alternatīvs ūdeņraža iegūšanas un uzglabāšanas veids.

Tika izveidota eksperimentāla testsistēma baktēriju ūdeņraža producēšanas kinētikas novērošanai un analīzei, izmantojot *E.coli* LMKK 332 standartcelmu, kas tiek izmantots kā indikators ūdens tīrības analīzēm. Tika salīdzināta ūdeņraža gāzes koncentrācija baktēriju kultūras barotnē (šķidrā fāzē) un gāzes fāzē.

Ūdeņraža veidošanās kinētika parādīja, ka šķidrā fāzē fermentācijas sākuma ūdeņradis veido pārkonzentētus šķīdumus glikozi saturošā barotnē ar baktēriju *E.coli* LMKK 332. Lai optimizētu testsistēmu un padarītu procesu rūpnieciski izdevīgu, nepieciešams pilnveidot analīzes mērījumus gāzes fāzē, kā arī apsvērt alternatīvu metožu izmantošanu ūdeņraža savākšanai tieši no barotnes (šķidrās fāzes).

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