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Can a Fermentation Gas Mainly Produced by Rumen *Isotrichidae* Ciliates be a Potential Source of Biohydrogen and a Fuel for a Chemical Fuel Cell?

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Bacteria, fungi, and protozoa inhabiting the rumen, the largest chamber of the ruminants' stomach, release large quantities of hydrogen during the fermentation of carbohydrates. The hydrogen is used by coexisting methanogens to produce methane in energy-yielding processes. This work shows, for the first time, a fundamental possibility of using a hydrogen-rich fermentation gas produced by selected rumen ciliates to feed a low-temperature hydrogen fuel cell. A biohydrogen fuel cell (BHFC) was constructed consisting of (i) a bioreactor, in which a hydrogen-rich gas was produced from glucose by rumen ciliates, mainly of the *Isotrichidae* family, deprived of intra- and extracellular bacteria, methanogens, and fungi; and (ii) a chemical fuel cell of the polymer–electrolyte type (PEFC). The fuel cell was used as a tester of the technical applicability of the fermentation gas produced by the rumen ciliates for power generation. The average estimated hydrogen yield was *ca.* 1.15 mol H_2 per mole of fermented glucose. The BHFC performance was equal to the performance of the PEFC running on pure hydrogen. No fuel cell poisoning effects were detected. A maximum power density of 1.66 kW/m^2 (PEFC geometric area) was obtained at room temperature. The maximum volumetric power density was 128 W/m^3 but the coulombic efficiency was only *ca.* 3.8%. The configuration of the bioreactor limited the continuous operation time of this BHFC to *ca.* 14 h.

Keywords: Rumen, *Isotrichidae*, biohydrogen, fermentation, glucose, fuel cell

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The rumen is the first and the largest chamber of the complex stomach of the ruminants. It is considered a highly specialized anaerobic ecosystem with very dense populations of microorganisms forming its biocoenosis. The main participants of the ruminal biocoenosis are bacteria (10^{10}–10^{12} cells/ml of rumen fluid), archea including methanogens (10^8–10^9 cells/ml), fungi, and protozoa (*ca.* 10^6 cells/ml) [8, 38, 46]. Among the protozoa, the most abundant and the most important are the ciliates, of which the most numerous are the representatives of the family *Ophryoscolecidae* (order *Entodiniomorphida*) followed by the *Isotrichidae* (order *Vestibuloferida*). The former one is represented by *Entodinium caudatum*, *Eudiplodinium maggii*, *Polyplastron multivesiculatum*, *Diploplastron affine*, *Eremoplastron dilobum*, and *Epidinium ecaudatum caudatum* and the latter one by *Dasytricha ruminantium*, *Isotricha prostoma*, and *I. intestinalis*.

The ruminal *Ophryoscolecidae* and *Isotrichidae* differ distinctly in their morphology and nutritional physiology. For example, the cells of the former are covered with a rigid pellicle and the ciliature is reduced to only one zone, the adoral ciliary zone (representatives of the genus *Entodinium*), or to two zones, the adoral and the dorsal ciliary zones (the rest of *Ophryoscolecidae* ciliates). In addition, the caudal end of the cells of these ciliates can be equipped with spines and/or lobes. Conversely, the pellicle of the ruminal *Isotrichidae* is very elastic and the cells are ovoid or ellipsoidal. Moreover, all of the surface of their cells is covered by cilia arranged in rows [46]. The *Ophryoscolecidae* ciliates prefer insoluble polysaccharides (starch grains and cellulose-rich green plant particles) [46]. Conversely, the *Isotrichidae* ciliates clearly prefer the soluble carbohydrates and they do not utilize cellulose [44]. Both groups of the rumen ciliates ferment carbohydrates

to acetic, butyric, lactic, and propionic acids; however, different proportions of the acids are observed for the *Isotrichidae* and *Ophryoscolecidae* families [1, 9, 27, 31, 33, 41, 43, 44]. Both the *Isotrichidae* and *Ophryoscolecidae* ciliates synthesize and store amylopectin in their cells and release hydrogen and carbon dioxide, the gaseous products of carbohydrate fermentation. However, the representatives of the former group seem to metabolize carbohydrates more intensively than the latter group. For example, the *Isotricha prostoma* and the small *Dasytricha ruminantium* were able to produce over 270 and 130 pmol short-chain fatty acids per protozoal cell per hour, respectively [44]. Conversely, the production rate of volatile fatty acids by large representatives of the *Ophryoscolecidae* such as the *Eudiplodinium maggii* and the *Diploplastron affine* did not exceed 45 pmol/cell/h [27, 43].

Hydrogen is also released by numerous species of rumen bacteria and fungi [32, 38]. Thus, the rumen can be envisioned as a natural hydrogen-producing bioreactor. It is well known that under natural conditions, all the hydrogen is immediately used by ruminal methanogens to produce methane in energy-yielding processes [6].

Little attention was focused to date on the potential use of the ruminal ciliates as biohydrogen producers and on the possibility to use the biohydrogen for a biotechnological purpose. The best known and the most intensively studied biological processes leading to hydrogen release are bacterial dark fermentation and photofermentation, as well as direct and indirect biophotolysis in microalgae [7, 12, 14, 19, 20, 24]. In this contribution, for the first time, we show that the hydrogen-rich fermentation gas produced by the ruminal *Isotrichidae* ciliates deprived of fungi, bacteria, and methanogens and incubated *in vitro* is able to feed a low-temperature hydrogen fuel cell. The *Isotrichidae* seemed to be more suitable for BHFC experiments than the *Ophryoscolecidae* ciliates because of (i) more intensive carbohydrate metabolism, (ii) better resistance to vigorous shaking, and (iii) better tolerance of incubation with antibiotics (our unpublished data).

A system in which a hydrogen-rich fermentative gas, produced in a bioreactor by microorganisms, is transported to a proper chemical fuel cell and used there as the fuel, is a special case of a microbial fuel cell, but is not often described in the literature [22, 36, 39, 40]. Microbial fuel cells (MFCs) convert the chemical energy of natural, organic compounds directly into electric energy with the aid of living microorganisms. Generally, metabolic cell processes associated with anaerobic oxidation of nutrients, mainly carbohydrates, can supply fuel for direct oxidation in an anode compartment, or they can be a source of electrons directly or indirectly transferred to an anode. To date, several different ways have been proposed to use microorganisms for producing electrical energy [4, 22, 23]. Systems with the hydrogen bioreactor and the chemical fuel cell separated are often considered not to be typical MFCs because the fuel cell is in fact a chemical one. However, the hydrogen is still generated by growing microorganisms [4]. It has been recently proposed to give such systems a separate name: "biohydrogen MFCs" or "HMFCs" [22]. It appeared to us that building a biohydrogen fuel cell (BHFC) around the ruminal ciliates could be interesting as a demonstration because, in contrast to fermentative bacteria, the ciliates are potential producers of a relatively clean hydrogen-rich gas; that is containing only small amounts of substances, such as H_2S harmful to low-temperature fuel cells (our unpublished data). Moreover, it is possible to prepare a methanogen-free suspension of the ciliates with the use of antibiotics [16, 33, 41]. Thus, there is a clear path to clean hydrogen production using the ciliates. No such possibility exists in the case of the rumen bacteria because of their sensitivity to antibiotics.

So far, there has been one report on using the rumen microorganisms, mainly bacteria, to construct a biologically assisted power generation device. Rismani-Yazdi *et al.* [34] contacted a mixture of rumen microorganisms with graphite creating an anode for an all-in-one MFC capable of extracting electrons from cellulose. There was also a concept of using rumen microorganisms as biocatalysts in a RUDAD (rumen-derived anaerobic digestion) process leading to biogas production from various plant materials [13, 18].

MATERIALS AND METHODS

Microbiological Part

The rumen ciliates were isolated from the natural rumen fauna of two rumen-fistulated cows fed a hay concentrate supplemented with sugar beet pulp and molasses, which stimulate the growth of the hydrogen-producing ciliates of the *Isotrichidae* family. The animals were fed twice daily and water was available all day. To prepare a suspension of active ciliates free of fungi, bacteria, and methanogens, 5–6 l samples of the rumen fluid were taken from the rumen about one hour after afternoon feeding. The fluid was filtered through a nylon 250-μm-pore-size filter, and the filtrate was collected in a cylindrical vessel immersed in a water bath at 40°C. This step removed fungi because fungi adhere to food particles in the rumen. Immediately after the collection, the filtrate was saturated with a stream of CO_2 and was allowed to stand for 30 min at 40°C. During this period, the residual food particles floated to the top of the vessel, while the protozoa sedimented at the bottom. The sediment was transferred to a smaller glass vessel and suspended in a warm (40°C) "*caudatum*"-type solution of (g/l) K_2HPO_4 (6.3), KH_2PO_4 (5.0), NaCl (0.65), $CaCl_2 \cdot 6H_2O$ (0.09), $MgSO_4 \cdot 7H_2O$ (0.09), and CH_3COONa (0.75) [5]. The pH of the solution was 6.84. The warm solution used to suspend the protozoa was saturated with a stream of CO_2 to remove the oxygen. The sedimentation procedure was repeated five times to remove the majority of extracellular bacteria and methanogens. Of the sedimented protozoa, the representatives of the family *Isotrichidae* adhered to the walls of the vessel. Because of this, it was

possible to remove the majority of the *Ophryoscolecidae* ciliates by delicate suction. The protozoa of interest were transferred to an incubation flask and suspended in a warm (40°C) culture medium composed of (g/l) K_2HPO_4 (1.0), $NaHCO_3$ (5.0), NaCl (6.0), $CaCl_2 \cdot 6H_2O$ (0.2), and $MgSO_4 \cdot 7H_2O$ (0.2) [15]. The pH of the medium was 7.76. The medium contained chloramphenicol, streptomycin, and ampicillin, each at 50 μg/ml, to eliminate the remaining intra- and extracellular bacteria and methanogens [3, 16, 27, 31], and the suspension was incubated overnight at 40°C under continuous mixing with a stream of CO_2 [28]. On the next day, the protozoa were again separated by sedimentation. The sediment was transferred to a measuring cylinder and diluted with the culture medium saturated with CO_2 (see above). A 5-ml sample of the suspension was taken to determine the number and the viability of the protozoa (see below), whereas the remaining part was divided into 8 equal portions that were put into 8 incubation flasks. The flasks with the suspension of protozoa were immediately flushed with a stream of CO_2 and the suspension was diluted to the final volume of 800 ml in each flask by adding warm (40°C) CO_2-saturated culture medium [15] containing antibiotics (see above) at 50 μg/ml each. The incubation flasks were then placed in two water baths (40°C) and shaken vigorously. A saturated glucose solution (120 g in 100 ml of water) was injected into the flasks in 5-ml/flask doses whenever a lowering in the production rate of gas was noted.

Samples for counting of the ciliates were taken from the freshly prepared suspension, as described above, and from each incubation flask at the end of the BHFC experiment. The samples were fixed with a 4% aqueous solution of formaldehyde added in a 1:1 (v/v) proportion. The ciliates were counted under an optical microscope according to Michałowski [29]. Images of ciliates were taken using an Olympus BX-51 optical microscope equipped with an Olympus DP-50 camera.

Short-chain carboxylic acids accumulated in the culture medium were determined qualitatively and quantitatively according to the gas chromatography method of Ziołecki and Kwiatkowska [48] (Philips, PU 4410, FID detector).

Fermentation Gas Conditioning, Metering, and Analysis

The gas evolving in the individual bottles was combined and, using a peristaltic pump, directed to the anodic chamber of a polymer electrolyte fuel cell (PEFC). Before entering the anodic chamber, the gas was passed through two wash bottles connected in series. The first one was filled with a 1 M aqueous Na_2CO_3 solution, and the second one with 1 M aqueous H_2SO_4 solution. The first bottle served as an absorber of traces of H_2S and carboxylic acids present in the produced gas. In the second bottle, traces of NH_3, also present in the gas, were trapped. H_2S and NH_3 have a detrimental effect on the functioning of the anode catalyst and the polymer electrolyte membrane of the employed PEFC, respectively.

The rate of gas production was determined by metering the gas exiting the anodic chamber of the PEFC under atmospheric pressure and noting the simultaneous current of the PEFC. Using the first Faraday's law, the fuel cell current was recalculated to a rate of H_2 consumption by the PEFC (neglecting traces of carboxylic acids, H_2 was the only "consumable" in the gas). The total rate of gas production was obtained by adding the rate of gas at the anode exhaust to the rate of H_2 consumption in the PEFC.

At specific moments of the experiment, the nonconditioned fermentation gas was collected in gas-tight gas pipettes. H_2, CO_2, and CH_4 were the principal gaseous metabolites quantified by subsequent gas chromatography (Shimadzu GC-14B, Carboxen 1010 PLOT column, TCD detection). In one gas sample, H_2S, short-chain carboxylic acids, and NH_3 were also determined (Hewlett Packard 6890, pre-estrification of acids, polar 0.3-μm capillary column, and FID or AED detection).

Electrochemical Part

The PEFC consisted of a membrane-electrode assembly (MEA) made by applying thin catalyst layers directly to a Nafion 117 membrane (Du Pont de Nemours, Wilmington, U.S.A.) after Wilson and Gottesfeld [47]. The geometric active area of the MEA was 5 cm^2. The anode catalyst used was Pt–Ru black with a Pt-to-Ru atomic ratio of 1:1 (from E-TEK, Somerset, U.S.A.). The catalyst loading in the anode catalyst layer was *ca.* 8 mg/cm^2 (geometric area). The cathode catalyst was Pt black (E-TEK) at a loading of 4 mg/cm^2 (geometric area). The MEA was sandwiched between gas-diffusion layers (carbon cloth-type; E-TEK), serpentine flow fields, and endplates. The latter two were homemade.

The fermentation gas was supplied to the anode as described above. The cathode was fed ambient air, humidified by passing it through a water-filled wash bottle with the use of the peristaltic pump. The temperature of the cell was maintained at 24–26°C.

Two types of polarization tests were run by means of a model 6051A DC electronic load (Agilent, Santa Clara, U.S.A.): (i) application of a constant voltage across the endplates of the fuel cell with monitoring of the current and the effective cell voltage over time, and (ii) steady-state polarization curve recording in the voltage-driven mode. During the latter test, the voltage of the cell was stepped down in 50-mV steps from the open circuit voltage (OCV) to a fixed low-voltage limit. At each applied voltage, the system was equilibrated for 60 s.

The PEFC used in the BHFC experiment was characterized before and after the BHFC experiment by recording steady-state polarization curves using pure hydrogen and pure synthetic air as the anode and cathode gas feeds, respectively. The PEFC temperature, pressure, flow rates, and humidification conditions of the pure gases were the same as during the BHFC experiment. During these polarizations, the internal resistance of the fuel cell was measured using a model 1260 impedance/gain-phase analyzer (Solartron Analytical, Farnborough, U.K.).

RESULTS

Biohydrogen Producers

The following description presents the results of a single representative rumen-BHFC experiment. The BHFC performance shown below is the result of a series of experiments, through which a choice of the right protozoa, an optimization of the PEFC for operation on the fermentation gas, and a matching of the size of the bioreactor and the size of the fuel cell were made.

At the beginning of the BHFC experiment, the total concentration of the *Isotrichidae* ciliates in each flask was *ca.* 2.1×10^4 cells/ml and their total number in all the flasks was *ca.* 1.34×10^8. The ciliates from the genera *Isotricha*

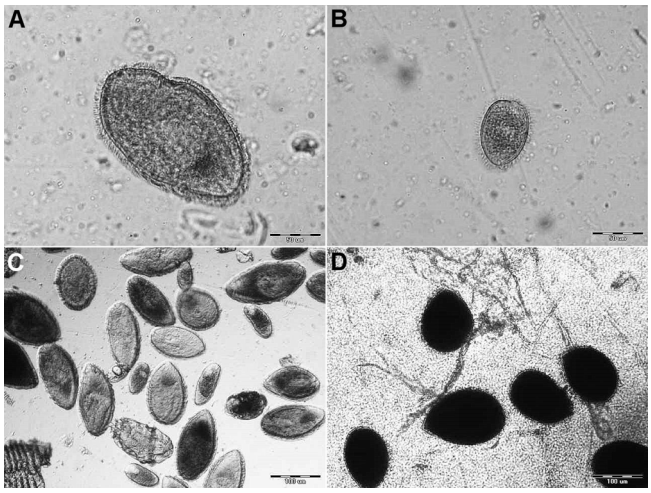

Fig. 1. Microscopic views of *I. intestinalis* (**A**), *D. ruminantium* (**B**), and the mixed population of *Isotrichidae* ciliates used to generate H_2 at the beginning (**C**) and at the end (**D**) of the BHFC experiment.

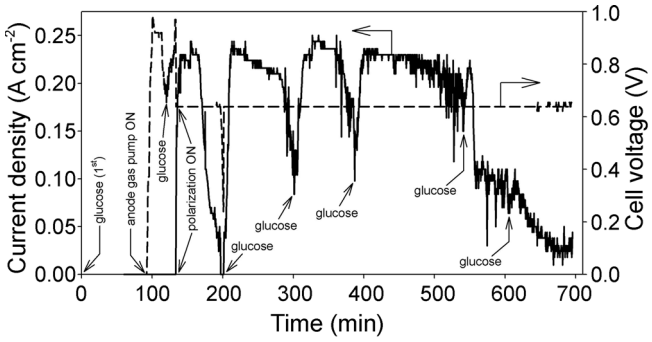

Fig. 2. Time record of the ruminal-BHFC voltage and current density.
The starting moments of anode gas pumping and (constant-voltage) polarization of the PEFC, as well as points of addition of glucose portions to the suspension of the protozoa are indicated by arrows. Plot-attached arrows assign the plots to their appropriate vertical axes. See the Material and Methods section for other details.

(Fig. 1A) and *Dasytricha* (Fig. 1B) constituted about 80% and 20% of the total number of the *Isotrichidae* ciliates, respectively. The ciliates from the family *Ophryoscolecidae* were also present in the suspension of the incubated protozoa. Their concentration at the beginning of experiment was *ca.* 3.5×10^3 cells/ml. Thus, they constituted about 14% of the total number of ciliates in the incubated suspension. Because of their small number and limited ability to utilize simple sugars [44, 46] compared with the *Isotrichidae*, the contribution of the *Ophryoscolecidae* ciliates to H_2 production and, hence, to the operation of the BHFC was likely insignificant. For the methodology, however, it is important to emphasize that the *Ophryoscolecidae* ciliates formed over 90% of the total number of protozoa in the rumen fluid taken from the cows. This shows that the procedure to prepare the suspension of the *Isotrichidae* resulted in elimination of the vast majority of *Ophryoscolecidae* ciliates. Most of the eliminated *Ophryoscolecidae* were the small ciliates from the genus *Entodinium*, which typically constitute 70–80% of the total number of ciliates in the rumen [46]. A microscope image of the mixed population of protozoa used to generate H_2 in the present experiment is in Fig. 1C.

From chromatographic analysis of gas samples collected throughout the BHFC experiment, it was found that the fermentation gas was composed of CO_2, H_2, water vapor, and traces of impurities (see section below on biohydrogen production and condition of *Isotrichidae* ciliates during experiment). Methane was not detected (the detection limit of the method was <50 ppm). This and our previous observation from optimization studies that the rate of gas evolution from such cultures was proportional to the number of ciliates present indicate that the removal of bacteria and methanogens was successful. Other investigators earlier found a similar effect of antibiotics [3, 16, 27, 31, 33, 41]. Thus, in our opinion, hydrogen was indeed produced by the protozoa and not by residual bacteria.

Continuous Operation of BHFC

Changes of the BHFC current density and voltage during the experiment conducted in the constant-voltage regime are in Fig. 2. The experimental time shown on the horizontal axis corresponds to the real time of the experiment minus a number of breaks for glucose addition, polarization curve recording, and fermentation gas sampling. These breaks included, the total BHFC experiment duration was 14 h.

The experiment was initiated by addition of the first portion of glucose to the flasks with the protozoa suspension and this moment corresponds to experimental time 0 min. Gas evolution in the flasks began soon after the first glucose addition. The natural pressure build up in the incubation flasks was not enough to drive the produced gas past the impurity-absorbing wash bottles and into the PEFC. Therefore, at 90 min of experiment, the anode gas began to be forced out of the flasks by means of a peristaltic pump set to a nominal flow of 60 cm^3/min (at ambient pressure). The pump remained on from then on. Note that because the nominal output of the pump was more than twice the effective production of the fermentation gas (see later), the protozoa fermented glucose at a relatively constant reduced pressure of *ca.* 5.5×10^4 Pa. Mandal *et al.* [26] have shown that reducing the total pressure above a culture of *Enterobacter cloacae* enhanced the rate of biohydrogen production. The lowered activity of gaseous metabolites makes the fermentation process more thermodynamically favorable [19, 20]. Such an effect is common for bacterial systems and should be also expected for protozoa. Note that the partial hydrogen pressure in the

rumen is also very low because of the immediate hydrogen consumption by methanogens. The concentrations of H_2, CO_2, and CH_4 in the rumen fluid are 0.6–5.8 μM, 190–2,800 μM, and 25–250 μM, respectively [21]. As a result of switching on the anode gas pump, the OCV of the PEFC immediately rose to 0.93–0.97 V but then started to fall slowly. After addition of a new portion of glucose at 120 min of experiment, the OCV returned to the high values.

At 130 min of experiment, the PEFC voltage was externally lowered to 0.638 V. The fuel cell instantly responded with a current density of *ca.* 0.225 A/cm^2, which remained stable for a period of 40 min. From then on, the current density began to fall and reached 0 A/cm^2 at *ca.* 200 min. Addition of new glucose restored the current density to the previous level. Such depletions of glucose and restorations of the initial current density level were observed two more times, at *ca.* 300 and *ca.* 390 min of experiment. This behavior suggests that the incubated protozoa readily metabolized glucose. After addition of glucose at *ca.* 390 min, the current density remained high for a longer period of time than before (*ca.* 150 min). Moreover the rate of current density decrease was smaller this time. The next glucose addition at *ca.* 540 min caused a brief restoration of the high current density, but after another 10 min the current density suddenly stepped down to *ca.* 0.100 A/cm^2. The last addition of glucose at *ca.* 610 min had a similar effect on the current density. Just after the addition, the current density rose a little, but then it fell quickly to a new low level of *ca.* 0.03 A/cm^2. Since this was a marginal current density value, the experiment was stopped at this point.

Biohydrogen Production and Condition of *Isotrichidae* Ciliates During Experiment

The ruminal BHFC running on glucose could work quite well for some time. However, at a certain point, the performance of the fuel cell decreased abruptly. The measured rate of fermentation gas production together with the rate of H_2 production calculated using the results of chromatographic gas analysis is given in Fig. 3 on the same time scale as the one in Fig. 2. Our preceding studies on obtaining sufficient numbers of protozoa to produce biohydrogen showed that the fermentation gas contained 7% water vapor, 35–40% hydrogen, and 55–60% carbon dioxide (four independent assays).

The changes of the rate of total gas production matched well, in their timing, the changes in current density from Fig. 3. Initially (*i.e.*, until 450 min experimental time), in periods just after fresh glucose addition, the rate of fermentation was steady and high, as assessed by the steady rate of gas production at *ca.* 30 cm^3/min. In these periods, one might expect an approximately linear decrease of the concentration of glucose and, correspondingly, a linear increase of the concentration of the soluble metabolites (short-chain acids). Notably, the addition of glucose at

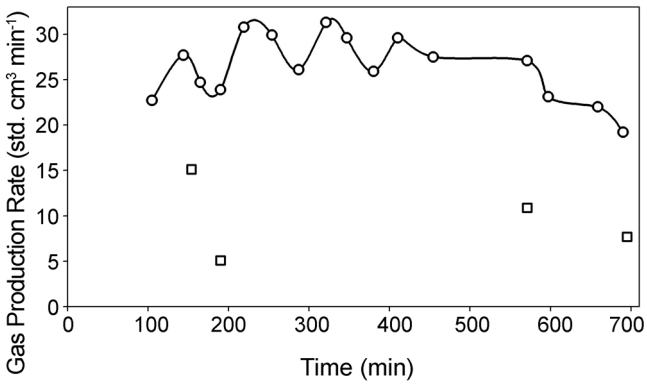

Fig. 3. Time record of the rate of total gas production by the *Isotrichidae* ciliates during the BHFC experiment (circles and line).
H_2 production rate is also shown based on the composition of the gas from chromatography (squares). The experimental timeline is the same as in Fig. 2. See the Materials and Methods section for other details.

540 min of experiment no longer increased the rate of gas production to *ca.* 30 cm^3/min (ambient pressure) as did the previous glucose additions. Towards the end of the experiment, the gas production rate gradually fell despite new glucose was introduced into the flasks. Clearly, the protozoa stopped converting glucose.

From the counting of the ciliates at the end of the experiment, it was found that the total number of protozoa from the *Isotrichidae* family decreased by about 49% to 1.0×10^4 cells/ml and those from the *Ophryoscolecidae* family by about 47% to 1.6×10^3 cells/ml. In Fig. 1D, one can notice the strongly decreased transparency of the still living ciliates at the end of the experiment, and this was due to abnormal synthesis of storage amylopectin. This process, together with the vigorous shaking of the flasks, could result in bursting of many individuals. Microscopic examination of the samples taken from the incubation flasks after the experiment confirmed such an interpretation: numerous loose grains of amylopectin were found in the fluid.

Determination of the major carboxylic acids in the post-experiment suspension revealed 0.0142, 0.0321, and 0.0432 M of acetic, butyric, and lactic acids, respectively. At the beginning of the experiment, no organic acids were detected by gas chromatography. For example, the maximum concentrations of acetic and butyric acids in the rumen fluid of sheep fed a hay-concentrate diet were 0.0794 and 0.0139 M, respectively [30]. The concentration of lactic acid in cattle fed a starch-rich diet may reach 0.018 M [42]. It is noteworthy that this marked increase in acid concentrations was not able to lower the pH in the incubation flasks below the values tolerated by the protozoa (*i.e.*, below 5.0) [46]. The relevant measurements performed at the beginning and the end of the experiment showed that a decrease in the pH, from 7.76 to about 6.90, had taken place during the incubation period. Such a small

acidification of the incubation medium was possible because of the high buffering capacity of the Hungate salt solution used to suspend the protozoa in the bioreactor.

BHFC Performance

From comparison of Fig. 2 and 3, we can conclude that the relative changes in the total gas production caused much higher relative changes of the PEFC current density. A slightly better qualitative correspondence was observed between the current density and the rate of H_2 production. Yet, the fuel cell responded overly also to the changes in the rate of H_2 supply. The supplied stream of H_2 expressed in A/cm^2 was always at least twice the observed current density. This was due to the fact that, by design of this experiment, the fuel cell operated at low relative flows of the fermentation gas, in which H_2 was substantially diluted with CO_2. Such effects were demonstrated before for PEFCs operating at low flows of H_2 diluted with an inert gas [37]. To confirm that the PEFC operation in this BHFC experiment was subject to an H_2 transport limitation, polarization curves were recorded three times during the experiment (Fig. 4). A reactant transport limitation on a polarization curve of a fuel cell is best inferred from the limiting current. The values of the limiting current were 0.33, 0.41, and 0.10 A/cm^2 for the polarization curves recorded at 160, 327, and 603 min of experiment, respectively. After consulting Fig. 3, we see that the limiting current values correlated well with the measured total rates of gas production.

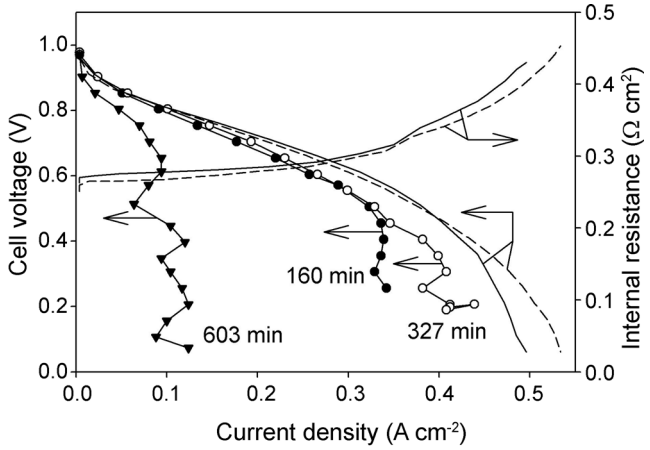

Fig. 4. Steady-state polarization curves of the ruminal BHFC (lines with symbols) and of the PEFC used in the BHFC experiment supplied with pure H_2 and pure air (lines only).
In the case of the PEFC running on the pure gases, a simultaneous record of the internal fuel cell resistance is also provided. The pure-gases PEFC polarization data obtained before (solid lines, no symbols) and after (dashed lines, no symbols) the BHFC experiment is shown. Experimental times (on the timeline from Fig. 2), at which the BHFC polarization curves were recorded, are indicated in the figure. Plot-attached arrows assign the plots to their appropriate vertical axes. See the Materials and Methods section for other details.

It is notable that the BHFC polarization curve at 327 min of experiment did not differ much from the polarization curves recorded with pure H_2 and pure air, particularly in the kinetically controlled region; that is at high cell voltage values. This shows that the PEFC used could operate on the hydrogen-rich fermentation gas just as efficiently as on pure H_2. This is a remarkable result, considering that the fermentation gas contained substantial levels of strong catalyst- and membrane-poisoning substances and that the removal of these impurities from the gas by absorption was certainly not complete. The levels of the impurities determined in one sample of the fermentation gas were 7 ppm H_2S, 190 ppm butyric acid, 10 ppm propionic acid, 6 ppm acetic acid, and 700 ppm NH_3. With these levels of catalyst poisons, a deterioration of performance for typical hydrogen PEFCs would be immediate. Moreover, the low PEFC operation temperature was favorable for poisoning. The resistance of this PEFC to catalyst poisoning can be explained by the fact that the catalysts and loadings used were typical for high-performance MEAs designed to directly oxidize light organic molecules, such as methanol. Hence, the MEA was highly tolerant to catalyst poisons. In the case of NH_3, which has the tendency to bind strongly with protons in the proton-conducting membrane lowering its conductivity, no harmful effect was observed on the time scale of the experiment, probably because of a relatively slow transport of the NH_3 to the membrane. Further confirming the good match between the PEFC used and the ruminal fermentation gas is the fact that the pure-gases polarization curves (including the internal resistance plots) recorded before and after the BHFC experiment almost overlapped (Fig. 4). This showed that the PEFC performance was unaffected by the BHFC operation.

From the best-recorded BHFC polarization curve at 327 min of experiment (Fig. 4), a maximum power density of 1.66 kW/m^2 (PEFC geometric area) was calculated. The value translates to a maximum volumetric power density (bioreactor+PEFC) of 128 W/m^3. This value is comparable with high values obtained with certain MFCs [2], although it is not better than the MFC record values [10, 11]. Since the volume of the PEFC was negligible compared with the volume of the bioreactor, employing a smaller bioreactor and a more concentrated suspension of the ciliates could raise this volumetric power density figure. The overall good BHFC power density in this work was thanks to the good performance of the PEFC (Fig. 4, lines only). It closely matched the performances reported for PEFCs with commercial MEAs tested under comparable operating conditions [17, 35].

Hydrogen Yield and Coulombic Efficiency

The average hydrogen yield in the bioreactor was estimated on the basis of an approximate balance of carbon for the whole experiment involving the products of glucose

fermentation. The balance includes the determined amounts of evolved CO_2 (0.47 mol C) and generated short acids (1.83 mol C) plus the CO_2 dissolved in the bioreactor liquid (0.15 mol C). Assuming no other carbonaceous products of fermentation were formed, the amount of glucose fermented could be calculated (0.47+1.83+0.15= 2.45 mol C=0.41 mol glucose). Considering the total amount of glucose added to the flasks during the experiment (1.07 mol glucose), it was found that 38% of the total glucose was fermented, whereas the rest was either converted to the amylopectin or stayed in solution. The comparison of the amount of evolved H_2 (0.47 mol H_2) with the fermented glucose gave the number of 1.15 mol H_2 per mol glucose. The number is in very good agreement with literature data on glucose fermentation by *I. prostoma* [32].

The estimation of the coulombic efficiency was done by dividing the total charge generated by the PEFC during the whole experiment (0.38 mol electrons) by the maximum charge potentially available from the glucose fermented during the whole experiment, which has been calculated above (0.41 mol glucose×24 mol electrons per mol glucose =9.84 mol electrons). A coulombic efficiency of 3.8% was obtained.

Discussion

The main aims of the present study were (i) checking if a fermentation gas produced by the rumen ciliates is a fuel compatible with a state-of-the-art PEFC and (ii) a comparison of PEFC operation on pure hydrogen and on the fermentation gas. In other words, the PEFC served as a tool for checking the quality of the fermentation gas produced by the rumen *Isotrichidae* ciliates.

In our BHFC studies to date, we tried to generate hydrogen with the aid of the ciliates as well as with the aid of selected anaerobic bacteria fed molasses (unpublished results) and to use the hydrogen as a fuel for the PEFC. From comparison of these two options, it appears that the bacteria give better hydrogen yields and their culture is much more sustainable. At the same time, the fermentation gas is more "dirty" (*i.e.*, it contains higher levels of sulfur compounds and longer-chain acids), so the risk of poisoning the PEFC is higher than with the ciliates.

Both the hydrogen yield and the coulombic efficiency numbers obtained for the described BHFC are low when compared with other studies on biologically assisted H_2 [7, 20] or power generation [39]. This results from two things. Firstly, the energetic efficiency of fermentation is much lower than the energetic efficiency of respiration. The *Isotrichidae* ciliates fermentation is thought to be similar to the *Clostridium*-type fermentation. However, the two enzymes, which are key to this metabolism (*i.e.*, the pyruvate:ferredoxin oxidoreductase and the hydrogenase responsible for conversion of pyruvate to acetyl coenzyme A and hydrogen) are this time located in hydrogenosomes [46]. This type of fermentation (when leading only to acetate, H_2, and CO_2) is capable of providing a maximum theoretical number of 4 mols H_2 per mol glucose, or one third of all electrons available from glucose [7, 20]. In our case, in which longer chain products of glucose fermentation were formed, roughly only one twelfth of all electrons available from glucose were recovered as H_2 in the bioreactor. Production of electricity in systems employing fermentative organisms is commonly associated with coulombic efficiency numbers of less than 10% [25]. Secondly, the PEFC converted only a fraction of its H_2 supply to electricity (about a half). The rest exited to the atmosphere. The utilization of H_2 in the PEFC could be raised by adequate system design.

After a period of relatively steady metabolic activity of the protozoa culture, we have observed a significant slowing of the overall metabolism that caused a drop of the BHFC performance. The possible reasons for it were (i) an overly accumulation of storage carbohydrates (protozoan amylopectin) inside the ciliate cells, and (ii) accumulation of soluble metabolites (*i.e.*, the short-chain carboxylic acids) in the suspension [33, 41, 45]. It has been suggested in the literature that the *Isotrichidae* ciliates lack a mechanism to regulate the storage of amylopectin, which may lead to rupture of the cells when they are incubated with sugars at high sugar concentrations [33, 41]. About 75–80% of the sugars taken up by the *Isotrichidae* can be converted to amylopectin [44]. During *in vitro* fermentation of glucose by *I. prostoma* and *D. ruminantium*, Prins and van Hoven [33, 41] have also observed high concentrations of lactic acid. Under natural conditions, the released acids are absorbed *via* the rumen wall or utilized by other microorganisms inhabiting the rumen. No such lowering mechanisms were present in our experiment and this had to result in increase of the acids' concentrations. We postulate that if any inhibitive effect of the acids was present, it could have been through specific biochemical interaction of excess lactic or butyric acid with the metabolic processes of the protozoa, rather than through lowering of the medium pH. Our result is in line with observations of others that the *in vitro* conditions are generally not favorable for these ciliates [46]. One should remember that the rumen is a specific ecosystem with strong symbiotic relations between all the rumen microorganisms. These relations are often poorly known.

Looking for traces of impurities accumulating on the PEFC electrodes, an additional sensitive test was run. Cyclic voltammetry of the PEFC anode and cathode was recorded immediately after the BHFC experiment (data

not shown). Results of the test showed that the electrodes were free of any strongly adsorbed impurities that influence current production.

The presented BHFC performance was equal to the performance of the PEFC running on pure hydrogen. However, considering the entire system and looking at it critically, we faced the problems of the low coulombic efficiency, the short life time related to the lack of sustainability of the *Isotrichidae* ciliates culture, and an overall negative energy balance due to the need of shaking, pumping, and maintaining the bioreactor temperature at 40°C. Furthermore, the use of antibiotics to eliminate methanogenesis is a serious limiting factor for a hypothetical application of those rumen ciliates because of environmental concerns. Unfortunately, there are currently no other methods to keep down the symbiotic methanogens.

From the point of view of a potential application, the problem of utilization or recycling of nongaseous fermentation products is also vital. The same problem is also faced in biohydrogen production from bacterial fermentation and several solutions have already been proposed, such as the use of the soluble products as raw materials in subsequent chemical syntheses or as substrates for other biological processes.

Nevertheless, in this contribution, a fundamental possibility of using the ciliates as producers of biohydrogen that can be used for a technical purpose (electricity generation) was demonstrated. We hope this work will direct the attention of the biological community towards the rumen microorganisms and their potential role as biohydrogen producers. In our view, the effort should go in the direction of employing the ruminal *Ophryoscolecidae* ciliates, because they are capable of generating hydrogen from starch and cellulose.

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