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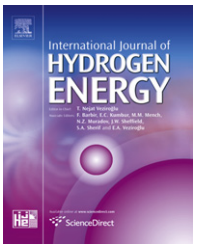

Selection of natural bacterial communities for the biological production of hydrogen

Rodrigo Enrique García [a,*], *Verónica Laura Martínez* [a], *Juan Isidro Franco* [b], *Gustavo Curutchet* [c]

[a] *General Directorate for Research and Development, Argentine Navy, Laprida 201, B1638AEJ, Vicente López, Buenos Aires, Argentina*
[b] *Hydrogen PEM Fuel Cell Laboratory, Institute of Scientific and Technological Research for Defense, San Juan Bautista de La Salle 4397, B1603ALO, Villa Martelli, Buenos Aires, Argentina*
[c] *Environmental Analysis Laboratory, General San Martin National University, Colectora de Av. General Paz 5445 between Albarellos and Av. de los Constituyentes, Quonset 23, B1650KNA, San Martín, Buenos Aires, Argentina*

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ABSTRACT

Current processes used for the production of hydrogen consume a great part of the energy they produce and/or depend on fossil fuel consumption, making them inefficient and harmful to the environment. Obtaining hydrogen from living systems by fermentation of organic matter considered waste is a promising alternative for the future. Especially when you take into account that the biological production of hydrogen is intrinsically linked to the degradation of said organic matter. In this paper, we explore the efficiency of different bacterial communities (also called consortia) for anaerobic fermentation of carbohydrates. The evaluated consortia were obtained from soil, commercial compost and sludge from a sewage treatment plant. The cultures that produced the highest amounts of hydrogen were those in which the inoculums used came from sludge and compost. Both reached a maximum accumulated concentration of approximately 30% of biological hydrogen in the gas mixture on day 8 of the fermentation process, as estimated by gas chromatography.

1. Introduction

Hydrogen gas is an energy carrier; this means that it can store energy for later use. Systems that function on H_2 energy are clean since its combustion produces mainly water vapor as waste material. It is non-toxic to living systems if it leaks to the atmosphere and it does not pollute soil or ground water. It can be used to generate power in fuel cells, which are more efficient than internal combustion engines [1].

But this gas does not occur naturally in its molecular form on Earth; therefore, all H_2 must be produced. There are several resources available to produce it, for example natural gas and coal. The most attractive production technologies are the sustainable ones which include resources that are renewable such as solar, wind, hydro, geothermal and fermentation of biomass. This diversity of production methods and applications turns H_2 into a great promise as an energy carrier for the future [2].

One of the processes that is emerging as very important is anaerobic fermentation. This is a metabolic organic matter degradation process performed by certain living systems. It produces energy for the microorganism and methane that is

* *Corresponding author*. Tel./fax: +54 (0) 114797 5346.
E-mail addresses: rodrigo.egarcia@gmail.com (R.E. García), vmartinez@citedef.gob.ar (V.L. Martínez), hfasoli@citedef.gob.ar, jfranco@citedef.gob.ar (J.I. Franco), gcurut@gmail.com (G. Curutchet).
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released to the environment, along with other products among which is H_2. This type of fermentation is called *methanogenic* or *anaerobic digestion*; given that it is the metabolic path of choice when oxygen is not present as a final electron acceptor. Some bacteria that exist and are capable of fermenting this pathway are obligate anaerobes, and some others are facultative anaerobes. These naturally occurring bacterial communities, called consortia, are able to degrade biomass to a gaseous mixture of compounds by acting in successive steps; in this way, the waste for some is the substrate for others (Fig. 1) [3]. The H_2 producing bacteria can be naturally found in environments where this type of degradation occurs, along with other species of bacteria.

In this work, three types of consortia were evaluated: soil collected from the DGID grounds, commercial compost used as fertilizer and sludge from a wastewater treatment plant located on a corvette from the Armada Argentina (Argentine Navy). A high ratio of H_2 producing bacteria is expected to exist in these consortia because they are all anaerobic environments, where organic matter degradation naturally occurs [4,5]. Some of the most well-known bacterial species that produce hydrogen and have already been identified in other studies are expected to be found in these consortia, such as *Clostridium* and *Enterobacter* [6].

After a heat pre-treatment [7] and incubation under anaerobic conditions, we expect to eliminate most methanogenic species and retain fermentative sporulating bacteria able to produce H_2, since the former synthesize methane by incorporating hydrogen, and therefore, decrease the H_2 content in the gas mixture.

The most efficient consortium will be selected out of the three, and a production optimization phase will begin along with a second line of research in collaboration with CITEDEF (Institute of Scientific and Technological Research for Defense).

2. Materials and methods

2.1. Bacterial consortia

Consortia selection criteria consisted of non-sterile easy to obtain materials that occur in nature and where there is a high degree of anaerobiosis, preferably with active degradation. That is why we selected a sample of soil collected at the DGID premises at a depth of about 15 cm [4], commercial compost used to fertilize crops and sludge from a sewage treatment plant [5]. This plant is located on board the "Guerrico" corvette that belongs to the Argentine Navy.

2.2. Pre-treatment

To eliminate as many unwanted microorganisms as possible, such as methanogenic bacteria, and to select spores from the hydrogen producing bacteria the consortia were pretreated with heat, submerging them in a bath at 70 °C for 50 min.

2.3. Incubation

The pre-treated broths were added to 100 ml of rich growth medium with sucrose as carbon source.

This growth medium was prepared using 30 g of sucrose, 2 g of ammonium bicarbonate, 1.2 g of monopotassium phosphate, 200 mg of magnesium sulfate heptahydrate,

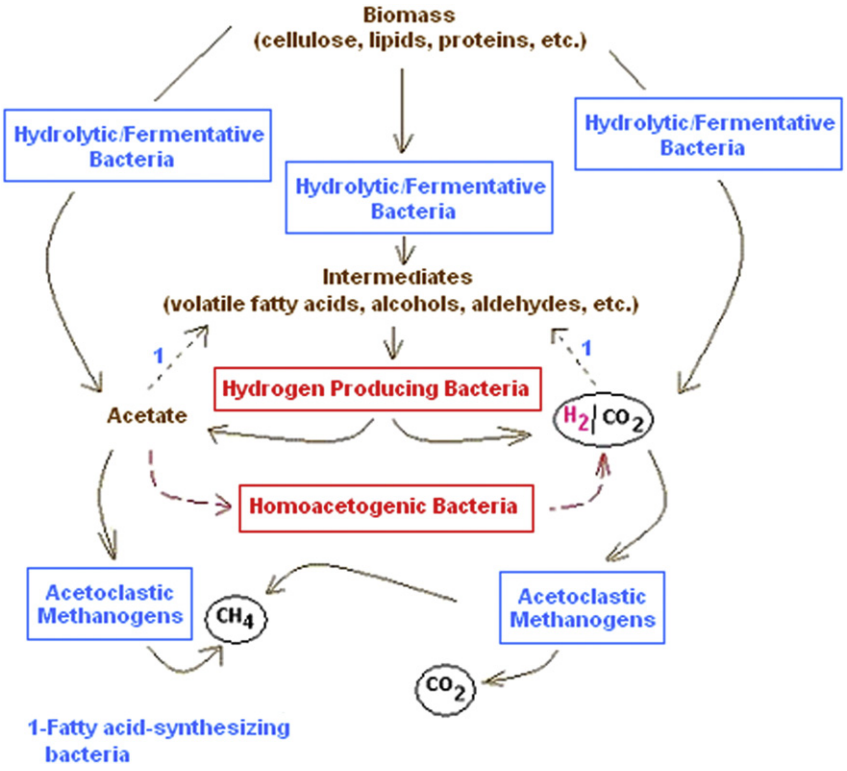

Fig. 1 – Carbon flow in methanogenesis, also called anaerobic digestion.

200 mg sodium molybdate dihydrate, 200 mg calcium chloride dihydrate, 200 mg of manganese sulfate monohydrate and 200 mg of iron per liter. The solution was buffered with 0.05M (molar) MES (J.T. Baker, Phillipsburg, NJ).

These trials were performed in triplicate, plus one control (blank). "Blank" trials contained bacteria but no carbon source or nutrients; this means they were diluted in the same volume of water. Such preparations were poured into 500 ml Erlenmeyer flasks and the oxygen content was removed by a purge with nitrogen gas. Then, the flasks were sealed with airtight rubber stoppers and fabric electrical tape.

They were kept in an incubator at 38 °C until days 2 (48 h), 3 (72 h), 7 (168 h) and 8 (192 h) when they were transferred to another laboratory for chromatographic determination of the percentage of biological hydrogen in the gas mixture. The day when preparation and initiation of cultures took place was recorded as day 0 (0 h). Due to time constraints inherent to the other mentioned laboratory, the chromatographic analyses were performed on the day of transfer for all samples, except for the detailed below:

- Sludge samples, day 3 (72 h): of all four flasks, two samples were analyzed the day of transfer; the third sample and the blank were analyzed the following day.
- Compost sample, day 3 (72 h): all samples were analyzed one day after the transfer.
- All samples for all consortiums removed from the incubator on day 8 were analyzed on day 11.

The flasks that were not analyzed the same day of transfer were stored at room temperature until measurement.

2.4. Percentage of H_2

2.4.1. Chromatography

The percentage of hydrogen in the gas mixture was determined by gas chromatography on a Hewlett Packard 5890 chromatograph, in the Analytical Services Laboratory of the National Atomic Energy Commission (CNEA), using nitrogen gas as carrier and a column HP- MOLESIEVE 30 m × 0.536 mm × 50.00 microns. This column is nonpolar and it can detect hydrogen, among other gases such as neon, argon, oxygen, nitrogen, methane and carbon monoxide. For each sample the maximum concentration of H_2 was observed after 1 min and a half since the start of the analysis, ending the run after 3 min. A sample of gas from an Erlenmeyer inoculated with compost was run for 1 h to see other possible compounds in the mixture.

2.4.2. Volume in syringe

This method involves the direct procedure of extracting gas from a flask with a 30 ml syringe and immediately injecting it in a PEM cell of 0.5V ± 0.01 V connected to an electric motor. The time the engine takes to stop and the final volume of gas in the syringe are recorded. A simple equation yields the percentage that the consumed gas represents. Both duplicates were measured using this method; the blanks were not evaluated. Furthermore, this method was validated in a previous study work [8].

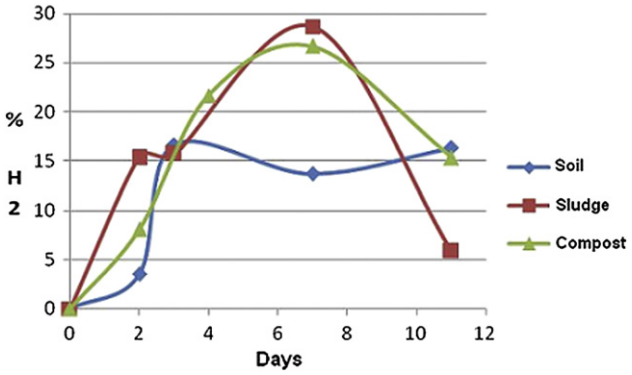

Fig. 2 – Percentage of H_2 gas present in culture flasks of bacteria harvested from different sources. Red Squares: sludge from waste water treatment plant. Green triangles: compost. Light blue diamonds: Soil. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.5. Functional evaluation of H_2

To evaluate the possibility of using the H_2 generated by bacteria in a practical application the gas was fed directly into a PEM fuel cell of 0.5V ± 0.01 V manufactured by the company *Horizon Fuel Cell Technologies*. The gas was extracted from the flasks piercing the stopper with a syringe and injecting it into the cell which was connected to a small electric motor.

2.6. Viability in time

An aliquot consisting of 10 ml of sludge was incubated for 120 h (5 days) in 100 ml of growth medium prepared in the manner described above. After this time, the H_2 content was evaluated by the volume in the syringe method, and another aliquot of 10 ml of this culture was inoculated in fresh growth medium. This method of H_2 percentage evaluation and aliquoting was repeated for more than 90 days according to the following scheme. Stock culture 1 was directly inoculated with fresh sludge from the treatment plant and aliquoted regularly over a period of 2088 h (95 days). The 10 ml aliquot used to start Stock 2 was obtained from a previous subculture, frozen a month earlier. This culture was aliquoted regularly over 2328 h (97 days).

Table 1 – Mean of the percentage of H_2 gas present in flasks with bacterial cultures harvested from different sources.

Day	% H_2 (soil)	% H_2 (sludge)	% H_2 (soil)
0	0	0	0
2	3.50	15.33	8.10
3	16.67	15.70	N/A
4	N/A	N/A	21.67
7	13.67	28.67	26.67
11	16.33	5.87	15.33

Blanks not shown. N/A: not available (days that were not included in the analysis scheme).

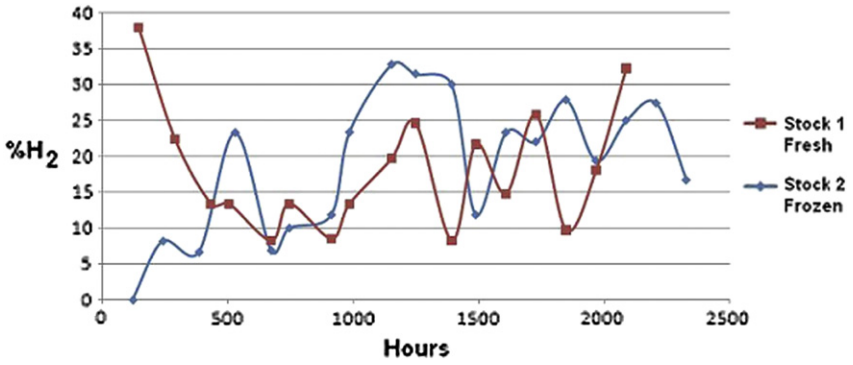

Fig. 3 – Two cultures remained viable and produced H_2 for over 90 days. Red squares: Stock 1, aliquot of fresh sludge maintained for 95 days. Light blue diamonds: Stock 2, aliquot of frozen sludge maintained for 97 days. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.7. *Temperature*

To evaluate the effect of temperature on the culture an aliquot of the sludge from the treatment plant was incubated in fresh growth medium in the manner described above. The Erlenmeyer flasks were incubated for 192 h (8 days) at the temperatures of 35 °C and 45 °C, and for 216 h (9 days) at 38 °C. We evaluated the percentage of H_2 at different times through the method of measuring the volume in the syringe.

2.8. *Carbon source*

To evaluate the possibility of the consortium to grow and produce H_2 on another substrate, Erlenmeyer flasks were inoculated in the same manner as described in 2.3 but replacing sucrose with glucose in the medium and incubating for 192 h (8 days). The percentage of H_2 in these flasks was analyzed through chromatography and the method of measuring the remaining volume in the syringe.

3. Results and discussion

3.1. *Selection of consortia*

Results of chromatographic analysis showed the expected behavior for the blanks with an H_2 percentage that did not exceed 0.15% in any flask, being in most cases less than 0.05%.

To construct the graphs of H_2 evolution (Fig. 2) the mean of the three triplicate flasks was calculated for each point, excluding blanks (Table 1). There was only one point deviated from the mean, which occurred on day 2 in a flask with soil, which was excluded from the analysis according to the Grubbs test for outliers ($P < 0.05$).

The trend observed for the compost and sludge consortia is an increase of accumulated hydrogen in the flask until it reaches a maximum and then decreases. The trend for the soil consortium is an increase of accumulated hydrogen until a plateau is reached (which is less than the other consortia) and stays stable.

Sludge consortium from the water treatment plant was the most efficient since it reached 50% of its production (14.34% H_2 in the gas mixture) in the shortest time. That is, before day 2 as shown in Table 1.

The soil consortium was the least efficient, showing a 3.5% of H_2 in the gas mixture on day 2.

Furthermore, the sludge was also more effective in reaching the maximum on day 7, with an average of 28.67% of H_2. The maximum obtained with the compost consortium was slightly lower with 26.67% on day 7. The soil consortium was also the least efficient, showing a 3.5% of H_2 in the gas mixture on day 3.

The results show a significant decrease in biological hydrogen production on day 11 for the sludge (5.87% of H_2) and compost (15.33% of H_2). This happens despite the fact that the flasks remained airtight. The concentration of H_2 for the soil consortium remained relatively constant from day 3 to day 11 when you can see a percentage of H_2 of 16.33%.

The 1 h chromatographic analysis did not detect other compounds in the gas mixture.

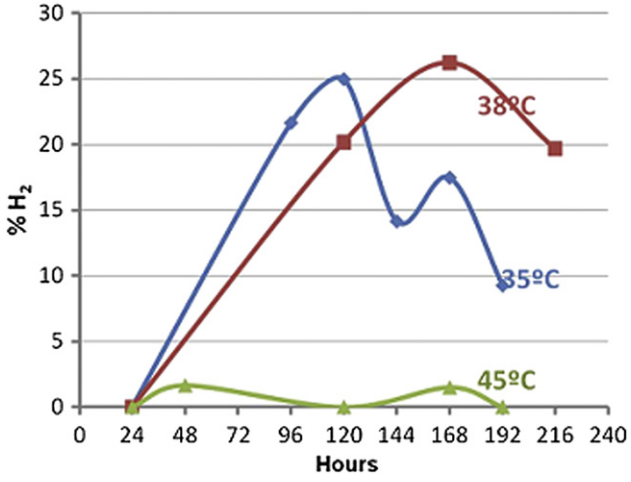

Fig. 4 – Comparison of the effect of temperature on the sludge consortium. All consortia started from a frozen aliquot. Red squares: 38 °C. Light blue diamonds: 35 °C. Green triangles: 45 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

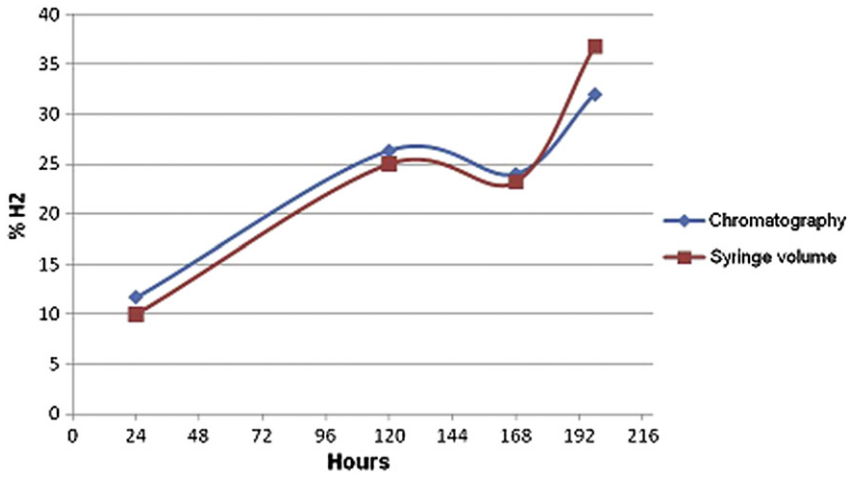

Fig. 5 – Hydrogen percentage from a culture grown on glucose. Values in Table 2. Light blue diamonds: Percentage measured by chromatography. Red squares: Percentage assessed according to the method of volume in syringe. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The biogas extracted from the flasks and injected into the PEM fuel cell manufactured by the company Horizon Fuel Cell Technologies, actually did run the electric motor connected to it, as it was confirmed by direct observation.

3.2. *Viability of cultures in time*

As can be seen in Fig. 3, both stock cultures 1 and 2 remained viable and capable of producing hydrogen for over 2000 h (95 and 97 days, respectively). In both cultures there is an oscillation in the concentration of accumulated hydrogen, high concentration values can be appreciated, followed by low values and then high values again.

It also clearly shows that the culture initiated from fresh sludge immediately produced a considerable amount of H_2, while the culture started from a frozen aliquot starts producing H_2 after the first 120 h.

3.3. *Effect of temperature*

Fig. 4 shows that the sludge consortium stops producing H_2 almost completely at a temperature of 45 °C.It is very likely that this is due to the death of the bacteria that make up the consortium.

At 38° and 35 °C an optimum production is observed. At 35 °C the percentage of accumulated H_2 reaches the 25% maximum after 120 h, while at 38 °C it takes 168 h. However, at 35 °C the accumulated percentage is significantly reduced to less than 10% at 192 h, compared to 20% at 216 h for 38 °C.

Table 2 – Hydrogen percentage values produced from a sludge culture on sucrose, as measured by chromatography and the remaining volume in a syringe method, as described in Materials and Methods.

Flask	Time (hrs)	% H_2 (chromatography)	% H_2 (syringe volume)
1	24	11.67	10
2	120	26.33	25
3	168	27	23.2
4	192	32	36.8

3.4. *Glucose as carbon source*

Sludge consortium can effectively grow and produce H_2 using glucose as a carbon source, as was expected (Fig. 5, Table 2).

In this experiment we observed a steady increase in the amount of H_2 accumulated inside the flask throughout the whole incubation period (192 h), without actually seeing a decrease as seen in other experiments above (for example, incubation with sucrose at different temperatures, Fig. 4).

In this experience, the concurrency in the percentage values obtained by chromatography and by the syringe method can also be appreciated.

4. Conclusions

The obvious conclusion that these results show is that the selected consortia are indeed capable of producing hydrogen using sucrose as an energy source, and also glucose in the case of sludge. It was found that the gas mixture can generate electricity when fed directly into a PEM fuel cell. The possibility of directly using biological H_2 without purification could be highly advantageous as it avoids the cost of processing and purification.

The consortia that reached a higher volume of accumulated H_2 were sludge from a water treatment plant and commercial compost. It was decided to focus the study in the sludge consortium which was the one that displayed a high production speed and accumulated the largest quantity of H_2. The high production capacity observed with the sludge consortium is probably due to the fact that these organisms are, to a greater or lesser extent, subjected to an anaerobic degradation process in their natural environment, making its culture already enriched in anaerobic bacteria whose anaerobic metabolism continues in the experiments, instead of

having to "adjust" from a more aerobic environment, as is the case of compost and soil.

Growth and viability observed over three months suggest that there is a high possibility of growing this consortium in a continuous bioreactor. It would be possible to optimize the biological production of hydrogen to steadily obtain a high percentage, and based on preliminary results, the key to achieving this would be in controlling pH.

The temperature chosen for the trials was 38 °C, although 35 °C is also interesting. More research would be needed in the future to determine what happens to production over longer periods of incubation and ambient temperatures.

Furthermore, it would also be interesting to observe a prolonged incubation with glucose as carbon source, because this work does not show a decrease in hydrogen production in this medium. One possible explanation is that glucose is a more direct source of carbon than sucrose.

In the future, the incubation conditions will be changed to determine the most favorable ones. Glucose as carbon source will be evaluated and different initial conditions of pH and culture temperature will be assessed. This will allow the culture to become enriched with the most appropriate microorganisms and to select growth conditions that maximize production.

Finally, the calibration of an experimental method of measuring the percentage of H_2 using a PEM fuel cell will be designed. To do this, the data obtained from the measurements of H_2 performed with the cell will be recorded and checked against the results of the analysis of the same samples by gas chromatography.

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