

Biohydrogen production in a continuous liquid/gas hollow fiber membrane bioreactor: efficient retention of hydrogen producing bacteria via granule and biofilm formation

Marie Renaudie^{a,b}, Christine Dumas^a, Stéphane Vuilleumier^c, Barbara Ernst^{a*}

^aUniversité de Strasbourg, CNRS, IPHCUMR7178, F-67000 Strasbourg, France

^bFrench Environment and Energy Management Agency, Angers, France

^cGénétique Moléculaire, Génomique, Microbiologie (GMGM) UMR 7156 CNRS-
Université de Strasbourg, Strasbourg, France

*Corresponding author: Laboratoire de Reconnaissance et Procédés de Séparation
Moléculaire (RePSeM), IPHC, UMR CNRS 7178, ECPM 25 rue Becquerel, 67087
Strasbourg Cedex 2, France, barbara.ernst@unistra.fr

Keywords

Hydrogen, bioreactor configuration, submerged membrane, hydrogen producing
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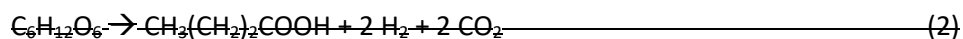
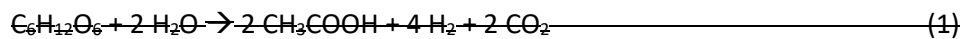
Abstract

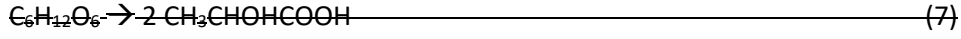
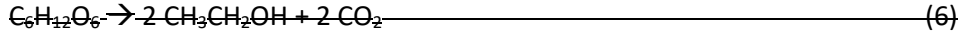
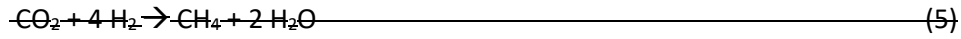
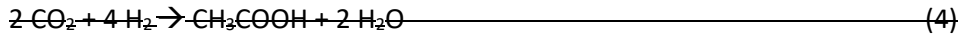
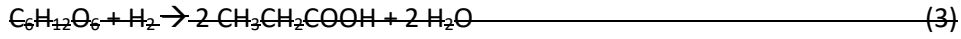
The aim of this work was to develop a continuous liquid/gas membrane bioreactor (L/G MBR), i.e. a fermenting module including hollow fibers for membrane for L/G separation, for biohydrogen production by dark fermentation. Originally seeded with sludge from a wastewater treatment plant, the L/G MBR underwent a complete stop for eight months. It was then operated without further reseeded. In the present experiment,

performed 551 days after the last reseeded, average hydrogen yield of 1.1 ± 0.2 mol per mol glucose added and hydrogen productivity of 135 ± 22 mL/L/h were reached, with acetate and butyrate as the main metabolite products. DNA sequence analysis revealed that *Clostridium beijerinckii*, *Clostridium pasteurianum* and *Enterobacter* sp. were dominant in liquid outlet, in a biofilm on the surface of the hollow fibers and in microbial granules. The L/G MBR has potential for the concentration and the long-term maintenance of an active hydrogen-producing bacterial community without need for reseeded.

1 Introduction

Dark fermentation is a process by which hydrogen is produced by bacteria from biomass under anaerobic conditions. It has been extensively studied at lab scale for the valorization of organic waste at mesophilic temperature without needing a light source (Etchebehere et al., 2016; Guo et al., 2010). *In vivo*, a hydrogen yield (HY) of 4 mol_{H₂}/mol_{hexose} is expected through the acetate pathway (Eq. (1)). With mixed culture, multiple metabolic pathways are coexisting in the reactive medium reducing the HY (Rafieenia et al., 2020). Butyrate pathway, as acetate pathway, is co-producing hydrogen with a lower HY (2 mol_{H₂}/mol_{hexose}) (Eq. (2)). Metabolic pathways for propionate production (Eq. (3)), acetate production by homoacetogenesis (Eq. (4)) or methane production (Eq. (5)) are hydrogenotrophic (consume the produced hydrogen). Finally, other metabolic pathways are concurrent from the substrate point of view (consume the substrate without hydrogen coproduction) i.e. solvent production (mainly ethanol (Eq. (6))) or lactate (Eq. (7)). By consuming the substrate or the hydrogen, all these various pathways are decreasing the final HY obtained.





Previous studies at pilot scale have focused on the use of co-cultures or mixed cultures, either by dark fermentation alone (Kim et al., 2011; Ren et al., 2006), in combination with photo-fermentation to maximize the hydrogen yield (HY) (Zhang et al., 2018), or together with anaerobic digestion to enhance methane production (Lee and Chung, 2010).

The retention of hydrogen-producing bacteria (HPB) in continuous systems (Li and Fang, 2007), and the prevention of changes in microbial composition (Badiei et al., 2011) represent major challenges for long-term operation of bioreactors for hydrogen production. An efficient gas extraction is essential to avoid the inhibition of hydrogen production due to its accumulation in the bioreactor (Clion et al., 2015; Kim et al., 2006).

Diverse membrane-based technologies have been used to address these bottlenecks. An extra membrane gas separation unit (gas-gas separation) in order to separate the hydrogen from the carbon dioxide produced can be put in series with a bioreactor without direct impact on bioproduction except that of gas extraction and the possible CO₂ recycling for enhanced mass transfer via bioreactor gas sparging (Bakonyi et al., 2018, Nemestothy et al. 2020). Solid/liquid membrane bioreactors (MBR) allow to decouple HPB retention from the hydraulic retention time (Kim et al., 2011; Lee et al., 2010; Shen et al., 2009). However, membrane fouling (Lee et al., 2010; Oh et al., 2004; Shen et al., 2010, 2009), likely the result of adhesion of polymeric substances of

microbial origin at the membrane surface (Jung et al., 2011), often leads to bioreactor operation failure. Dynamic membranes, consisting of a biofilm on a solid/liquid microfiltration membrane may limit membrane fouling (Park et al., 2017). Liquid/gas membrane bioreactors (L/G MBR), with a hydrogen-permeable membrane located within the culture medium of the bioreactor were developed to mimic the action of H_2 consuming methanogens (Zheng et al., 2010). In another study, the use of a hollow fiber membrane improved hydrogen yield by 9%; however, the biogas diffused in the lumen of hollow fibers had to be removed by a vacuum pump, with additional energy cost (Liang et al., 2002). In order to meet both requirements of *in situ* hydrogen extraction and bacterial immobilization, a continuous liquid/gas membrane bioreactor (L/G MBR) composed of a hollow fiber module was recently developed in our laboratory (Ernst et al., 2015). The specificity of this reactor is to integrate in a single unit the bioproduction of hydrogen in the shell part of the module and the extraction of the latter through the membrane in the lumen of the fibers; this system then constituting a gas-liquid contactor.

In this study, we investigated biohydrogen production performance of the submerged hollow fiber L/G MBR, and the potential role of the membrane as a support for the retention of hydrogen-producing bacteria, by documenting bacterial composition over time, and in the different sections of the bioreactor.

2 Materials and methods

2.1 Bioreactor setup and operation

The L/G MBR (0.5 L working volume, Figure 1) consisted of a cylindrical-shaped hollow-fiber membrane module. The hollow fibers (pore diameter: 0.1 μm), usually

used for ultrafiltration, were made of polytetrafluoroethylene and sealed at each extremity of the membrane module. The useful external surface of the fibers is evaluated at 0.195 m².

The composition of the feed solution was as follows: 12 g/L glucose, 1.8 g/L KH₂PO₄, 35 g/L (NH₄)₂ SO₄, 2.5 g/L FeSO₄, 7H₂O, 0.5 g/L MgSO₄, H₂O, 0.5 g/L NiCl₂, 6 H₂O. The feed solution was filtered on a Büchner filter with pore size of 40µm and stored at 4°C. A stirred tank was used for storage of feed solution and kept cold (6°C). The feed solution was swept by N₂. Injection of feed solution into the bioreactor circuit was achieved using a peristaltic pump. Time zero of the fermentation experiment corresponded to the end of the filling phase of the entire circuit. The bioreactor was operated by continuous feeding throughout the experiment, applying a hydraulic retention time (HRT) of 12.5 h and an organic loading rate (OLR) of 0.9 g-glucose/L/h. During steady-state, NaOH (0.5 M) was added continuously to the liquid medium using a peristaltic pump, and its speed was adjusted manually as required to maintain the pH of the liquid outlet at 4.8 ± 0.1. The pH was measured using a multi-parameter analyzer. Temperature in the reactor was maintained at 37°C by heated water circulating within a heating coil placed around the membrane module.

The bioreactor had originally been seeded with heat-treated activated sludge from the anoxic zone of the Strasbourg Eurometropole wastewater treatment plant. Fresh activated sludge was collected and introduced in a stirred tank reactor equipped with a double jacket connected to a thermostated bath (Bioblock Scientific Polystat 5A). The sludge was agitated at 220 rpm in order to maintain the suspension and went through a thermal treatment at 70°C during 1 h under static air. At the end of the heat treatment, the temperature was then regulated at 37°C, the reactor was wrapped with an aluminum foil to be in dark conditions and the reactive medium was flushed by nitrogen to be

under anaerobiose. A model substrate (1.04×10^{-2} mmol/L of glucose) was used to feed the bacterial consortium. After 5h of fermentation, the hydrogen production was at its maximum and the reactive medium, adapted to dark fermentation was transferred in the virgin MBR where the fermentation occurs then in continuous (with a model feeding solution) for the first time. The bioreactor had been operated without reseeded for a series of successive fermentation tests lasting 7 to 15 days and then left unused for eight months. The experiment reported here describes the restart of hydrogen production 551 days after the original seeding of the membrane module, with eight months of process interruption after the last seeding.

In order to assess the bacterial development on the outer surface of the membrane without sacrificing the membrane module, three hollow fibers were added to the membrane module (figure 1) before the experiment to be cut after the experiment and used for the subsequent microbiological analyses. As the supplementary fibers were connected to the gaseous outlet, with the same orientation, no perturbation to the usual operation of the L/G MBR was expected, in terms of gas extraction and hydrodynamic behavior. Also, the induced augmentation of the total surface of the membrane was negligible when compared to the original total surface of the membrane; therefore no augmentation of the hydrogen production was expected.

2.2 Chemical analyses

2.2.1 Gas analyses

Gases in the gaseous outlet circulating in the lumen of the hollow fibers were analyzed by thermal conductivity detection using a gas microchromatograph (T3000, SRA, France), equipped with a molecular sieve column of 0.5 nm (10 m x 0.32 mm, 30 mm) and a PoraPLOT U column (8 m x 0.32 mm, 10 μ m) for the separation of N₂, CH₄, O₂,

H₂ and CO₂. Series of 3 analyses were performed every 10 min, with a sampling time of 10 s.

2.2.2 Organic acids and alcohols analyses

The liquid outlet was sampled at time zero, then during the fermentation (twice a day) and centrifuged (4500 rpm, 30 min). Supernatant and pellet were stored separately at -18°C.

Organic acids (citrate, pyruvate, succinate, lactate, formate, acetate, propionate, butyrate, valerate and isovalerate) and glucose were analyzed simultaneously by high pressure liquid chromatography using a variable wavelength detector and a refraction index detector. Supernatant samples were thawed, centrifuged for 15 min at 15000 rpm, and filtered at 0.2 µm. 20 µL of the resulting samples were injected onto a chromatographic column HiPlex-H (7.7 m×300 mm, 8 µm) (Agilent Technologies) heated at 65°C, eluted isocratically with 5 mM aqueous H₂SO₄ at 0.4 mL/min. Each sample was analyzed twice.

Alcohols (ethanol, butanol, propan-1-ol) were analyzed by gas chromatography using a flame ionization detector (7890A GC, Agilent Technologies). Helium (0.6 mL/min) was used as a vector gas. Supernatants were centrifuged during 15 min at 15000 rpm then filtered at 0.2 µm and diluted in an internal standard. The resulting mixture was injected (0.5 µL) in split mode onto a chromatographic column HP-INNOWax (30 m x 0.250 mm, 0.5 µm) (Agilent Technologies) heated at 45°C, eluted isothermally for 4 min and then by a 10°C/min ramp up to 200°C. Each diluted sample was analyzed 4 times.

2.3 Microbiological analyses

2.3.1 Sampling

Upon completion of the experiment (92 h), 7 mL of liquid outlet were sampled, the membrane module was totally emptied by the bottom opening (Figure 1), and the granules in the liquid fraction were collected. The additional hollow fibers were cut at both potting extremities and removed from the module via the top opening of the membrane module; they were then cut in three 6 cm long segments.

2.3.1 Scanning electron microscopy

Fiber and granule samples were then fixed by immersion in 2.5% glutaraldehyde and 2.5% paraformaldehyde in cacodylate buffer (0.1M, pH 7.4), and post-fixed in 1% osmium tetroxide in 0.1M cacodylate buffer for 1 hour at 4°C. The samples were dehydrated through graded alcohol (50, 70, 90, 100%), and critical point dried with hexamethyldisilazane. After mounting on stubs with conductive carbon adhesive tabs, the cells were coated with gold-palladium in sputter coater (BAL-TEC SCD 005), and then examined by XL SIRION 200 FEG Scanning Electron Microscopy (SEM) (FEI Company, Eindhoven, The Netherlands).

2.3.2 DNA sequencing

DNA (DesoxyriboNucleic Acid) was extracted from the centrifugation pellets, the granules and the fiber samples previously stored at -18°C, using the Fast DNA Spin Kit for Soil (MP) following the protocol recommended by the manufacturer, and stored at -18°C. The concentration of the extracted DNA was determined fluorometrically with the Qubit® dsDNA HS assay kit (Invitrogen, USA) following the instructions of the manufacturer.

Variable regions V4-V5 of the 16S ribosomal RiboNucleic Acid (rRNA) gene were amplified by polymerase chain reaction and sequenced using a MiSeq sequencer (Illumina) by a commercial provider (INRA Narbonne). The identification of the bacterial taxa was performed online using NCBI Nucleotide Blast and the 16S ribosomal RNA sequences database. The sequences presenting more than 97% identity were considered as belonging to the same phylotype. The bacterial diversity was estimated using Simpson's index (Mouillot and Leprêtre, 1999).

3 Results and discussion

The membrane bioreactor was originally seeded with heat-pretreated sludge from the WWTP of the city of Strasbourg. Several trial fermentations using the same feed solution of glucose substrate and nutrients were then carried out without further addition of bacterial biomass. Surprisingly, hydrogen production was maintained despite extensive rinsing and washing of the membrane bioreactor between trials. This suggested to us that the microbial populations may be stabilized on the surface of membrane fibers of the bioreactor. To investigate this idea, a new experiment was designed involving the fitting of an additional removable fiber to the membrane bioreactor to allow sacrificial analysis of the fibers after the experiment. The granules observed in the membrane bioreactor at the end of each fermentation test were also analyzed.

3.1 Evolution of gas and soluble metabolites productions

Bioreactor operation was resumed without reseeded for 551 days, with a process interruption of a year. A lag time of approximately 12 h was observed before onset of gas production (Figure 2a). This was accompanied by a pH drop from its initial value of

8.0, and its stabilization at 4.8 ± 0.1 (Figure 2a). The hydrogen production rate (HPR) reached a maximum of 4.3 mL/L/min between 12 h and 28 h. As evidenced by pH monitoring, conditions in the bioreactor then remained stable for over 70h, with sustained although somewhat irregular hydrogen production. Overall, an average HPR of 135 ± 22 mL/L/h, representing a hydrogen flow rate per unit of membrane surface of 346 ± 56 mL/m²/h, was obtained during monitored steady-state (45-92 h), with a stable H₂/CO₂ ratio (1.0 ± 0.1). Thus, efficient, stable and long-lasting H₂ production was achieved with our L/G MBR design, with continuous hydrogen extraction through hollow fibers, and without needing to seed a hydrogen-producing bacterial community each time the process is started. Our approach presents several advantages. In comparison with other technologies making use of a sweeping gas (Kim et al., 2006; Mizuno et al., 2000), there is no dilution of the produced gases with the L/G membrane configuration, which facilitates the subsequent gas purification step. Both HPR and HY remained stable at least up to 92h of fermentation. With a HRT of 12.5 h and an OLR of 0.9 g/L/h, a hydrogen production rate of 3.2 ± 0.5 L/L/d was reached in the same order of magnitude than Shen et al. (2006) (4.6 L/L/d with a HRT of 8h and and OLR of 0.9 g/L/g). By decreasing the HRT (at 0.8 and 3 h, respectively) and increasing the OLR (at 7.9 and 5 g/L/h, respectively), Kim et al. (2006) and Park et al. (2018) obtained higher hydrogen production rate (40 and 48.9 ± 4.6 L/L/d, respectively).

Glucose conversion started from the beginning of the experiment and remained almost complete from 29 h (Figure 2b). Butyrate and acetate were the major metabolites detected. They were produced in equimolar proportions until 20h, after which the butyrate/acetate ratio increased to approximately 2.2, as evidenced by the respective flow rates. In steady state, acetate flow rate is stable between 0.9 and 1.3 mmol/L/h when butyrate is stable between 2.3 and 2.7 mmol/L/h. Other organic acids and alcohols

(lactate, isovalerate, propionate, succinate, formate, ethanol) were produced in more variable and lower quantities (<0.3 mmol/L/h; Figure 2b, and data not shown). The slight increase in ethanol productivity observed at 77h correlated with a transient decrease in production of acetate and butyrate. Thus, the major co-products butyrate and acetate from the glucose feed confirm that the metabolism selected by this bioreactor design is well- tuned for H₂ production (Hawkes et al., 2007). The hydrogen production seems to occur mainly through the butyrate pathway which was already mentioned as an efficient pathway (Dauplain et al., 2020).

3.2 Evolution of the bacterial community in the liquid outlet

Respectively, 156, 49 and 62 OTU were detected at 20 h, 53 h and 92 h of fermentation. Simpson's index tended to diminish over time (0.54, 0.38 and 0.28 respectively at 20 h, 53 h and 92 h of fermentation), indicating that bacterial diversity increased in the liquid medium. The cumulated related abundances of *Clostridium*, *Enterobacter* and *Ethanoligenens*, known to produce H₂, in the bioreactor outlet were over dominant and very similar throughout the experiment (Figure 3): 92.4% at 20 h, 96.8% at 53 h and 95.6% at 92 h. At 20 h, *Clostridium beijerinckii* was the most abundant taxon (70.9%) followed by *Enterobacter* sp. (19.9%). The relative abundances of *Clostridium beijerinckii* and *Enterobacter* sp. then both decreased at 53 h (30.1% and 8.6% respectively), while conversely, *Clostridium pasteurianum* increased very significantly (53.9%). This trend continued until the end of the experiment (92 h), with the proportion of *Clostridium pasteurianum* further increased (78.6%), and that of *Clostridium beijerinckii* (5.8%) and *Enterobacter* sp. (4.7%) further reduced. Notably, the proportion of *Ethanoligenens harbinense*, another well-known hydrogen producer (Ren et al., 2009), steadily increased throughout the experiment, from 0.1% (at 20 h) to 3.3% (at 92 h). Thus, the dominant taxa observed in the liquid outlet (mainly

Clostridium pasteurianum together with *Clostridium beijerinckii*, *Enterobacter* sp. and *Ethanoligenens harbinense* to a lesser degree) are well-known H₂ producers (Cabrol et al., 2017; Hung et al., 2011). The composition of the bacterial community in the liquid medium evolved over time, but remained mainly composed of HPB *Clostridium beijerinckii* (predominant at the start of H₂ production with the highest flow rate) and *Clostridium pasteurianum* (highly dominant at the end of the experiment).

3.3 Bacterial composition in different sections of the L/G MBR

Operation of the L/G MBR was stopped at 92 h, while H₂ production was still in steady-state. Upon opening of the bioreactor, granules, both suspended and sedimented, were visible by eye, and suggested microbial development in the bioreactor. In contrast, no biofilm was detected by eye on the surface of the hollow fibers, although its presence was suspected. Further investigation of the fibers by scanning electron microscopy indeed confirmed biofilm development on the hollow fibers, although it was very thin (monolayer) and heterogeneous in appearance (Figure 4). Biofilm density was low and uneven in the top section (close to the liquid inlet) of the membrane module (Figure 4a), and significantly higher at the bottom (close to the liquid outlet) (Figure 4b). The majority of cells were rod-shaped, some undergoing sporulation, and no extracellular polymer matrix was apparent. Microbial granules (Figure 4c) were porous, also diverse in size, and appeared similar to previously reported H₂-producing microbial granules (Zhang et al., 2007). Cell morphologies in the granules were also mainly rods, but highly diverse in size (Figure 4d). The cells were mixed with clusters of particulate matter which may include dead cell material.

DNA analysis showed that the relative composition of bacterial communities varied in the different locations of the membrane module analyzed at the end of the experiment (Figure 4), although globally the same highly dominant taxa were observed as in the

liquid outlet through time (Figure 3). Especially, this similarity suggests that the biofilm that developed on the supplementary hollow fibers (Figure 1) was representative to the overall biofilm production. In the sample of the fibers located at the top and at the bottom of the membrane module, and in the granules, 211, 100 and 68 OTU were detected respectively. In addition, Simpson index indicated a closely similar overall bacterial diversity in the microbial granules (0.34) and in the liquid outlet (0.28), while the diversity was higher in the biofilm at the hollow fiber surface (0.12 at the bottom and 0.09 at the top). *Clostridium pasteurianum*, *Enterobacter* sp. and *Clostridium beijerinckii* were the dominant taxa in the granules and the liquid outlet (Figure 4), although their relative abundances differed in the two sections. *Clostridium pasteurianum* was much more abundant in the liquid outlet (78.6% versus 52.2% in the microbial granules). Conversely, *Enterobacter* sp. and *Clostridium beijerinckii* showed higher abundance in the granules than in the liquid outlet (27.6% and 12.7% versus 4.7% and 5.8%, respectively). Although less abundant, *Ethanoligenens harbinense* was also present in both compartments (1.1% and 3.3% respectively in the liquid outlet and in the granules). As mentioned above, the taxonomic diversity was superior in the biofilm of the hollow fiber membrane, but the bacterial composition differed at the two sampled locations of the biofilm. *Clostridium pasteurianum* predominated, but with different proportions at the bottom (73.7%, as at the liquid outlet) and on the top of the fiber (51.9%, as in the microbial granules). Other dominant taxa included *Clostridium drakei* (2.5% and 9.6% at the bottom and the top of the fibers, respectively), *Ethanoligenens harbinense* (6.7% and 4.0%) and *Clostridium beijerinckii* (3.5% and 4.2%). Bacterial strains belonging to these taxa were reported to have additional roles supporting H₂ production: *Ethanoligenens harbinense* by granule formation (Ren et al., 2009) and *Enterobacter* sp. by O₂ scavenging (Zhu and Béland, 2006). According to

Banu *et al.* (2018), *Enterobacter* sp. and *Clostridium* sp. play a role in the formation of granules. Also, other detected taxa with significant relative abundance were more numerous in the fiber's biofilm than in the granules or the liquid outlet, and included *Lactobacillus satsumensis*, *Lysinibacillus* sp. and *Stenotrophomonas* sp..

3.4 Interest of the L/G MBR

It appears that the operating conditions in the L/G MBR constrain the bacterial development to a few HPB in the liquid medium. Similar bacterial community profiles were also reported in previous studies using other types of MBR. In a gas/gas MBR seeded with sludge, *Clostridium pasteurianum*, *Enterobacter aerogenes* and *Lactobacillus casei* were reported to be selected (Bakonyi *et al.*, 2017). Notably, the relative abundance of *Clostridium* was higher in our study (72-87% against 30-40%), as a possible consequence of *in situ* H₂ extraction via the liquid/gas membrane. The influence of the type of bioreactor configuration on the type and relative abundance of selected bacterial taxa in MBR for hydrogen production is further exemplified by another study of a solid/liquid MBR (Park *et al.*, 2017), where *Clostridium butyricum* was over dominant (about 90% of the total community), with the presence of *Enterobacter* sp. and *Enterococcus* sp..

Remarkably and as a further advantage of our L/G MBR, good hydrogen production performances were readily restored even though the membrane module had not been reseeded for 551 days and the process had undergone a complete stop of eight months before several starts and stops of operation before the start of the experiment presented in this paper. It seems likely that part of the biofilm developing on the hollow fibers remains strongly attached to the membrane and serves as the seed culture for subsequent experiments. Indeed, the present work, by including additional membrane fibers for microbial analysis, allowed demonstrating that the main taxa detected in the liquid

outlet also colonized the fibers as a biofilm. The dominance of *Clostridium* in our bioreactor design makes sense from this point of view as well, as biofilm formation by *Clostridium* strains is well-documented (Pantaléon et al., 2014). The flexibility and stability of bioreactor operation afforded by a "self-seeding" membrane system as in our bioreactor may represent a clear asset for hydrogen production using organic waste as a substrate in the future.

As a final advantage of our L/G MBR, no bacterial washout was observed after more than 6 renewals of the reaction medium, possibly again as a consequence of HPB biofilm and granule formation. Bacterial immobilization in the form of granules has been used extensively in anaerobic wastewater treatment, e.g. for methanogenesis, as it allows applying higher organic loading rates and reducing the volume and surface of bioreactors (Kato et al., 2003). In contrast to multilayered methanogenic granules (Fang et al., 2002), H₂-producing granules were reported to be less complex, with composition and microstructure depending on the operating conditions (e.g. temperature, pH, substrate, presence and concentration of calcium and iron) (Zhang et al., 2007). It was previously highlighted that in a fluidized bed reactor, from 0 to 5 days (time scale of our test), there was a majority of biofilm and a minority of HPB granules, and the tendency was reversed progressively from 12 days of operation (Zhang et al., 2008), which suggests that the long-term operation of the L/G MBR could similarly combine high bacterial density through H₂-producing granule formation, together with an efficient continuous H₂ extraction. Technological development of H₂-producing granules was previously implemented in the Upflow Anaerobic Sludge Blanket (UASB) configuration, *i.e.* a column bioreactor with an ascending liquid flow towards a top liquid outlet (Ning et al., 2013). Its major drawback was heterogeneous liquid circulation (*i.e.* hydraulic shortcuts, dead volumes) and the time of formation of mature

granules. The UASB was improved by the development of the EGSB (Expanded Granular Sludge Blanket) bioreactor configuration, which aims to improve the liquid medium homogeneity by applying higher liquid fluxes (Bárcenas-Ruiz et al., 2016). A perspective of the present work would be to improve the dark fermentation process flexibility and efficiency by intensifying the EGSB process with a L/G membrane, acting both as an interface for an efficient *in situ* hydrogen extraction and as a reservoir of HPB for the formation of hydrogen producing granules during the start-up phase of the process.

5 Conclusion

During the steady-state operation of the L/G MBR, glucose was totally consumed; a HPR of 135 ± 22 mL/L/h and a HY of 1.1 ± 0.2 mol_{H₂}/mol_{glucose} were reached, with acetate and butyrate as the main metabolites products, testifying of an efficient bacterial production with the *in situ* extraction of hydrogen. The composition of the bacterial community ~~in the liquid medium~~ evolved over time, but remained mainly composed of HPB *Clostridium beijerinckii*, *Clostridium pasteurianum* and *Enterobacter* sp..The L/G membrane, acting both as an interface for ~~an~~ efficient *in situ* hydrogen extraction and as a reservoir of HPB for the formation of hydrogen producing granules ~~during the start-up phase of the process~~, could be used to intensify the EGSB configuration.

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Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

CRediT authorship contribution statement

Marie Renaudie: Investigation, Methodology, Writing - original draft, Christine Dumas: Methodology, Validation, Writing-Review, Stéphane Vuilleumier: Resources, Writing-Review, Barbara Ernst: Conceptualization, Supervision, Writing-Review&Editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure Captions

Figure 1: Schematic diagram of the L/G MBR.

Figure 2: Time course of (a) hydrogen and CO₂ flow rates and pH and (b) glucose and main metabolites (acetate, butyrate, ethanol) flow rates in the liquid outlet of the L/G MBR. Collection of gas data was suspended between 30 h and 45 h.

Figure 3: Evolution of relative abundance of dominant taxa (>5%) in the liquid outlet of the L/G MBR through time at 20 h (begin of steady-state), 53 h (mid-experiment) and 92 h (experiment end).

~~Figure 4: Scanning electron micrograph images of microbial colonization of the bioreactor at the end of the experiment. (a) hollow fiber surface, top of the membrane module (2500x); (b) hollow fiber surface, bottom of the membrane module (1000x); microbial granule at (c) 100x and (d) 2500x magnification.~~

Figure 4: Spatial distribution in the bioreactor at the end of the experiment (92 h) of relative abundance of major (>5%) taxa in the different compartments of the L/G MBR.

