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► To cite this version:

Marie Renaudie, Valentin Clion, Christine Dumas, Stéphane Vuilleumier, Barbara Ernst. Intensification and optimization of continuous hydrogen production by dark fermentation in a new design liquid/gas hollow fiber membrane bioreactor. Chemical Engineering Journal, 2021, 416, pp.129068. 10.1016/j.cej.2021.129068 . hal-03264359

HAL Id: hal-03264359

<https://hal.science/hal-03264359>

Submitted on 10 Mar 2023

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Intensification and optimization of continuous hydrogen production by dark fermentation in a new design liquid/gas hollow fiber membrane bioreactor

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Keywords

Membrane bioreactor, hydrogen, dark fermentation, continuous fermentation strategies, gas extraction, hydraulic retention time

Abbreviations

CSTR: Continuous Stirred Tank Reactor

EGSB: Expanded Granular Sludge Bed

DNA: DesoxyriboNucleic Acid

HPB: Hydrogen-Producing Bacteria

HPR: Hydrogen Production Rate

HRT: Hydraulic Retention Time

HY: Hydrogen Yield

L/G MBR: Liquid/Gas Membrane BioReactor

L/L MBR: Liquid/Liquid Membrane BioReactor

MBR: Membrane BioReactor

OLR: Organic Loading Rate

rRNA: ribosomal RiboNucleic Acid

SEM: Scanning Electron Microscopy

S/L MBR: Solid/Liquid Membrane BioReactor

UASB: Upflow Anaerobic Sludge Blanket

WWTP: Waste Water Treatment Plant

Abstract

A liquid/gas membrane bioreactor (L/G MBR) was developed to intensify the dark fermentation process. A hollow fiber membrane module was used to combine biohydrogen production, *in situ* liquid-gas separation and hydrogen producing bacteria retention in a single unit. The L/G MBR was seeded once and did not require further microbial input, as consistent average hydrogen yield of 0.97 ± 0.09 mol-H₂/added mol-glucose and hydrogen production rate of 106.5 ± 10.6 mL-H₂/L-medium/h were reached over a year. Different biogas extraction strategies showed that efficient *in situ* H₂ extraction is possible without sweeping gas in the lumen of the fibers, thus facilitating H₂ purification in an industrial setting. Modelling predicted an optimal hydrogen yield of 1.2 mol/mol-glucose added for a glucose concentration in the feed of 13.1 g/L, close to experimental hydraulic retention time of 8-10 h with an organic loading rate of 1.4 g-glucose/L-medium/h. No washout of hydrogen-producing bacteria was observed at low HRT (2 h), suggesting the possibility of further hydrogen production rate enhancement using an optimized organic loading rate. Acetate and butyrate were the main metabolites identified. *Clostridium* and *Enterobacter* dominated in the liquid outlet. The relative abundance of *Clostridium pasteurianum* increased with glucose concentration in the bioreactor, as opposed to *Clostridium beijerinckii* which was more abundant at low glucose concentration. The original hollow fiber L/G MBR configuration enabled the testing and selection of fermentation strategies that greatly simplified the implementation of the dark fermentation process by addressing its key operational

bottlenecks. Indeed, the L/G membrane surface served as a support and reservoir for the hydrogen producing bacteria across a wide range of HRT conditions.

1. Introduction

In recent years, membrane bioreactors (MBRs) have attracted world-wide attention as a sustainable technological solution for the treatment of organic effluents in a wide range of applications [1]. Originally developed for the treatment of wastewater or industrial effluent, anaerobic MBRs also bring about various opportunities and challenges for the co-production of energy (biogas) [2]. MBRs have been used for the intensification of hydrogen (H_2) production by the dark fermentation process since the early 2000s [3]. Several implementation strategies of a membrane module have been tested: (i) in a solid/liquid extraction mode either in an *ex situ* [4, 5] or an *in situ* [6] (Fig. 1a) configuration, to increase the concentration of bacterial biomass or suspended organic matter by removing the liquid phase, (ii) in a liquid/liquid (L/L) extraction mode, to selectively extract part of the liquid components (organic acids) of the medium in order to limit their negative impacts on fermentation, either in an *ex situ* [7] or an *in situ* [8] configuration (Fig. 1b), (iii) in an *ex situ* gas/gas (G/G) extraction mode, to selectively extract hydrogen from the gas stream leaving the reactor in order to obtain purified/concentrated hydrogen [9, 10] and finally in few studies [11-14] (iv) (Fig. 1c), in liquid/gas extraction mode, the membrane module can be directly placed in contact with the reaction medium in order to extract the gases produced therefrom (Fig. 1d). Keeping a low H_2 partial pressure in the bioreactor, H_2 being an inhibitor of the bacterial H_2 production, and avoiding the washout of H_2 producing bacteria (HPB) and are both essential requirements for efficient continuous production of H_2 by dark fermentation with regards to process efficiency. Several strategies aiming to lower H_2 partial pressure inside the bioreactor were previously assessed. The use of N_2 as a sweeping gas improved H_2 yield (HY) from 23 to 88%, and increased H_2 production rate (HPR) from 2.6- to 3.7-fold [10, 15-17]. However, this strategy triggers the need for a subsequent H_2/N_2 separation step and increases process

costs, which is a major drawback at industrial scale. The recycling of produced CO₂ as a sweeping gas has also been envisioned, given that H₂/CO₂ separation processes are more advanced technologically [18]. For instance, a G/G separation membrane coupled to a dark fermentation bioreactor allowed the retention of produced CO₂ and its reintroduction within the reactive medium, enhancing HPR by 25% compared to performance in a continuous stirred tank reactor (CSTR) [10]. Also, slight continuous pressure release (via vacuum) in a CSTR reactor enhanced H₂ production at low HRTs (4-8 h) [19]. The operation of a liquid/gas MBR in semibatch mode was also proposed, for the submerged H₂-permeable flat membrane to mimic H₂-consuming microorganisms, but H₂ production performances were not reported [20]. Similarly, HY was improved by 9% in a hollow fiber MBR (using a silicone rubber membrane) but at the expense increased process energy cost, as the produced gases were extracted using a vacuum pump [14].

Aiming to decouple HPB retention from hydraulic retention time (HRT), various configurations of immobilized biomass bioreactors were also tested, often taking advantage of the propensity of microbial cultures to progressively form biofilms in presence of a solid support. In continuous MBRs, the substrate is usually pumped at the bottom of a column bioreactor, so the liquid flowing towards an outlet positioned at its top end [21, 22]. The instauration of pH and nutrient gradients and the use of a single-use support are main issues of this configuration. An exchangeable cartridge arrangement was proposed to limit the pH and nutrient gradient [23]. The implementation of solid/liquid membrane bioreactors (S/L MBRs) have also been envisaged to increase HPB density and to limit the volume of dark fermentation bioreactors, but membrane fouling resulting from the adhesion of extrapolymeric substances was reported to impede process flow [4, 24]. The ability of some HPB to auto-aggregate and form granules was exploited in Up-flow Anaerobic Sludge Blanket Reactor (UASB) [25] and Expanded Granular Sludge Bed (EGSB) bioreactor configurations [26]

avoiding the need of either a solid support nor of a S/L membrane to be retained within the bioreactor. However, preferential flows, hydraulic short-cuts, dead volumes and the extensive length of time to form the granules remain bottlenecks to be addressed [3].

In order to meet both dark fermentation requirements of efficient hydrogen extraction and bacterial biomass immobilization, a continuous liquid/gas membrane bioreactor (L/G MBR) was previously developed in our laboratory [17] (Fig 1e). A hollow fiber module was used to extract produced gases continuously and to provide a support for the development of a HPB biofilm [11]. The liquid phase circulated outside the hollow fibers, while produced gases diffused to the lumen of hollow fibers. Contrary to S/L MBRs, the L/G MBR was not sensitive to membrane fouling because the biofilm does not limit gas diffusion through the membrane. HPB were retained both via quick biofilm and minimal granule formation, independently of time-consuming formation and stabilization of granules as reported in immobilized biomass bioreactors.

In the present article, we report several improvements of the H₂ production process by dark fermentation in the L/G MBR. After a one-time initial microbial seeding, long term operation of the L/G MBR was investigated, with and multiple process stops and restarts without further seeding. Different strategies of gas extraction were tested. Hydraulic retention time (HRT) and organic loading rate (OLR) were optimized. The impact of these modifications on the production of hydrogen and metabolites, and on the composition of bacterial communities growing in the membrane bioreactor were analyzed.

2. Materials and methods

2.1. Feed solution

The feed solution was composed of 12 g/L glucose, 0,175 g/L KH_2PO_4 , 3.5 g/L $(\text{NH}_4)_2 \text{SO}_4$, 0.25 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002 g/L $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$. The mix was then filtered on a Büchner filter with 40 μm pore size and stored at 4°C until needed. The feed solution was diluted when lower OLR were tested.

2.2. Bioreactor setup and operation

The L/G MBR (Fig.2a) was constituted of a cylindrical-shaped hollow-fiber membrane module and had a total working volume of 0.5 L. Ultrafiltration polytetrafluoroethylene (PTFE) hollow fibers were sealed at each extremity of the membrane module. The bioreactor was seeded as described previously [12] with fresh activated sludge introduced in a stirred tank reactor and went through a thermal treatment at 70°C during 1 h. The temperature was regulated at 37°C. After 5 h of fermentation, the hydrogen production was at its maximum and the reactive medium was transferred in the MBR where the fermentation occurs in continuous and corresponds to the 0-day test. Before each experiment, feed solution was injected into the bioreactor circuit within 30 minutes using a peristaltic pump. Fermentation starting time was defined as the timepoint when the filling of the bioreactor circuit was completed. During the fermentation test, the system was continuously fed using a peristaltic pump. pH of the liquid outlet was measured using a multi-parameter analyzer and maintained at 5.1 ± 0.3 by the addition of a solution of NaOH 0.5 M into the liquid medium using a peristaltic pump, which speed was adjusted manually. Temperature was maintained at 37°C with heated water circulating within a heating coil placed around the membrane module. The bioreactor was emptied and rinsed with water after each test.

HRT was initially set at 12h and OLR at 1g-glucose-L/medium/h. During HRT/OLR optimization tests, HRT was modified by acting on the rotation speed of the peristaltic pump. OLR was adjusted accordingly to reach its desired value by diluting the feed solution when necessary.

By default, sweeping gas (N₂) circulated at a flow rate of 11 mL/min within the lumen of the hollow fibers (Fig. 2b). When CO₂ was tested as sweeping gas, the same gas flow rate was applied. When no sweeping gas was used, the gas inlet was closed (Fig. 2c).

2.3. Chemical analyses

The liquid outlet was sampled at time zero, twice a day during fermentation then centrifuged for 30 minutes at 4,500 rpm. Supernatants and pellets were stored separately at -18°C.

2.3.1. Gas analysis

Gases were analyzed using a gas microchromatograph (T3000, SRA, France) equipped with thermal conductivity detectors, 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 µm) for separation of N₂, CH₄, O₂, H₂ and CO₂. Series of 3 analyses was performed every 10 min during L/G MBR operation. CO₂ production could not be quantified when CO₂ was used as a sweeping gas.

2.3.2. Analysis of organic acids, glucose and alcohols

Organic acids (citrate, pyruvate, succinate, lactate, formate, acetate, propionate, butyrate, valerate and isovalerate) and glucose were analyzed simultaneously in culture supernatants by high-pressure liquid chromatography coupled to a variable wavelength detector and a refraction index detector. Supernatants were thawed, centrifuged for 15 min at 15,000 rpm and filtered at 0.2 µm. Resulting samples were injected (20 µL) onto a chromatographic HiPlex-H column (8 µm, 7.7 m x 300 mm, Agilent Technologies, USA) placed in an oven

heated at 65°C. The mobile phase was composed of a solution of aqueous H₂SO₄ (5 mM) circulating at 0.4 mL/min. Each sample was analyzed twice.

Alcohols (ethanol, butanol and propanol) were analyzed by gas chromatography coupled to a flame ionization detector (7890A GC, Agilent Technologies). Helium (0.6 mL/min) was used as vector gas. Supernatants were centrifuged during 15 min at 15,000 rpm, filtered at 0.2 µm and diluted with an internal standard. Resulting mixtures were injected (0.5 µL) in split mode into a chromatographic HP-INNOWax column (0.5 µm, 30 m x 0.25 mm, Agilent Technologies, USA) heated at 45°C for 4 min then oven temperature ramped up at 10°C/min until it reached 200°C. Each sample was analyzed 4 times.

2.4 Microbiological analyses

2.4.1 DNA sequencing

DNA was extracted from culture pellets previously stored at -18°C using the Fast DNA Spin Kit for Soil (MP Biomedicals), following the protocol recommended by the manufacturer, and stored at -18°C. Concentration of 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 rRNA gene were amplified by polymerase chain reaction (PCR) and sequenced using a MiSeq sequencer (Illumina Inc., USA). The identification of bacterial taxa was performed using the online software NCBI Nucleotide Blast and the 16S rRNA sequences database. The sequences presenting more than a 97% identity were considered as belonging to the same phylotype. Bacterial diversity was estimated using the Simpson index, tending towards 0 in datasets of high diversity and towards 1 in datasets of low diversity [27].

2.4.2 Quantitative PCR

Analyses of the 16S rRNA gene by qPCR were performed with the TaqMan system as described previously [28], with 0.5 µL each of primers BAC338F (0.1 µM), BAC805R (0.25

μM) and labeled fluorescent TaqMan Tamra probe BAC516F ($0.05 \mu\text{M}$) in $20 \mu\text{L}$ final volume per reaction. Two-step amplification of target DNA was performed by 40 cycles of 7 s at 95°C and 25 s at 60°C after the enzyme activation step (2 min at 98°C).

2.4.3 Scanning electron microscopy (SEM)

A fermentation test was operated and hollow fibers were sampled and analyzed by SEM as described previously [12].

3. Results and discussion

In this work, we sought to enhance the efficiency of a novel H_2 producing L/G MBR process [11, 12]. One-time initial seeding with thermally-treated Waste Water Treatment Plant (WWTP) sludge was followed by long-term operation without re-inoculation. Different strategies of gas extraction were evaluated, and several combinations of HRT and OLR to determine an optimum in H_2 production performances were tested.

3.1. H_2 production without initial seeding of the membrane module

A first 4-day long test was performed with initial seeding of the membrane module with thermally treated WWTP anaerobic sludge (0 day), followed by other tests performed without reseeded, which were started 365, 418 and 432 days thereafter, respectively (Fig. 3).

Efficiency of H_2 productions were compared (Table 1) and analyzed as a function of produced metabolites and bacterial composition profiles.

3.1.1 Hydrogen production

In all tests, a brief lag phase (<6 h) was followed by an exponential increase of HPR up to a maximum of $2.5\text{--}3.0 \text{ mL-}\text{H}_2\text{/L-medium/min}$ at 13–25 h. Then, a pseudo-stable state was reached as HPR stabilized at $1.6\text{--}2.3 \text{ mL-}\text{H}_2\text{/L-medium/min}$ (Fig. 2a), during which HPR of all three tests operated more than a year after seeding the membrane module remained close ($\approx 1.7 \text{ mL-}\text{H}_2\text{/L-medium/min}$) and slightly lower than HPR of the seeding test. The pseudo-

stable state was maintained, with some pH variations (4.8-5.5, Fig. 2b). Average HY and HPR (Table 1) of the three tests performed after a year without reseeded the membrane module were consistent (0.97 ± 0.09 mol-H₂/mol-glucose added, 106.5 ± 10.6 mL-H₂/L-medium/h), despite an 8-month complete interruption in bioreactor operation. The hydrogen yield observed, lower than the *in vivo* theoretical maximal yield of 4 mol-H₂/mol-glucose, could be explained by different phenomenon described previously [29]: (i) occurrence of the butyrate pathway, lowering the maximal HY that could be reached (2 mol-H₂/mol-glucose), (ii) presence of hydrogen consuming bacteria (suspected due to the production of propionate) and (iii) presence of other bacteria consuming the substrate without coproducing hydrogen (production of acetate by homoacetogenesis, ethanol or lactate).

Therefore, robust continuous hydrogen production by dark fermentation in the L/G MBR was demonstrated, despite several bioreactor drainings and no HPB reseeded during the start-up phase. Such an operating mode had previously been tested in a trickling biofilter bioreactor inoculated with a pure culture of *Clostridium butyricum* [30] with successful restoration of bioreactor performances after draining. However, contamination of the pure culture was observed, an issue no longer relevant in our process strategy which implements a mixed culture. In our study, the development of a thin bacterial layer was observed on hollow fibers by SEM (Fig. 4). This bacterial community might have stayed active during fermentation and after draining and rinsing the membrane module, thus serving as a bacterial reservoir.

H₂ production performances of the L/G MBR were compared with those from previous reports (Table 2). Under close feeding conditions, HY and HPR of the L/G MBR were close to those obtained by Lee *et al.* [31] but lower than those of Shen *et al.* [24] which used a S/L MBR aiming to concentrate the bacterial biomass. Highest HPRs (>750 mL/L/h) were obtained as expected in studies using higher OLRs (5 g-glucose/L-medium/h) than in our study (1 g-glucose/L-medium/h) [5, 32]. The MBR described in Noblecourt *et al.* [32]

operated close to semi-batch conditions and was therefore not directly comparable to our continuous process. Remarkably, high values obtained in the study of Park *et al.* [5] using liquid/liquid separation to remove organic acids from the medium suggests that performances might be improved by increasing OLR. Worthy of note and in contrast to previously reported studies with S/L MBR, membrane fouling was not an issue with our L/G MBR, and additional cleaning of the membrane was not required; this allows to reduce the operational cost of the process [33]. In an industrial production scenario, the scale up of the L/G MBR process can benefit from the flexibility associated with the use of hollow fiber membrane modules, as previously documented for other processes [34]. Indeed, the size of the L/G MBR unit at pilot scale can be defined according to the flow of gas extracted, and multiple small-size modules can be implemented in parallel or in series, thus minimizing the HY loss due to the use of a single bigger unit. Also, in order to maximize substrate consumption, L/G MBR units can be added in series according to the quantity of biomass to be treated over a defined period of time. Compared to reference CSTR processes, an additional cost item would be the initial investment in membrane modules, which would depend on the number of membrane modules required [34]. Then, the other process costs would be the same: chemical inputs (e. g. sodium hydroxide) to compensate pH variations over time due to the coproduction of organic acids, electrical consumption to pump the liquid into the system and to regulate the temperature in the membrane modules.

3.1.2. Production of soluble metabolites

Glucose conversion was almost complete and metabolite distribution profiles (Fig. 5) were close in all tests. Acetate (0.8-1.6 mmol/L/h), butyrate (1.1-2.2 mmol/L/h) and ethanol (0.8-1.3 mmol/L/h) were the main compounds produced as expected, due to their association with H₂ production, in acetate-butyrate and acetate-ethanol metabolic pathways [35]. Low levels of formate (<0.5 mmol/L/h) were occasionally observed and represent as yet unexploited

potential source of H₂, as formate can be further transformed to H₂ and CO₂ [29]. Other metabolites unrelated to H₂ production (isovalerate, lactate, propionate, succinate) were found in low quantities (<0.1 mmol/L/h). Ethanol productivity decreased over time (Fig. 5), and the butyrate/acetate (B/A) molar ratio tended to increase (from 1.1 (365 days) to 2.0 (432 days)), suggesting a metabolic shift from acetate-ethanol to acetate-butyrate metabolism [36]. The observed high production of acetate may explain the low average HY (0.97 ± 0.09 mol/mol), due to homoacetogenesis, thereby consuming potential H₂ [37].

3.1.3. Bacterial community composition

Bacterial compositions of the L/G MBR liquid outlets of the test performed after seeding the membrane module with heat-treated activated sludge and the test performed 418 days after the initial seeding were compared (Fig. 6). In both cases, samples of the liquid outlet were collected at 68 h of fermentation (i.e. during the pseudo-stable state) operation. Total bacterial diversity was lower in the experiment performed without microbial reseeded (Simpson index of 0.20 at 418 days, compared to 0.03 at 0 day). In both experiments, *Firmicutes* (mostly genera *Clostridium*, *Ethanoligenens* and *Sporolactobacillus*) predominated in the liquid outlet, especially in the experiment after 418 days without reseeded (81% of total genus-level taxa, compared to 55% in the 0-day experiment, Fig. 6). The *phylum Proteobacteria* was slightly more abundant without initial reseeded (19% against 12, mainly including the hydrogen producing genus *Enterobacter*. *Clostridium* was the most abundant genus (21% and 50% for the 0 day- and 418 days-experiments, respectively). In the 418 days-experiment, initially minor or undetected *Ethanoligenens* (24%), *Enterobacter* (19%) and *Ruminococcus* (3%) appeared at significant levels.

The higher bacterial diversity observed in the experiment following the initial microbial seeding suggests that a lot of bacteria from the WWTP sludge used as *inoculum* were still present after thermal treatment and 68 h of bioreactor operation. After repeated draining and

rinsing of the L/G MBR, selective pressure from storage and operating conditions of the bioreactor led to the decrease in bacterial diversity reported. We hypothesize that living and active bacteria remained attached to hollow fibers after each process stop, including well-known HPB such as strict (*Clostridium*, *Ethanoligenens*) and facultative (*Enterobacter*) anaerobes. Indeed, the taxa detected were linked to different roles in H₂ production [29, 38]: as major H₂ producers (*Clostridium*, *Enterobacter*, *Ethanoligenens*), in improving H₂ production via microbial granule formation (*Ethanoligenens* [39, 40] and *Clostridium* [41, 42]), in maintaining an anaerobic environment by O₂ scavenging (*Enterobacter* [43]), and in increasing H₂ production by digestion of complex substrates (*Ruminococcus*, [44, 45]). Notably, the association of *Clostridium* with *Enterobacter* was observed repeatedly in past studies of biohydrogen production [46-48]. Only one genus-level taxon described to have a negative impact on H₂ production (*Sporolactobacillus*) was abundant in both samples and was significantly present in the initial test only. *Sporolactobacillus* is known to produce bacteriocins affecting HPB [49], which could contribute to explain the relatively low HPR at the beginning of this test (Fig. 3), despite otherwise good performances during the pseudo-stable state phase.

Thus, without reseeded of the L/G MBR, the selection over time of a robust and resilient functional with regard to H₂ production ecosystem was favored, in non-sterile conditions. An efficient start-up strategy of a L/G MBR dark fermentation process was thereby successfully implemented.

3.2. Effect of sweeping gas (N₂, CO₂) and gas free extraction on biohydrogen extraction

In order to improve the L/G MBR process, different approaches for gas extraction were also evaluated. The L/G MBR was operated with either N₂ or CO₂ as a sweeping gas circulating in the lumen of the hollow fibers. The latter was considered as a potential approach to recycle produced CO₂ as sweeping gas to avoid H₂ dilution by N₂ [10]. Moreover, the L/G MBR was

operated without sweeping gas (no sweeping gas (NSG)) to produce biogas (H_2 and CO_2) without any dilution. In this series of experiments, HRT and OLR were set at 13 h and 0.8 g-glucose/L-medium/h respectively.

3.2.1. Gas production

Globally, the extraction strategy impacted H_2 production (Table 3), while glucose conversion was similar and high (>97%) in all cases. The largest difference in HPR concerned lag time before hydrogen production, which was longer when the L/G MBR was operated without sweeping gas. We suppose that H_2 production started earlier than the lag phase measured, but was analyzed only when a sufficient transmembrane pressure was reached for the biogas to be transferred towards the lumen of the hollow fibers. However, in NSG conditions, lag time decreased with the number of experiments performed, possibly thanks to an increase of the wetting of membrane pores, resulting in a lowered transmembrane pressure threshold for the biogas transfer to happen.

HY, HPR and H_2/CO_2 ratio were often significantly higher without sweeping gas than with N_2 sweeping, whereas sweeping with N_2 yielded slightly higher HY and HPR in the pseudo-stable state than sweeping with CO_2 . These results markedly differ from previous reports of dark fermentation in bioreactors with other configurations. For example, HY increased by 88% and HPR 2.6-fold in a semibatch bioreactor swept with N_2 [17], and HY was improved by 23% in a CSTR with N_2 sweeping [15]. The difference observed can readily be explained by the fact that in these alternative bioreactor configurations, the sweeping gas bubbled through the liquid culture medium, whereas it circulated in the lumen of the hollow fibers in our L/G MBR configuration with controlled contact with the reactive liquid phase. Thus, the intensification of the process with an *in situ* L/G membrane separation in our design was a substitute for a sweeping gas. Notably, no apparent resistance to mass transfer was associated with our bioreactor configuration in steady state. Produced gases were therefore efficiently

extracted without dilution by a sweeping gas, while preserving both HY and HPR. In this operating mode, HPR was always equal to or higher than CO₂ production rate, unlike when N₂ sweeping was used (Table 3).

3.2.2. Production of soluble metabolites

Metabolite production was compared in the pseudo-stable state phase (Fig. 7) for three experiments performed either with N₂ sweeping (432 d), CO₂ sweeping (439 d) or no sweeping gas (551 d). Total metabolite productivities were similar (4.1 ± 0.4 mmol/L/h), but slightly higher with N₂ sweeping (4.5 mmol/L/h), and lower with CO₂ sweeping (3.7 mmol/L/h), as a smaller HPR was also observed for the latter (Table 3). Distribution of soluble metabolites was also similar under all conditions, the main metabolites being butyrate (~50%), acetate (~30%) and ethanol (~8%). Ethanol production was quite absent without sweeping gas. Other analyzed metabolites (butanol, propanol, succinate, propionate, lactate, isovalerate and formate) were produced at lower levels (<0.1 mmol/L/h).

3.2.3. Bacterial community composition

Samples of the liquid outlet of the three tests were collected at 68 h operation. Simpson diversity index was similar without gas sweeping (0.38) and with CO₂ sweeping (0.42), and higher than when N₂ sweeping was used (0.20). Thus, N₂ sweeping was associated with higher bacterial diversity within the membrane module. *Clostridium*, *Enterobacter* and *Ethanoligenens*, well-known HPB, were the major genus-level taxa identified (Fig. 8a), and irrespectively of gas extraction mode (91% to 96%). Strikingly, *Clostridium beijerinckii* was dominant (>57%) in the liquid outlet of the tests without gas sweeping and with CO₂ sweeping, and much less abundant with N₂ as sweeping gas (14%) (Fig. 8b). Conversely, *Clostridium pasteurianum* was much twice abundant with N₂ (28%) than without gas sweeping (14%), and was almost absent with CO₂ sweeping (0.4%). Similarly, *Ethanoligenens harbinense* was abundant with N₂ sweeping (24%) and almost absent without

gas sweeping or with CO₂ (<2%). Finally, *Enterobacter* sp. was abundant with CO₂ (26.1%) and less so with N₂ or without sweeping (19% and 16% respectively), possibly explaining the observed slightly lower HPR and total metabolite production by using CO₂ as sweeping gas. Thus, bacterial diversity differed more between the different extraction approaches that were tested than the very similar distribution of produced metabolites (Fig. 7) would suggest. Moreover, bioreactor operation without sweeping gas favored the development of HPB *Clostridium beijerinckii* (57%), *Clostridium pasteurianum* (14%) and *Enterobacter* sp. (16%) while limiting the diversity of secondary taxa. The slightly lower HPR obtained with CO₂ sweeping could be related to the absence of *Clostridium pasteurianum* and a higher abundance of *Enterobacter* sp.. Without sweeping gas, bioreactor operation was thereby oriented towards H₂ production, with slightly better HPR and low production in ethanol and in secondary metabolites. The L/G MBR design, substituting to other gas extraction strategies such as a sweeping gas which implies an additional dilution of the biogas, is therefore a valuable approach to facilitate subsequent gas purification.

3.3. Optimization of OLR and HRT

Glucose was almost to fully converted in our dark fermentation experiments, suggesting that HRT and OLR could be optimized in order to enhance HPR. In several previous studies of HRT impact, the experimental conditions applied resulted in simultaneously increasing the OLR and lowering the HRT, as the concentration of the feed solution was not modified [50-53]. Furthermore, this usually resulted in HY decrease due to inhibition of hydrogen-producing pathways [51]. Our objective in this study was (i) to determine the impact of HRT independently of the OLR and *vice versa*, (ii) to specify the operating limits of the L/G MBR configuration (decrease the HRT and find the optimal OLR for different HRT) and finally (iii) to follow the effects of the variation of HRT and OLR on the metabolism and the microbiology of the reaction medium. To do so, experiments were conducted between 538

and 733 days after seeding the L/G MBR, keeping constant either HRT (2-14h) or OLR (0.6-2.1 g-glucose/L-medium/h) by adjusting the feed concentration in the latter case.

3.3.1. Hydrogen production

Firstly, the L/G MBR was operated within a HRT range between 2 and 10 h at a constant OLR of 1.4 g-glucose/L-medium/h (Table 4). Remarkably, no HPB washout occurred even when a HRT of 2 h was applied (Fig. 9), even though the reactive medium was renewed more than 26 times after having reached the steady state without any HPR drop. This result differs with what was reported in a previous S/L MBR study [5], where a higher flow rate was applied. However, it might be due to the difference of configuration and volume of bioreactors (5.5 L compared to 0.5 L in our study) and a higher flow rate possibly leading to biofilm removal [5]. In addition, glucose conversion remained high (91%) in our study under these conditions (HRT = 2 h), while glucose conversion was previously reported to drop from 90% to 78% when HRT was lowered from 8 h (OLR = 1.3 g-glucose/L-medium/h) to 6 h (OLR = 1.7 g-glucose/L-medium/h) in a CSTR [50].

Overall, HPR ranged between 66.5 and 253.7 mL/L/h (Table 4). HPR increased for higher OLR values, for each series of HRT values (Fig. 10a). Maximal HPR was obtained for a HRT of 8 h and an OLR of 2.1 g-glucose/L-medium/h. However, this did not correspond to maximal HY (1.16 mol/mol), which was obtained with a lower OLR (1.4 g-glucose/L-medium/h) and the same HRT (Table 4). A double optimum in terms of HPR and HY was also reported previously [51], with maximal HPR of 187 mL/L/h for a HRT of 6 h (OLR = 2.5 g-glucose/L-medium/h) and HY of 1.62 mol/mol-glucose added for a HRT of 18 h (OLR = 1.2 g-glucose/L-medium/h).

HY ranged between 0.60 and 1.16 mol-H₂/mol-added glucose (Table 4). Experimental data of HY related to glucose concentration in the feed (mol-H₂/mol-glucose consumed) were interpolated with a second order equation (Fig. 10b). Best-fit parameters predicted an optimal

glucose concentration of 13.1 g/L for a maximal HY (1.22 mol/mol) in close agreement to the experimentally observed optimal HY obtained by combining a HRT of 8-10 h and an OLR of 1.4 g-glucose/L-medium/h. In contrast, the lowest HY (with minimal glucose conversion, 78.1%) was obtained for an OLR of 1.7 g-glucose/L-medium/h and a HRT of 14 h, *i.e.* at the maximal glucose concentration that was tested (23.8 g/L). Notably, HY was 1.5-fold superior with a slightly higher OLR (2.1 g-glucose/L-medium/h) and 2-fold lower HRT (8 h), *i.e.* corresponding to a glucose concentration of 16.8 g/L closer to the optimal glucose concentration, at equivalent glucose conversion (78.4%). Both HY (1,13-1,16 mol-H₂/mol-glucose) and HPR (210-225 mL/L/h) of the optimized L/G MBR process (TSH = 8-10 h, OLR = 1.4 g/L/h) compared to the performances obtained in a CSTR study realized under similar operating conditions. For instance, using glucose as a substrate, Kumar *et al.* [51], obtained a HPR of 139 mL/L/h and a yield of 0.67 mol/mol applying a HRT of 9 h and an OLR of 1.67 g/L/h.

Globally, glucose conversion in our experiments varied between 78.1% and 100% (Table 4). There seemed to be a limit of the amount of substrate that could be converted into hydrogen by the HPB in the L/G MBR since HY dropped when the glucose concentration was increased. Indeed, when the HRT was increased from 2 to 8 h and the OLR from 0.6 to 1.4 g-glucose/L-medium/h (with glucose concentrations of the feed solution remaining inferior to 13 g/L), glucose conversion was higher than 92%. On the other hand, glucose conversion was below 82% when the HRT was set to longer times, *i.e.* between 8 and 14h, and OLR between 1.4 and 2.1 g-glucose/L-medium/h (glucose concentration of the feed solution superior to 13 g/L). Thus, it seems that the lowest limit of operation of the L/G MBR was not reached in terms of glucose conversion to hydrogen. Therefore, it would potentially be possible to further lower the HRT while increasing the OLR to enhance the HPR while maintaining the HY. This

mode of operation might result in an increase of HPR, as observed previously [6], while avoiding issues of membrane fouling at high OLR, as reported e.g. with a S/L MBR [54].

In a majority of our tests, the H_2/CO_2 ratio did not vary much (from 0.87 to 1.09). When the HRT was increased, the H_2/CO_2 ratio decreased (Table 4). Indeed, for the lowest HRT tested (2.1h) with an OLR of 1.4 g-glucose/L-medium/h, the H_2/CO_2 ratio was 35% higher than the average value of all experiments. This might be explained by the larger NaOH amendment to maintain pH under this condition, as CO_2 transfer is enhanced when OH^- increases in the medium ($CO_{2(g)} + OH^- \leftrightarrow CO_{2(liq)} + OH^- \leftrightarrow HCO_3^-$) [55]. Moreover, in order to reduce the HRT, the liquid flow rate was increased. As a result, a higher content of dissolved CO_2 was evacuated via the liquid outlet, thus reducing the proportion of gaseous CO_2 in the lumen of the fibers, which resulted in an increase of the measured H_2/CO_2 ratio.

3.3.2. Production of soluble metabolites

Butyrate and acetate were the dominant metabolites under all tested conditions (Fig. 11a). Formate, lactate, succinate and ethanol were also produced in smaller quantities. In the experiments generating the highest HY (HRT = 8-10 h and OLR = 1.4 g/L/h) with a high HPR, high butyrate productivity (> 50 mol% of all metabolites produced) was observed.

At this OLR (1.4 g/L/h), both HY and HPR increased with HRT (Table 4), while metabolites productivities and distributions remained close, except for a rise in butyrate production at 10 h HRT and production of acetate at 5 h HRT (Fig. 11a). It might be possible that improved H_2 extraction at high HRT limited acetate production by homoacetogenesis.

For an equivalent HRT (8h), the total quantity of metabolites produced (mainly acetate and butyrate) was directly proportional to the OLR (Fig. 11b) and therefore to the concentration of glucose in the liquid medium. The butyrate/acetate molar ratio varied between 1.5 and 2.5 and increased with the OLR whatever the HRT (Fig. 11c).

3.3.3. Bacterial community composition

Samples were collected at the liquid outlet after 68 h dark fermentation for the following five HRT and OLR combinations: 2 h and 1.4 g/L/h, 8 h and 1.0 g/L/h, 8 h and 1.4 g/L/h, 8 h and 2.1 g/L/h, 14 h and 0.8 g/L/h. Samples were analyzed by sequencing of PCR amplicons of the 16S rRNA gene and by qPCR. Simpson index was similar for all samples, suggesting similar bacterial diversity for all conditions. Independently of HRT, a positive correlation ($R^2 = 0.90$) was found between OLR and 16S rRNA gene copy number used as a proxy of bacterial biomass (Fig. 12a). Thus, bacterial density increased when the higher OLR were applied. Therefore, further increasing the OLR, thus the bacterial concentration in the liquid medium and potentially HPB, might be a potential solution to enhance HPR. There was no correlation between the HRT and the concentration of bacteria of the liquid medium. The abundance of *Clostridium pasteurianum* increased when the glucose concentration was augmented, unlike that of *Clostridium beijerinckii*, which was more abundant at low glucose concentration (Fig. 12b) with linear regression coefficient of $R^2 = 0.98$ and $R^2 = 0.94$, respectively for *Clostridium pasteurianum* and *Clostridium beijerinckii* (HRT = 14 h OLR = 0.8 g/L excluded). *Clostridium*, *Ethanoligenens* and *Enterobacter* were the major genera detected in all experiments (Fig. 13a). Notably, *Lactococcus*, which is not a HPB, developed at both minimal and maximal glucose concentrations (2.8 and 16.8 g/L) showing a derive in bacterial growth (less oriented toward H₂ producers) for glucose concentration away of the optimum (13.1 g/L) previously determined. Indeed, the modeled optimal HY seemed highly correlated with a maximal abundance of HPB in the liquid outlet (*Clostridium beijerinckii*, *Clostridium pasteurianum*, *Clostridium carboxidivorans*, *Ethanoligenens harbinense* and *Enterobacter* sp., Fig. 10b). The maximal HPR was obtained at the highest OLR applied (2.1 g/L/h), which corresponded to a glucose concentration of 16.8 g/L in the field (slightly higher than the glucose concentration for optimal HY), despite a drop in glucose consumption (78.4%). In

this condition, *Clostridium pasteurianum* (58%) and *Enterobacter* sp. (13 %) were the main detected HPB (Fig. 13b). Analysis of bacterial composition evidenced that such optimal conditions favored development of *Clostridium pasteurianum* (74.8% of analyzed sequences), representing more than $4.5 \cdot 10^7$ 16S rDNA copies in the liquid outlet of the L/G MBR (Fig. 12b, Fig. 13b). We hypothesize that this taxon was responsible for the favorable HY and HPR obtained in the range of 11.2 to 16.8 g/L glucose.

Conversely, at low glucose concentrations (2.8 g/L), lower HY (-25% of the optimal HY) was obtained. *Clostridium pasteurianum* was less abundant in this condition (1.1%), at the benefit of *Clostridium beijerinckii* and the non-HPB *Lactococcus* sp. (Fig. 13b) going with the lower hydrogen performances observed. At a HRT of 2 h, significantly higher production of bacterial biofilm and granules were observed (data not shown). Future experiments with a low HRT for a longer operating time and a higher OLR could determine whether HPB taxa would be favored under these conditions.

4. Conclusions

An innovative L/G MBR configuration for H₂ production by dark fermentation, using a hollow fiber membrane module, was successfully implemented and its operation strategy was improved on several aspects. The intensification of the hydrogen production process in this bioreactor is twofold: *in situ* hydrogen extraction and immobilization of a hydrogen-producing biofilm, serving as a bacterial reservoir during the process in operation and shutdown phases. Indeed, after seeding with anoxic thermally treated WWTP activated sludge, the L/G MBR was repeatedly operated over a year under similar conditions (HRT = 12 h and OLR = 0.8 g/L/h), and without further bacterial reseeded. Despite repeated draining and rinsing, consistent HY of 0.97 ± 0.09 mol-H₂/mol-glucose added, and HPR of $106.5 \pm$

10.6 mL-H₂/L-medium/h, were reached. Acetate, butyrate and ethanol were the main metabolites produced. The diversity of the bacterial community of the liquid outlet was lower during the operation of the L/G MBR without reseeded compared to its operation right after the WWTP seeding stage. Moreover, it was mainly composed of HPB. Reseeding was successfully suppressed, thus greatly simplifying the L/G MBR process operation for subsequent H₂ production.

Several biogas extraction strategies were tested with sweeping gas (N₂ or CO₂) circulating in the lumen of the fibers or without sweeping gas. In the last situation, metabolism and bacterial development were oriented towards H₂ production, and a slightly better HPR (128.1 ± 27.4 mL-H₂/L-medium/h) was reached. Therefore, gas purification from the mixture of H₂ and CO₂ gases produced was greatly facilitated in the L/G MBR configuration since sweeping gas diluting the biogas produced was no longer required.

Unlike most of the continuous dark fermentative studies reported in the literature, we aimed to decouple the effect of the hydraulic residence time of the substrate (HRT) from that of the substrate feed rate (OLR) to describe the impact of these two key parameters. Thus, a range of HRT (2-14 h) were tested for the same OLR (1.4 g/L/h), and conversely, several OLR (1.0-2.1 g/L/h) were tested for the same HRT (8 h). Increasing OLR enhanced the HPR, but was detrimental to the HY at a glucose feed concentration above 13.1 g/L. An optimal hydrogen yield of 1.22 mol/mol-glucose added for a glucose concentration in the feed of 13.1 g/L was predicted by modelling of the experimental data, which was close to the experimentally determined optimum at experimental HRTs of 8-10 h combined with an OLR of 1.4 g/L/h.

Under these conditions, the development of hydrogen-producing bacteria (*Clostridium*, *Enterobacter* and *Ethanoligenens*) was enhanced. *Clostridium beijerinckii* developed preferentially at low glucose concentration and HRT without washout, while *Clostridium pasteurianum* dominated when higher glucose concentration and HRT were used. Thus, the

development of hydrogen-producing bacteria in the L/G MBR was successfully controlled by the relevant choice of operational conditions to apply.

To sum up, the original hollow fiber L/G MBR configuration enabled the testing and selection of fermentation strategies that greatly simplified the implementation of the dark fermentation process by addressing its key operational bottlenecks. Indeed, the L/G membrane surface served as a support and reservoir for the hydrogen producing bacteria. At the same time, *in situ* extraction without any apparent resistance to the gas transfer was enabled.

Acknowledgements

The authors thank Olivier Théobald (ADEME) for his comments on this study, Christelle Gruffaz for assistance with DNA extraction and Anne-Clémence Aun for assistance with HPLC/GC analysis. The French Agency for Ecological Transition (ADEME) with Région Alsace, and ADEME with Université de Strasbourg are thanked for doctoral fellowships to VC and MR, respectively. Special thanks from BE to G., D. & S. Gougeon.

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Tables

Table 1. HY, HPR, H₂/CO₂ ratio, related to the time elapsed since seeding of the L/G MBR with thermally-treated anaerobic sludge.

Time elapsed since the last seeding (d)	Calculation area	Average pH	HY (mol/mol) (added glucose)	HPR (mL/L/h)	H ₂ /CO ₂
0	26-68 h	5.2	1.09	153.0	1.27
365	48-100 h	5.4	0.95	102.0	0.97
418	30-96 h	5.5	0.90	98.8	0.77
432	30-68 h	5.0	1.07	118.6	0.90

Table 2. Comparison of HY and HPR in our study with values reported for dark fermentation membrane bioreactors.

Reactor mode	Separation, membrane	T°C	pH	OLR		HY (mol/mol)	HPR (mL/L/h)	Reference
				HRT (h)	(g/L/h) (glucose)			
Continuous	L/G, hollow fiber, PTFE	37	4.7-5.5	14	0.8	1.0 ± 0.1	108 ± 8	This study
Continuous	S/L, hollow fiber, PVDF	23	5.5	8	0.9	1.8	187	[24]
Continuous	S/L, flat sheet, PE	35	5.5	9	2.7	0.9	101 – 107	[25]
Semi-sequential	S/L, hollow fiber, PVDF	35	6.0	13	5.0*	1.6	751	[27]
Continuous	L/L, polyester mesh	37	5.5-6	3	5.0	3.0	2141	[28]

*calculated

Table 3. HY, HPR, glucose conversion, lag time (λ) and H₂/CO₂ ratio obtained with tests performed either with gas sweeping (N₂ or CO₂) or without gas sweeping.

Sweeping	Time elapsed since the last seeding (d)	HY (mol/mol) (added glucose)	HPR (mL/L/h)	λ (h)	Glucose conversion (%)	H ₂ /CO ₂
N ₂	432	1.07	118.6	4.6	97.7	0.90
CO ₂	439	0.86	94.3	4.8	na	na
NSG	380	1.03	98.7	45.8	na	1.05
NSG	453	1.21	132.7	15.5	98.4	1.01
NSG	551	1.29	153.0	10.9	100.0	1.03

NSG: no sweeping gas; na: not analyzed

Table 4. Effect of different combinations of OLR and HRT on average HY, HPR, H₂/CO₂ ratio and glucose conversion.

OLR (g/L/h)	HRT (h)	Feed glucose concentration (g/L)	HY (mol/mol) (added glucose)	HPR (mL/L/h)	H ₂ /CO ₂	Glucose conversion (%)
0.6	5	3.0	0.79	66.5	1.09	97.7
0.8	14	11.2	1.00	108.1	0.87	na
1.0	8	8.0	1.05	112.0	1.00	98.2
1.4	2	2.8	0.86	170.8	1.34	91.9
1.4	5	7.0	1.00	184.4	1.04	100
1.4	7	9.8	1.02	186.3	0.89	95.6
1.4	8	11.2	1.16	225.3	0.98	92.8
1.4	10	14	1.13	210.0	1.02	81.9
1.7	14	23.8	0.60	137.5	0.93	78.1
2.1	8	16.8	0.92	253.7	0.97	78.4

na: not analyzed

Figures

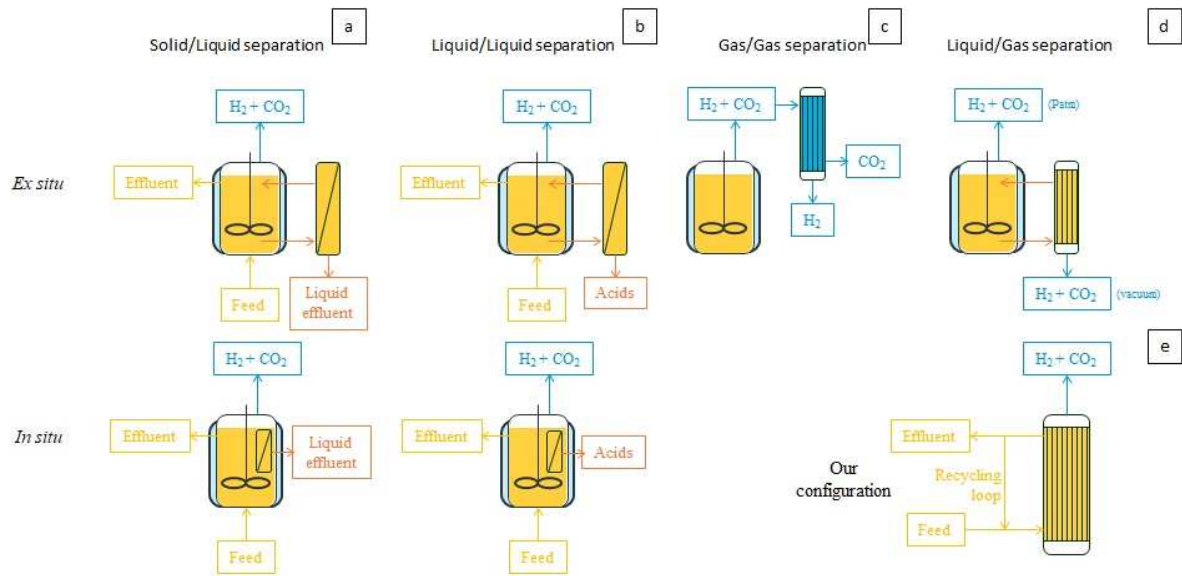


Figure 1. Different configurations of membrane bioreactors with *ex situ* and *in situ* solid/liquid (a), *ex situ* and *in situ* liquid/liquid (b), gas/gas (c), *ex situ* liquid/gas (d) and *in situ* liquid/gas separation (our design) (e).

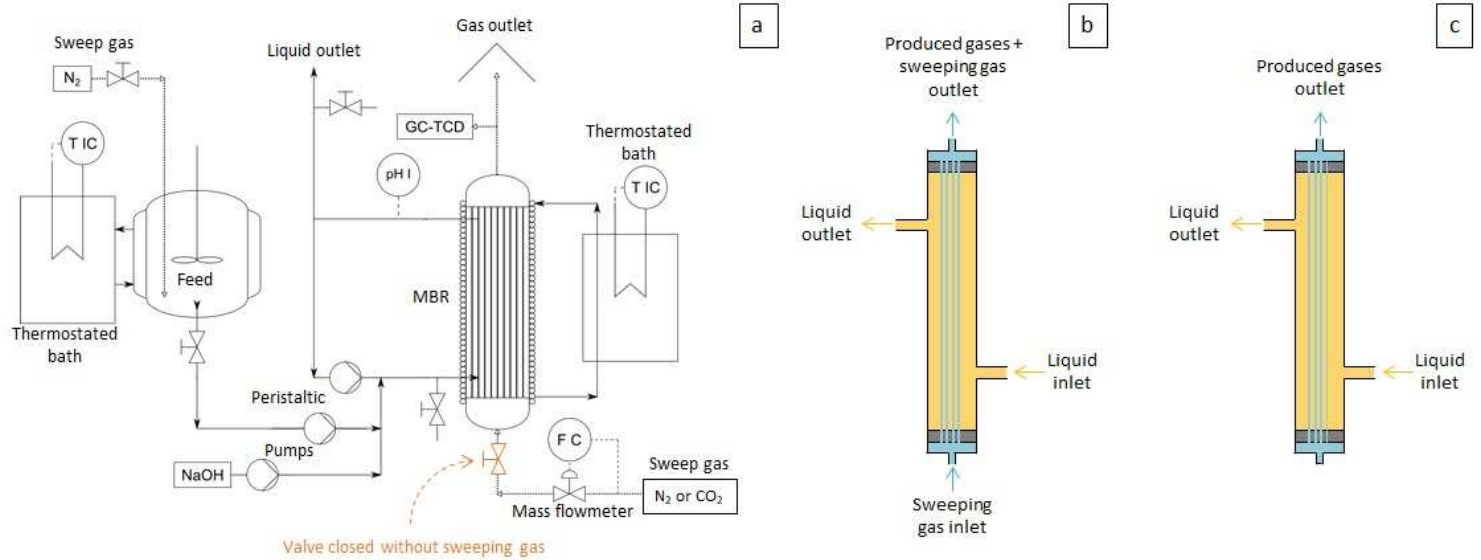


Figure 2. Experimental set-up of the L/G MBR (a), MBR configuration using sweeping gas (b) and MBR configuration without sweeping gas (c).

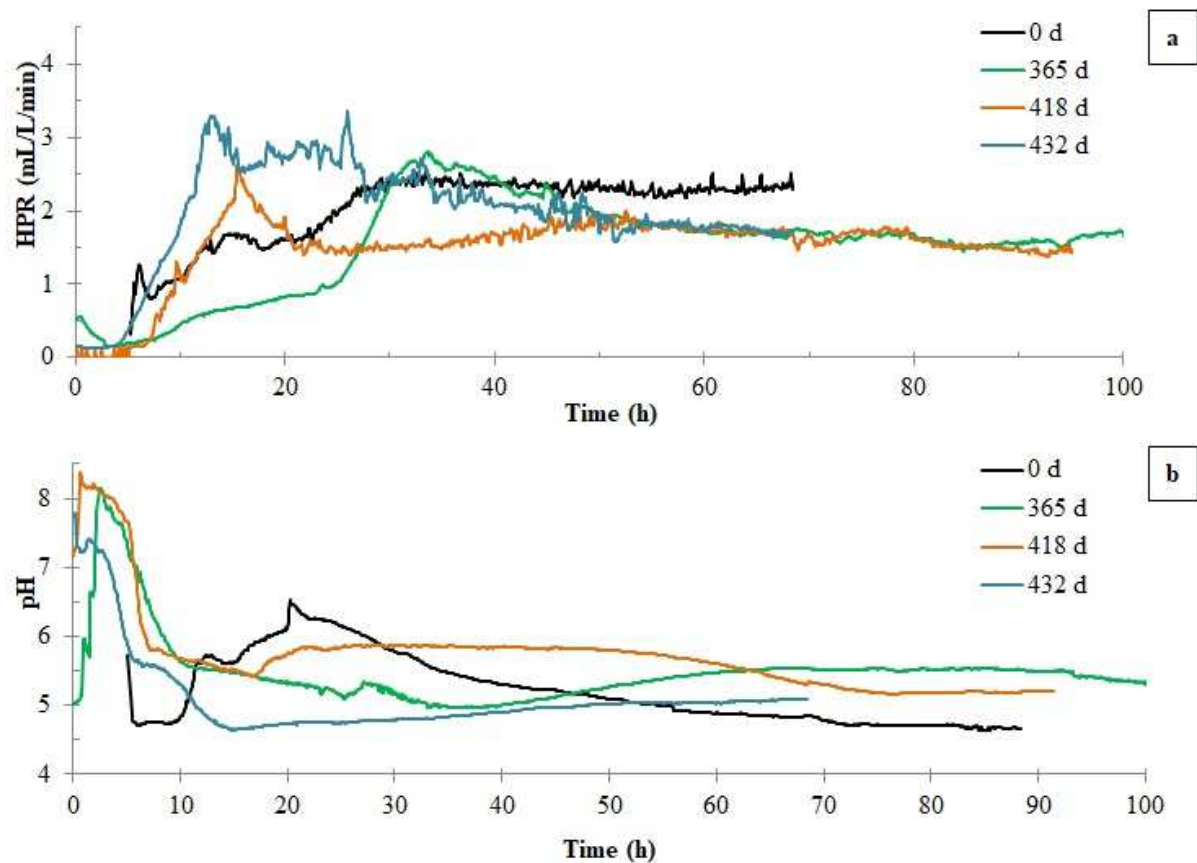


Figure 3. Evolution of hydrogen production rate (a) and pH (b) across 432 days (d) in the liquid outlet of the L/G MBR seeded at day 0 with WWTP sludge, without subsequent reseeded. Analysis was performed at 0, 365, 418 and 432 days.

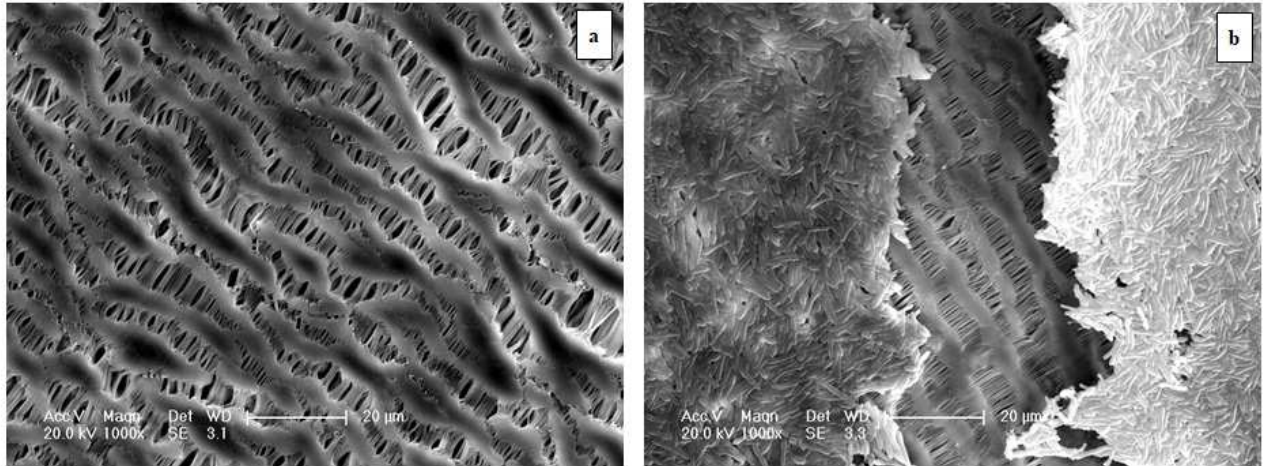


Figure 4. Scanning electronic micrograph of the bare hollow fiber surface (x1000) (a) and of the bacterial layer developed on the hollow fiber (x1000) (b).

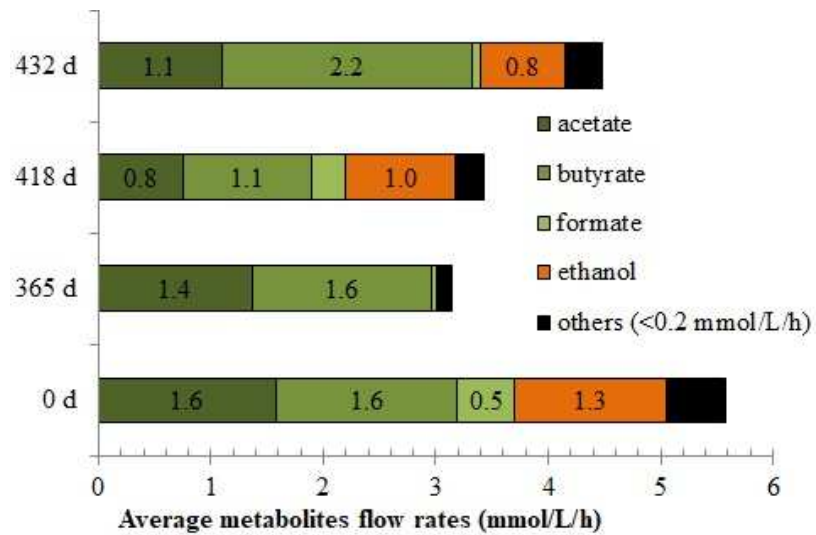


Figure 5. Evolution of flow rates of major metabolites (acetate, butyrate, ethanol) in the liquid outlet of the L/G MBR seeded with WWTPs sludge (0 days) and tests performed after 365, 418 and 432 days without reseeded.

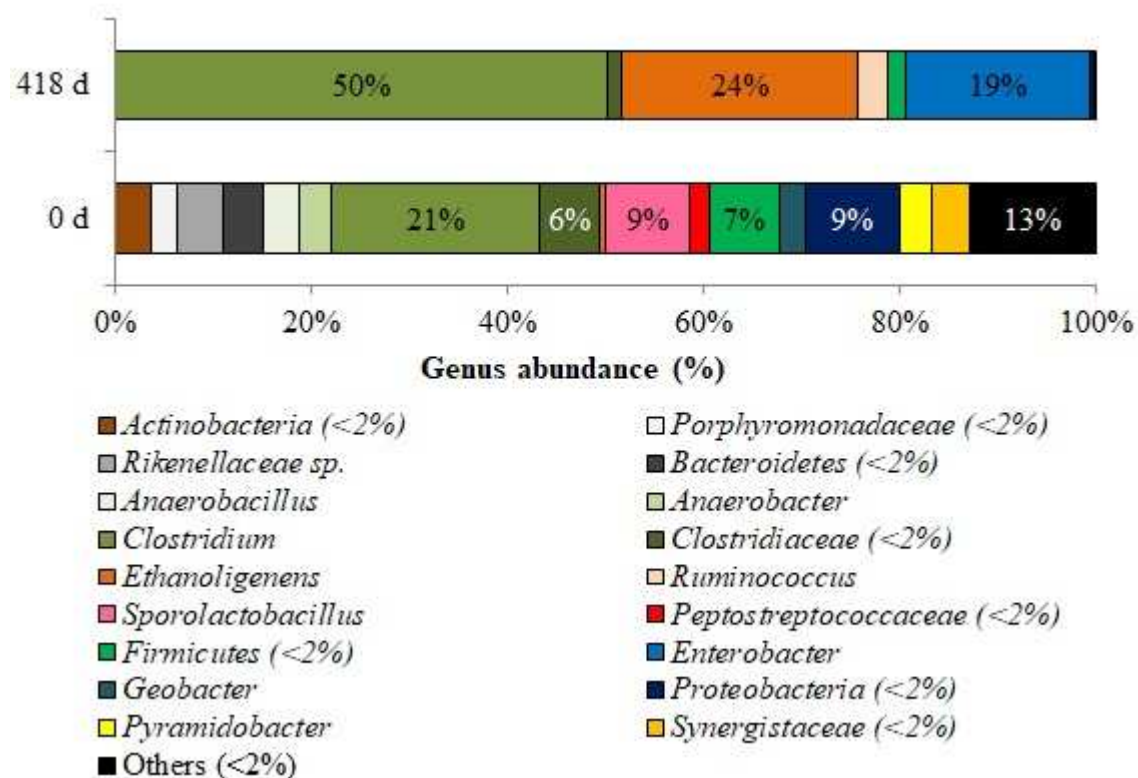


Figure 6. Relative abundance of main taxa (> 5%) in the liquid outlet of the L/G MBR at seeding with WWTP sludge (0 d) and after 418 days (418 d). Minor taxa (<2%) are grouped and presented under family/phylum denomination.

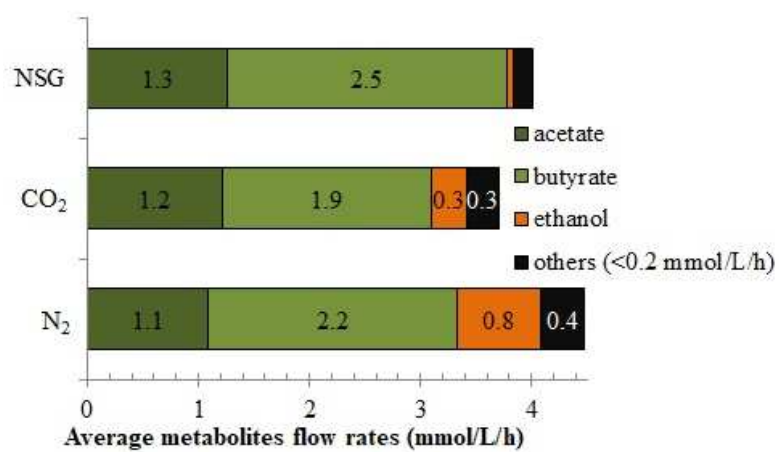


Figure 7. Evolution of flow rates of major metabolites (acetate, butyrate, ethanol) in the liquid outlet of the L/G MBR, operated without sweeping gas (NSG), with CO₂ sweeping (CO₂), or with N₂ sweeping (N₂).

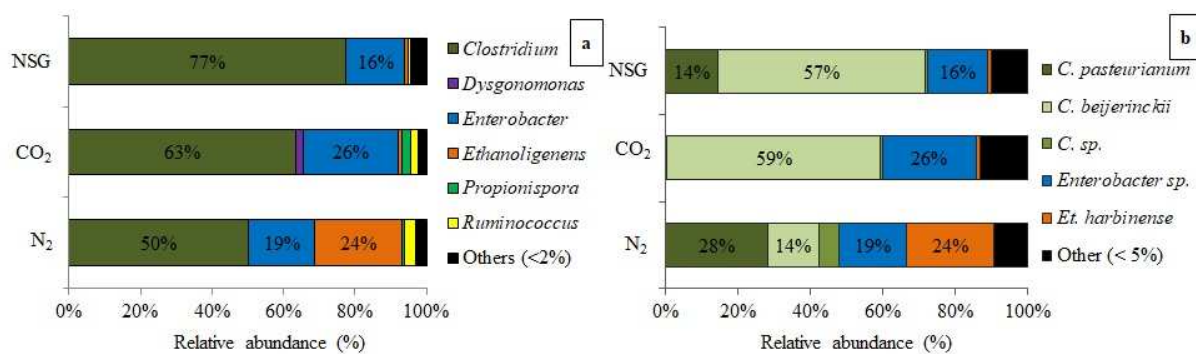


Figure 8. Evolution of relative abundance of major genus-level taxa (> 2%) (a) and of major species-level taxa (>5 %) (b) in the liquid outlet of the L/G MBR, operated without sweeping gas (NSG), with CO₂ sweeping (CO₂), or with N₂ sweeping (N₂).

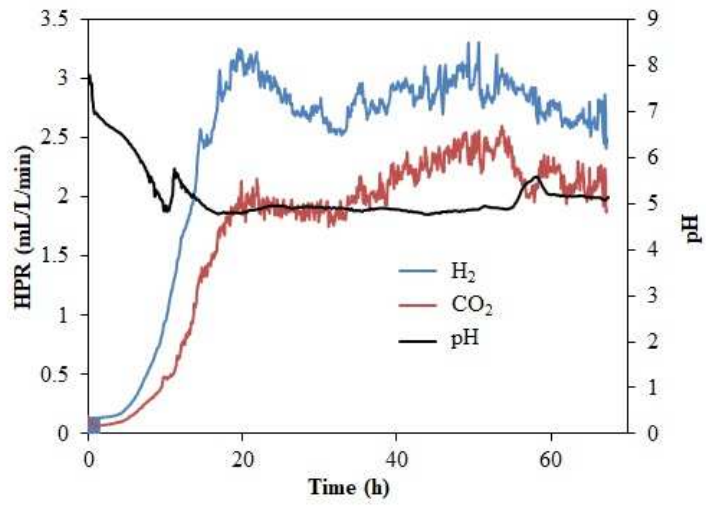


Figure 9. Evolution of hydrogen and CO₂ production rate, and pH in the liquid outlet of the L/G MBR (HRT = 2h; OLR = 0.8 g/L/h)

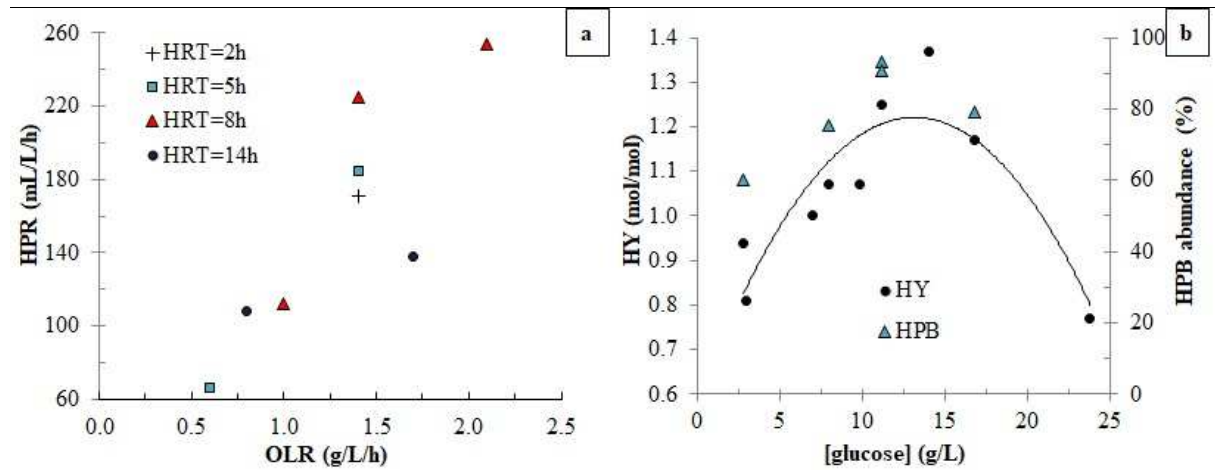


Figure 10. Correlation between HPR and OLR using different HRT (a), and between HY and relative HPB abundance (sum of *Clostridium beijerinckii*, *Clostridium pasteurianum*, *Clostridium carbodixivorans*, *Ethanoligenens harbinense* and *Enterobacter* sp. relative abundances), as a function of glucose concentration in the feed (b). Fitting the data to a second order polynomial ($y = ax^2 + bx + c$) yielded best-fit coefficients $a = -0.0037$, $b = 0.0974$, and $c = 0.5845$ ($R^2 = 0.80$).

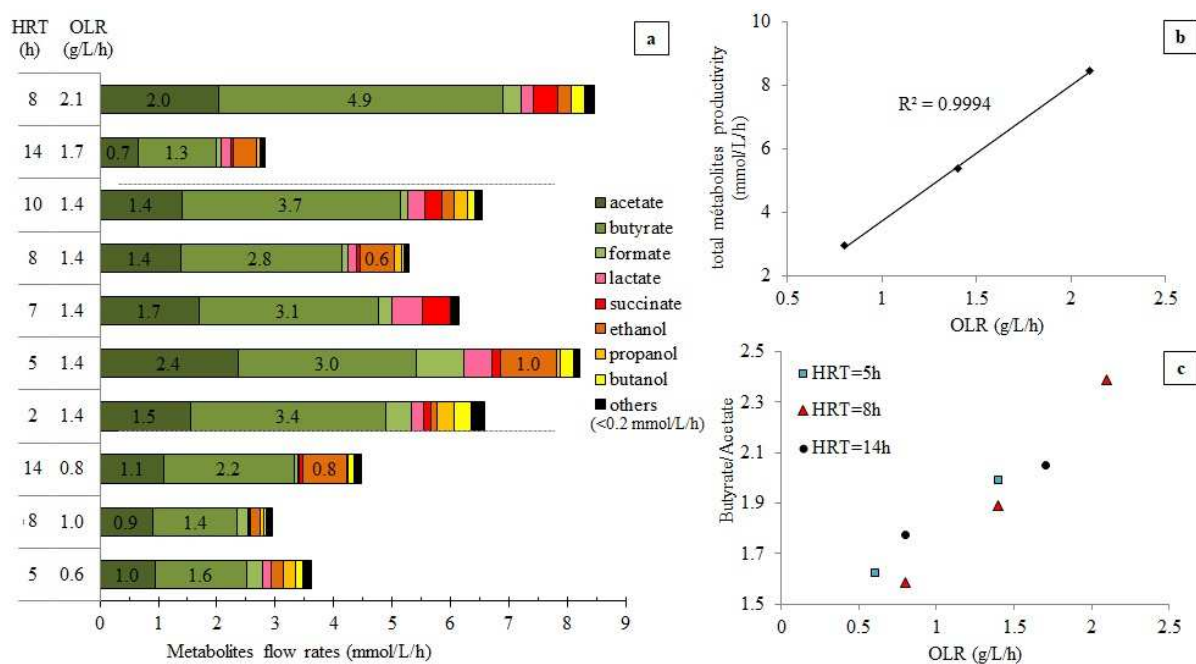


Figure 11. Metabolite production rates in the L/G MBR operated with different HRT/OLR combinations (a). Correlation between total metabolite productivity (sum of acetate, butyrate, formate, isovalerate, lactate, propionate, succinate, valerate, ethanol, propanol, butanol) and OLR at 8h HRT (b). Correlation between butyrate/acetate ratio and OLR for different HRT (c).

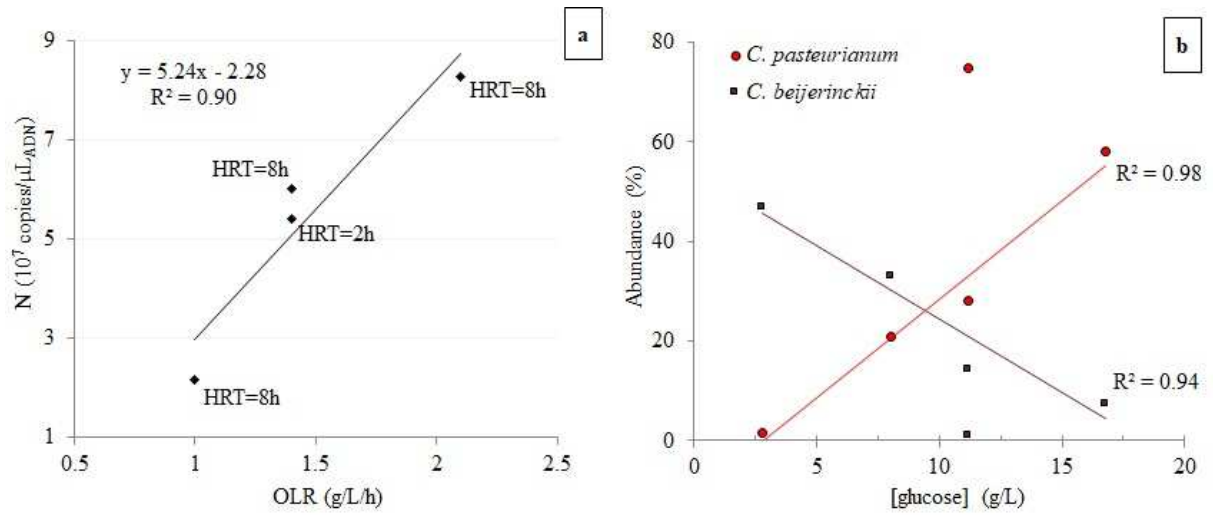


Figure 12. Correlation between OLR and total number of copies of the 16S rRNA gene in the liquid outlet, at 2h and 8h HRTs (a), and between glucose concentration in the feed and relative abundance of *Clostridium pasteurianum* and *Clostridium beijerinckii* (b).

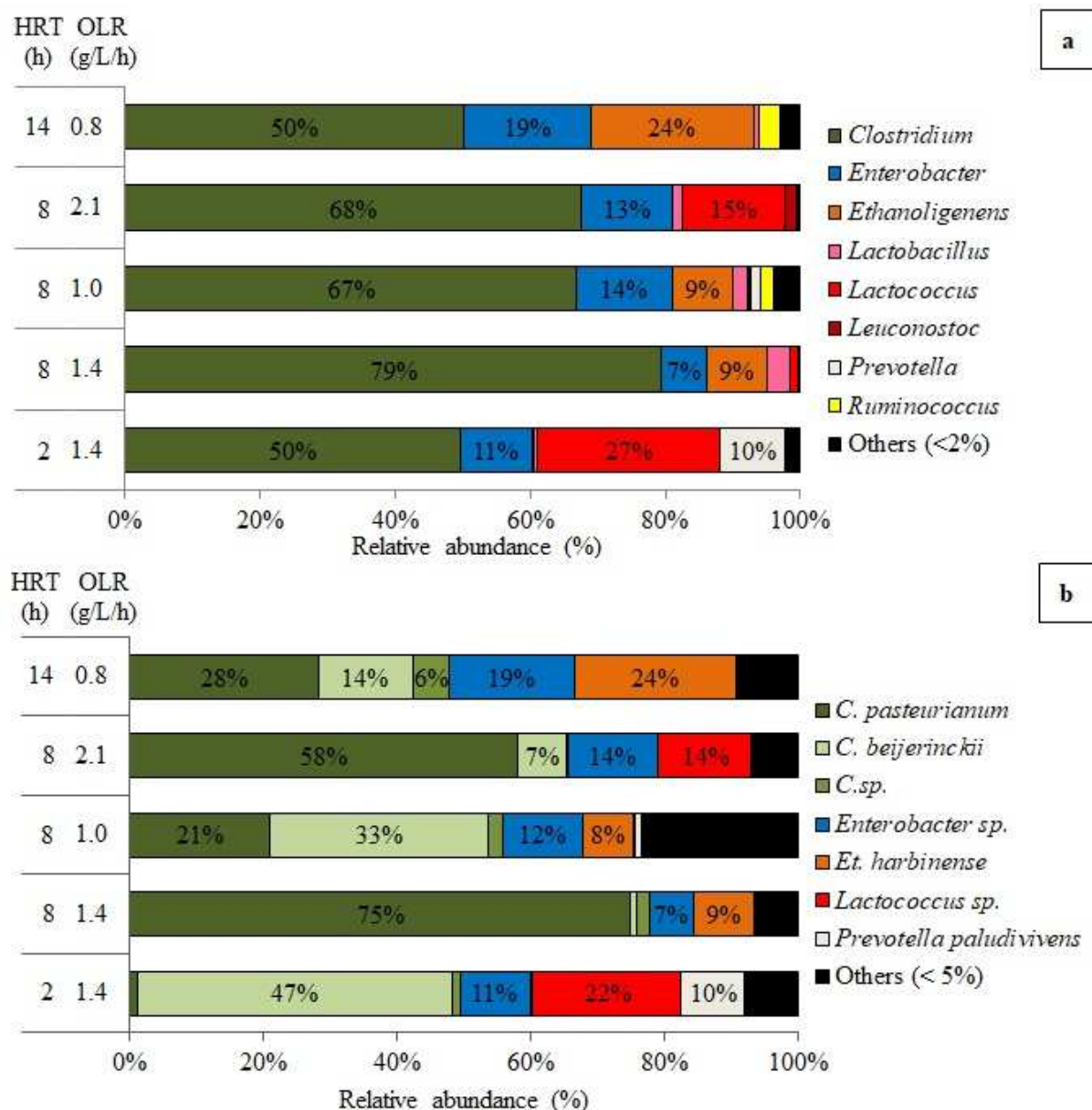
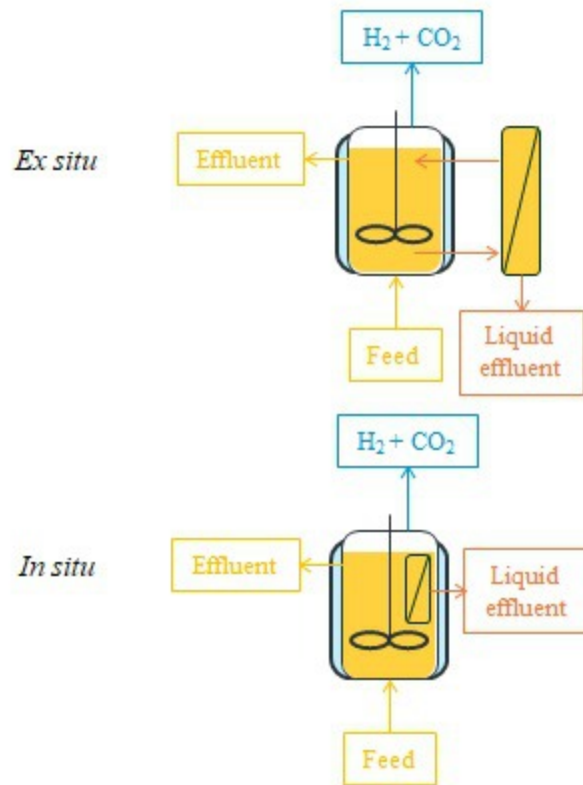


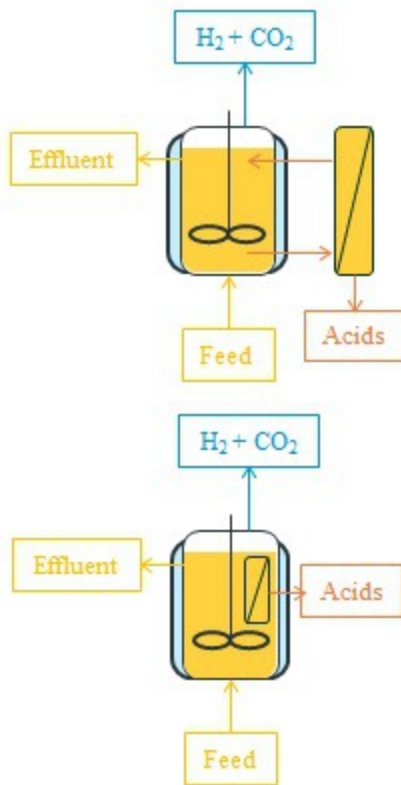
Figure 13. Relative abundance of major genus-level taxa (> 2%) (a) and of major species-level taxa (>5 %) (b) in the liquid outlet of the L/G MBR operated under different HRT/OLR combinations.

Different configurations of membrane bioreactors for H_2 production by dark fermentation

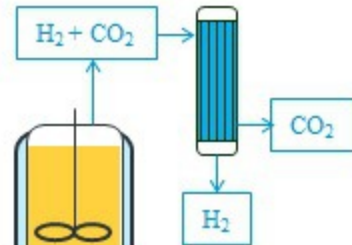
Solid/Liquid separation



Liquid/Liquid separation



Gas/Gas separation



Liquid/Gas separation

