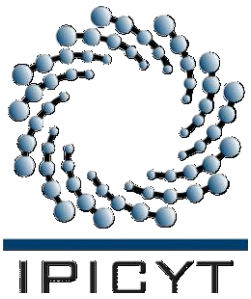

INSTITUTO POTOSINO DE INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA, A.C.

POSGRADO EN CIENCIAS AMBIENTALES

Effect of inoculum pretreatment on hydrogen production in high cellular density reactors and evaluation of interactions between their microbial functional groups

Tesis que presenta

Crhistian Cisneros Pérez

Para obtener el grado de

Doctor en Ciencias Ambientales

Director de la Tesis:

Dr. Elías Razo Flores

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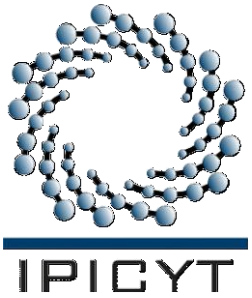

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DOCTOR EN CIENCIAS AMBIENTALES

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Crhistian Cisneros Pérez

sobre la Tesis intitulada:

Effect of inoculum pretreatment on hydrogen production in high cellular density reactors and evaluation of interactions between their microbial functional groups

que se desarrolló bajo la dirección de

Dr. Elías Razo Flores

El Jurado, después de deliberar, determinó

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SECRETARIA ACADEMICA

Dedicado a mi mamá, hermanas y toda la familia, por su apoyo.
A Eunice, por su presencia y hacer todo más bello.

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Resumen

Efecto del pretratamiento de inóculo en la producción de hidrógeno en reactores de alta densidad celular y evaluación de las interacciones entre sus grupos funcionales microbianos

Palabras clave: biohidrógeno, DGGE, pretratamientos, inóculo, ecología microbiana

Se considera que el hidrógeno tendrá una gran relevancia como combustible en el futuro próximo, llegando incluso a hablarse de una economía basada en el hidrógeno, ya que además de ser usado de manera directa es posible usarlo como acarreador intermediario de energía. Sin embargo, aunque la tecnología para su aprovechamiento tiene ya varias décadas de desarrollo, su producción aún depende en gran medida de los combustibles fósiles a los que tendría que suplantar. Uno de los métodos más prometedores es la fermentación oscura, por medio de la cual se aprovechan las características fisiológicas de bacterias creciendo en condiciones anaerobias. Hay una gran variedad de parámetros que influyen en la producción de biohidrógeno por fermentación oscura, como el tiempo de residencia hidráulica, la velocidad de carga orgánica, pH, tipo de sustrato y características del inóculo. La mayoría de estos parámetros han sido ya estudiados y descritos en la literatura. Sin embargo, poco se ha logrado esclarecer acerca de la interdependencia y demás interacciones entre los distintos componentes de la comunidad microbiana anaerobia que permitn altas producciones de hidrógeno. Estas comunidades existen naturalmente en los lodos anaerobios de las plantas de tratamiento y se intercalan con las productoras de metano. Por esto, las fuentes más comunes de obtención de inóculo para producción de metano son las naturales y/o de plantas de tratamiento, a las que es necesario dar un tratamiento previo para inhibir toda actividad metanogénica. En esta tesis se evaluaron dos diferentes pretratamientos como estrategias de enriquecimiento para obtener comunidades hidrogenogénicas, el choque térmico y el lavado celular. Los reactores de biomasa inmovilizada tienen la capacidad de alcanzar mayores concentraciones de biomasa microbiana, permitiendo mayor eficiencia en la producción de biohidrógeno. Usando reactores granulares de lecho expandido (EGSB), los máximos rendimientos molares (0.92 mol H_2/mol de hexosa) y de velocidad volumétrica de producción de hidrógeno (4.23 L H_2/L-d), con una carga orgánica de 36 g de glucosa/L-d a un tiempo de retención de 10 h con lodo tratado por lavado celular. En reactores anaerobios de lecho fluidificado (AFB), el lodo tratado por lavado celular produjo valores máximos de volumen de producción y rendimiento molar de 7 L H_2/L-d y 3.5 mol H_2/mol hexosa, respectivamente a una carga de 60 g de glucosa y 6 h de tiempo de retención. En ambos casos el lavado celular produjo mejores rendimientos y desempeño en general del reactor que lo obtenido usando inóculo tratado térmicamente, a pesar de ser este último el más ampliamente reportado. Ambos reactores mostraron alta abundancia de miembros de los taxa *Clostridia*, *Enterobacteriaceae* y bacterias ácido lácticas. En reactores EGSB, miembros de la familia *Enterobacteriaceae* en conjunto tuvieron una abundancia relativa mayor, pero los aumentos en la producción de hidrógeno estaban relacionados con el aumento en la presencia de miembros del género *Clostridium.* En el caso de reactores AFB, *Clostridium* fue el principal componente de la comunidad. Las bacterias ácido lácticas, principalmente de los géneros *Lactobacillus*, *Sporolactobacillus* y *Lactococcus* estuvieron presentes durante la

operación de ambos tipos de reactores, estando relacionados en ambos casos con disminuciones en la producción de hidrógeno. En general, los reactores AFB mostraron mayor abundancia de *Clostridia* en comparación con EGSB, indicando una selección positiva por parte del carbón activado granular usado como soporte inerte y el régimen hidrodinámico obtenido por la configuración del reactor. El papel de los diferentes grupos funcionales ecológicos en la producción de hidrógeno también fue estudiado, observando que aunque *Clostridium* es el principal productor de hidrógeno, otros géneros como *Megasphaera* pueden también participar activamente aún cuando el sustrato principal se ha consumido, aumentando la producción y mejorando el desempeño. Aunque la configuración del reactor tiene un efecto directo e inmediato en el rendimiento del hidrógeno, es la estructura y composición de la microbiota que constituye la biomasa que determina el potencial para la producción de hidrógeno en condiciones adecuadas.

Abstract

Effect of inoculum pretreatment on hydrogen production in high cellular density reactors and evaluaton of interactions between their microbial functional groups

Key words: biohydrogen, DGGE, pretreatment, inoculum, microbial ecology

It is considered that hydrogen will have great relevance as a fuel in the near future, even going so far as to speak of a hydrogen-based economy, since besides of being used directly it is possible to use it as an energy intermediary carrier. However, although the technology for its use has already several decades of development, its production still depends to a great extent on the fossil fuels. One of the most promising methods is the dark fermentation, by means of which the physiological characteristics of bacteria growing in anaerobic conditions are exploited. There are a variety of parameters that influence biohydrogen production by dark fermentation, such as hydraulic residence time, organic loading rate, pH, type of substrate and characteristics of the inoculum. Most of these parameters have already been studied and described in the literature. However, little has been achieved to clarify the interdependence and other interactions between the different components of the anaerobic microbial community that allows high rates in hydrogen production. These communities exist naturally, and are intercalated with methane producers. For this reason, the most common source of inoculum for the production of methane are natural ones and / or treatment plants, which need to be pre-treated to inhibit all methanogenic activity. In this thesis two different pretreatments were evaluated as enrichment strategies to obtain hydrogenogenic communities, thermal shock and cell wash-out. The immobilized biomass reactors have the capacity to reach higher concentrations of microbial biomass, allowing greater efficiency in the production of biohydrogen. Using expanded granular sludge bed (EGSB) reactors, maximum molar yield (0.92 mol H_2 / mol hexose) and volumetric hydrogen production rate (4.23 L H_2/L-d) with an organic loading rates of 36 g glucose/L-d at HRT of 10 h with cell wash-out pretreated sludge. In anaerobic fluidized bed (AFB) reactors, sludge that was pretreated with cell wash-out produced maximum volumetric and molar yield values of 7 L H_2/L-d and 3.5 mol H_2/mol hexose, respectively, at 60 g glucose/L-d and HRT of 6 h. In both cases cell wash-out produced better yields and overall performance of reactor than that obtained using thermally treated inoculum, despite being the latter the most widely reported in the literature. Both reactors showed abundance mainly in members of *Clostridia*, *Enterobacteriaceae* and lactic acid bacteria taxa. In EGSB reactors, members of the family *Enterobacteriaceae* as a whole had a higher relative abundance, but increases in hydrogen production were related to an increase in the presence of members of the genus *Clostridium*. In the case of AFB reactors, *Clostridium* was the main component of the community. Lactic acid bacteria, mainly of the genera *Lactobacillus*, *Sporolactobacillus* and *Lactococcus* were present during the whole operation of both types of reactors, being related in both cases with decreases in hydrogen production. In general, AFB reactors showed greater abundance of *Clostridium* compared to EGSB, indicating a positive selection by the granular activated carbon used as an inert support and the hydrodynamic regime obtained by the reactor configuration. The role of the different functional ecological groups in hydrogen production was also studied, noting that although *Clostridium* is the main producer of hydrogen, other genera such as *Megasphaera* can also actively participate even when the main substrate has been consumed, increasing

production and improving the performance. Although the reactor configuration has a direct and immediate effect on the hydrogen yield, it is the structure and composition of the microbiota that constitutes the biomass that determines the potential for the production of hydrogen under suitable conditions.

Chapter 1

Hydrogen production by dark fermentation: process conditions

Summary

Biohydrogen production, especially by dark fermentation, is considered a promising alternative to conventional fuels and it belongs to the next generation of energetics in which the economy will be based. It has a high energy content (122 kJ/g), it is carbon neutral and it can be produced through organic waste valorization. In the last years, several studies have reported advances on this topic, mainly evaluating different reactor conditions, substrates and inoculum sources. There are many known factors that contribute to obtaining high and stable hydrogen production rates, but by far, the best studied parameters are operational conditions. A high cell concentration seems to be the most important condition in order to obtain an acceptable stability and good performances. Fixed biomass reactors allow to support high organic loading rates while diversity in the bacterial community allows to maintain stable processes in the presence of sudden operational changes. In this chapter, appropriate operational conditions that regulate fermentative process in hydrogenogenic communities are examined, as well as the main strategies in biohydrogen production.

1.1 Introduction

The current global economy is dependent on fossil fuels and all perspective studies show that they are depleting. Moreover, their use producing energy poses several environmental problems such as pollution and production of greenhouse gases. One attractive option is to use a biological process to produce a biofuel. Among the various biofuel options, biohydrogen gas is an interesting energy carrier due to its potentially higher efficiency of conversion to usable power, practically non-existent generation of pollutants and high energy density [1–3]. Therefore, research on this renewable clean-energy resource has become a priority for politics and scientists in the last years [3–5]. Instead of fossil fuel, hydrogen production from biomass has to be proven as sustainable and renewable. All processes of biological hydrogen production are dependent on the presence of hydrogen-producing enzymes, such as pyruvate-ferrodoxin hydrogenase or pyruvate-formate lyase [6–8] and the ultimate electron donor is an organic compound such as a sugar. As the source of the organic compound is biomass, fermentative bioH_2 can be considered renewable, since the biomass itself originated from photosynthesis.

Hydrogen can be produced by dark fermentation where anaerobic bacteria growth on carbohydrate rich substrates giving organic end products, principally acids (lactic, acetic, butyric, etc.) and alcohols (ethanol, butanol, etc.), hydrogen and carbon dioxide [1,3,8,9]. Pure cultures found to produce hydrogen from carbohydrates include species of *Enterobacter*, *Bacillus* and *Clostridium*. The pure substrates used include glucose, starch and cellulose. Process conditions including inoculums have a significant effect on hydrogen yield as they influence the fermentation end products [6].

Biohydrogen production usually follows two main metabolic pathways: (i) the acetate pathway (Eq. 1.1), and (ii) the butyrate pathway (Eq. 1.2).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \qquad (1.1)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \qquad (1.2)$$

Depending on the pathway, the theoretical biogas composition is around 67% of H_2 (acetate pathway) or 50% of H_2 (butyrate pathway). This indicates a very interesting potential of H_2 production from carbohydrate-rich wastewater [1,2,5], although in practice, usually a

mixture of both pathways is obtained, along with other acids, giving low yields and production rates, as a direct consequence of the thermodynamics of known metabolic processes [1]. Types and relative proportions of products depend on organisms, environmental conditions (pH, temperature and the partial pressure of hydrogen) and the oxidation state of the substrate being degraded.

Since the process of fermentative hydrogen production are under the influence of several factors such as inoculum, substrates, inorganic nutrients, operational condition, etc., it is beneficial that an appropriate experimental design would be used to study the effects of various factors on the process, understanding easily its effect and even optimizing its performance [7].

1.2 Reactors

Possible improvements to biohydrogen production have been sought through specialized bioreactor configuration [1], producing systems with more robust, reliable performance that are stable over long periods of time (months) and resistant to short-term fluctuations in operational parameters. Batch mode fermentations have been shown to be more suitable for initial optimization studies [10–15].

Two main typologies of reactors are commonly employed for continuous and semi continuous experiments: (i) suspended biomass reactors, in which the fermentative bacteria form suspended flocs of biomass that are continuously mixed with wastewater, and (ii) immobilized biomass reactors, where the fermentative bacteria grow self-immobilized by a granulation process or immobilized on the surface of a carrier material [5]. Among the suspended biomass systems, the most used for H_2 production are the anaerobic sequencing batch reactor (ASBR) and the continuous stirred tank reactor (CSTR), whereas among the immobilized biomass systems the most used are the anaerobic packed bed reactor (APBR), expanded granular sludge bed (EGSB) and the anaerobic fluidized bed reactor (AFBR) [5].

CSTRs are the most commonly used continuous reactor systems, due to its simple construction, ease operation and effective homogeneous mixing. However, in these reactors, hydraulic retention time (HRT) controls the microbial growth rate and therefore HRTs must be greater than the maximum growth rate of the desired organism(s), or lower than the unwanted microbial community growth rate, because faster dilution rates cause wash-out of cells. The immobilized biomass reactor overcomes the limitations of CSTRs

and offers several advantages for a practical bioprocess. Because microbial growth and the concentration of microbial biomass are rendered independent of HRT, high cell concentrations can be achieved, fostering high volumetric production rates, and high throughput is possible, allowing the use (and treatment) of dilute waste streams with relatively small volume reactors [1,3,16,17].

1.2.1 Granulation based reactors

Anaerobic granules are particulate biofilms, formed spontaneously by self-immobilization of anaerobic bacteria in the absence of a support material, where each granule is a functional unit comprising all different microorganisms necessary for anaerobic degradation of organic matter [18]. Shear stress, caused by the operational hydrodynamic conditions in bioreactors, is thought to be one of the most important factors promoting granule formation [19]. A major drawback in granule based processes is the long start-up period, which generally requires several months for the formation of hydrogen-producing granules [20]. The link between digester performance, and the physiological and structural characteristics of the anaerobic sludge is particularly relevant in up-flow anaerobic sludge blanket (UASB), expanded granular sludge bed (EGSB), or internal circulation (IC) reactors [17].

1.2.2 Biofilm based reactors

A biofilm is a complex coherent structure of cells and cellular products, like extracellular polymers, immobilized on a substratum that can be a static solid surface (static biofilms) or suspended carriers (particle supported biofilms) [19]. Cells growing on the biofilms have an increased resistance and better survival rates under harsh environmental conditions relative to the planktonic bacteria [21], and an increased ability to resist invading microbial communities [22].

The anaerobic fluidized bed reactor (AFBR) with attached biofilm has been widely used as a biological treatment system for wastewater with high efficiency and low HRT due to its potential advantages, e.g., high concentration of biomass attached to a dense carrier and good mixing characteristics [21,23,24].

However, rapid hydrogen-producing culture growth and high organic loading rate might limit the application of biofilm biohydrogen production, since excessive growth of

fermentative biomass would result in wash-out of support carrier [25].

1.3 pH and buffer composition

The pH play a pivot role in biological processes, because it triggers the signaling network, regulating enzyme activities, the metabolism pathways, microbial community structure and diversity, etc. [3,7,10,26]. Buffering compounds in media formulation becomes more important when batch mode is being employed and there is no external solution to reduce pH variations during fermentations. In batch mode, optimal pH for hydrogen production lie between 4.2 and 8.0, depending on the inoculum source and its previous history [7], but below 6.0 when pure cultures are used [27]. However, when continuous mode is employed, optimal pH around 5.5 has proven to be effective to obtain an acetate/butyrate fermentation type [8,26,28,29]. The composition of culture media had a strong effect on hydrogen production, kinetics and also on the microbial diversity [10,11,30,31].

There are many reports about biohydrogen production using complex substrates, and most of the times the use of a carbonated compounds as buffer mineral media is preferred [31–35], because conventionally it has been successfully used in anaerobic digestion processes. Since in dark fermentation there is an intrinsic generation of organic acids that react with the bicarbonate, it could generate additional dissolved CO_2, decreasing the buffer capacity [10]. Phosphate-based buffer mineral media have the advantage of having greater capacity to reduce pH variations and to be the source of phosphate required to generate energy as ATP and other anabolic compounds.

1.4 Hydraulic retention time and organic loading rate

Hydraulic retention time (HRT) controls the microbial growth rate (dilution rate of the reactor); hence, HRTs must be greater than the maximum growth rate of the microorganisms because faster dilution rates cause wash-out in suspended biomass systems [3,36]. OLR or HRT adjustment is a common strategy for operators; shorter HRT and higher OLR may raise the hydrogen production rate [37], but just in reactors with immobilized biomass, since they can overcome the wash-out problem and result in high cell concentrations, fostering high volumetric production rates, in which HRT may exert a significant hydrodynamic selection on the mixed-microbial populations [3,20,36–38,16]. In the same way, rapid hydrogen-producing culture growth and high organic loading rate

might limit the application of biofilm biohydrogen production, since excessive growth of fermentative biomass would result in wash-out of support carrier [25].

1.5 Inoculum

Microorganisms that are capable to produce H_2 are part of the microbial communities from several natural and artificial habitats such as soil, wastewater, sludge from wastewater treatments plants, compost, etc., thus these materials can be used as potential source of inoculums for fermentative H_2 production [7]. In the most effective systems, consortia are selected for growth and dominance under non-sterile conditions and usually show high stability and resistance to transient unfavorable changes in the bioreactor environment [19]. The potential of various pure cultures has been exploited to produce hydrogen using a variety of substrates. Members of *Clostridium* and *Enterobacter* were most widely used as inoculums for fermentative H_2 production [3,7,9]. These experiments were conducted in batch and glucose was used as substrate. However H_2 production from organic wastes is more desirable as it is a feasible process for full scale applications with the goal of waste reduction and energy production [7,27]. There are also a number of potential advantages of using microbial consortia instead of pure cultures. Hydrogen fermentations at full scale will have to be carried out under non-sterile conditions using readily available complex feedstock with only minimal pretreatment. Microbial consortia address these issues as they have been selected for growth and dominance under non-sterile conditions. As complex communities, they are also likely to contain a suite of the necessary hydrolytic activities, and they are potentially more robust to changes in environmental conditions [1,36,27]. The use of microbial consortia has indeed been proven useful in reactor systems that yield high volumetric rates of hydrogen production, as discussed above. However, as complex communities their composition can vary over time, with changes in process parameters and from reactor to reactor, as has been shown by molecular (16S rRNA genes) studies [1,10,26,29,39]. A possible way to overcome this might be to construct a designed consortium [1,9,36,27] with the goal of creating a community of diverse members, each contributing a unique and essential metabolic capacity. The total community metabolic range would be greater than any individual member, while at the same time mutual interdependence would assure stable maintenance of individual members. However, little is known about the complex interactions that occur in natural consortia or how stable

synthetic microbial communities could be built [1,3,9,20,32].

1.6 Inoculum pretreatments

Treatments applied to the inoculum had been used in previous studies as methods for increasing hydrogen production by altering the microbial communities present in the starting mixed population [3,20,28].

The various pretreatment methods reported for enriching H_2-producing bacteria from mixed cultures mainly include heat-shock, acid-base shock, aeration, freezing and thawing, chloroform, sodium-2-bromoethane sulfonate or 2-bromoethane sulphonic acid and iodopropane [7]. Different pretreatment processes have different properties, and comparisons of different pretreatment methods to get a better one for a given process has been conducted by many researches [3,5,7,15,26,43].

Most of the studies about pretreatment methods had been conducted in batch modes, in which heat-shock has been used as preferred method. During heat-shock, spore-formers hydrogen-producing bacteria are principally selected, but those hydrogen producers that are not capable to sporulate are eliminated along with hydrogen consumers. However, kinetic control has been proposed in continuous mode [7], accomplishing the same function on the basis of differences in microbial duplication times between bacteria and archea by means of short hydraulic retention times [41–43], during which the operational condition favor fermentative bacteria, within which are the hydrogen-producers.

1.7 Community structure

A good understanding of the structure of hydrogen-producing microbial communities is vital for attempts to optimize H_2 production [21,26,27,40,44]. Examination of the biodiversity within hydrogen-producing consortia are mainly based on 16S rRNA gene sequence analysis. The techniques most commonly used for analyzing the diversity of hydrogen-producing microbial communities are polymerase chain reaction-denaturing gradient gel electrophoresis (PCR- DGGE) and direct sequencing of the DNA extracted from DGGE bands or cloning and sequencing of the DNA from these bands [20,24,28,29], terminal restriction fragment length polymorfism (T-RFLP) using reference strains [45]. However, these strategies often lead to an underestimation of the true bacterial diversity as a result of co-migration of different DNA fragments during electrophoresis and

concentrating only on the most prominent DGGE bands, peaks or sequences [19]. Overall, three physiological groups of microorganisms have been observed: high-yield hydrogen producers (*Clostridium, Kosmotoga, Enterobacter*), fermenters with low-hydrogen yield (mostly from *Veillonelaceae*), and competitors (*Lactobacillus*) [26]. Often, these functional groups are found in hydrogen producing reactors, but the knowledge about interactions at any level is still poor. Because of this, biohydrogen production is usually fluctuating or even unstable at various time scales, possibly due to the constant dynamic changes in the microbial community between the different levels of hydrogen-producers, competitors and hydrogen-consumers [7,22,26,36,45].

1.8 Justification

The use of anaerobic digestion is one of the technologies that stand out for their efficiency in obtaining renewable energy sources such as biofuels, most notably biohydrogen. Advances in the fundamental understanding of the biochemistry and microbiology of anaerobic processes has led to successful applications, which show great promise to overcome the limitations associated with anaerobic processes. The EGSB and anaerobic fluidized bed reactors (AFBR) have proven to be efficient and successful in their scaling to industrial level. The establishment of benchmarks for optimal operation in anaerobic hydrogen production, environmental conditions created inside the reactor, as well as identification of microorganisms responsible, interactions, dependencies, community structure, function and physiological ecology in general, will allow to understand hydrogen-producer bacterial communities and improve this process.

1.9 Scope and structure of the thesis

1.9.1 Hypothesis

Different inoculum pretreatments will develop different hydrogenogenic bacterial communities, affecting the performance of immobilized-cell reactors in terms of hydrogen production. EGSB and AFB reactors will allow to operate lower hydraulic retention times and higher organic loading rates, increasing the H_2 production.

The production of hydrogen in EGSB and AFBR will be different because the diverse selection of microorganisms in each reactor.

Microorganisms present in hydrogen producing reactors have different hydrogen producing

capacities and their interaction inside the reactor lead to different performances.

1.9.2 General objective

The aim of this work was to identify the most appropriate inoculum pretreatment and operational parameters for high volumetric hydrogen production rates in EGSB and AFB reactors and understand the interaction of the different functional groups present in the reactors.

1.9.3 Specific objectives

a) To determine operational conditions that yield higher volumetric hydrogen production rates using EGSB and AFB reactors.

b) To determine which inoculum pretreatment is the most suitable in order to obtain a more adequate hydrogenogenic bacterial community, in terms of VHPR and HY.

c) To determine the activity that perform the major components of the bacterial community that were present in the original methanogenic inoculum, the biomass obtained after pretreatments, and the biomass resulting from each operational condition by identifying them, relating to the reactor used, as well as the performance observed.

d) To determine the possible interactions between identified components in the microbial communities and their physiological activity, and its relationship with the reactor performance.

1.9.4 Structure of the thesis

In Chapter 2, two different inoculum pretreatments (heat-shock and cell wash-out) were assayed, in a fermentation carried out in EGSB reactors, in order to study the effect of pretreatments in the granular microbial communities and its dynamics while hydraulic retention time and organic loading rate were modified. Community structures of both reactors were analyzed and compared using PCR-DGGE.

In Chapter 3, the same strategy was employed, but on a microbial community based on a biofilm developed on AFB reactors, and applying a starvation period before to return to previous operational conditions, in order to observe resilience of the communities and performances.

In Chapter 4, pure strains from different ecological functional groups were employed to

observe their interactions in co-cultures after learning about their kinetics and metabolic characteristics as mono-cultures. The data obtained in batch assays were then used to construct co-cultures in continuous mode using CSTRs.

The global results obtained in this work are presented and discussed in Chapter 5, with final conclusions, perspectives and recommendations.

1.10 References

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Chapter 2

Inoculum pretreatment promotes differences in hydrogen production performance in EGSB reactors

Summary

Hydrogen production by dark fermentation is one of the most promising methods for obtaining clean energy. The inoculum pretreatments allow selecting bacteria that have better performance in hydrogen production, because the selection of pretreatment limits the presence of some species while favoring others. In order to elucidate the inoculum pretreatment influence during the operation of two EGSB reactors, two pretreatments were assayed: heat shock and cell wash-out. Different organic loading rates (24 to 60 g glucose/L-d) and hydraulic retention times (10 to 4 h) were applied to both reactors to determine population dynamics along 100 days of operation. Reactors exhibited differences in both volumetric hydrogen production rate and molar yield but with cell wash-out pretreatment showing better performance than heat shock pretreatment. Maximum molar yield (0.92 mol H_2/mol hexose) and volumetric hydrogen production rate (4.23 L H_2/L-d) were obtained with organic loading rates of 36 g glucose/L-d at HRT of 10 h in EGSB reactor inoculated with cell wash-out pretreated sludge. The microbial community of the reactors samples were analyzed by 16S rRNA genes profiles and the predominant bands were excised and their DNA sequence determined. *Clostridium* and representatives of *Enterobacteriaceae* were dominant, with a strong presence of *Lactobacillus* genus. The whole result indicate that the inoculum pretreatment have a strong initial effect during early stages of fermentation, after which the operating conditions have a greater impact on reactor performance.

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2.1 Introduction

Biological hydrogen production by dark fermentation is a promising alternative to produce energy, because it has several advantages: a vast potential of organic wastes to be used as substrates, no need for media sterilization, robustness, adaptation capacity of the microbial community and suitability for continuous process, and inherent potential to be scaled-up for commercial purposes [1-3]. Theoretically, facultative anaerobic bacteria can produce two moles of hydrogen per mol of glucose, whereas strict anaerobes produce four [3-5]. Therefore, the main objective and challenge in research and development of dark fermentation is mainly focused on obtaining high yields of hydrogen in stable conditions [4,5]. This can be achieved using mixed cultures rather than pure ones, since they do not require sterile conditions and their diversity guarantee certain adaptability to operational changes [6,7].

Mixed cultures are complex microbial communities, as are the sources from which they are obtained. It is expected that those communities have a potential hydrolytic activity, and robustness to cope with environmental changes [8] because various similar and complementary metabolic pathways occurs simultaneously, even during the degradation of the same substrate. Many studies have suggested that the population dynamics in a bioreactor may be governed by chaotic shifts between functionally redundant organisms [9,10]. This allows the community to adjust to shocks and disturbances, giving to the whole system a functional diversity and environmental specificity available in the community, based on their strength or resistance (when the population maintains abundance over time), resilience (when the population returns to their abundance after a disturbance) and redundancy (when the population is replaced by another one with the same function) [10,11]. However, there are several problems associated with the use of mixed cultures: as complex microbial communities, their composition varies with time, responding to changes in process parameters and from reactor to reactor, as it has been shown by many molecular studies [12-16]. In order to obtain high performances in dark fermentation systems, the activity of different types of H_2 consumers must be inhibited, sheltering hydrogen producers. With this in mind, seed sludge for dark fermentation reactors is often subjected to some kind of previous treatments, such as heat, electric current, air or chemicals [2,6,17,18]. Since many mesophilic H_2 producing bacteria are spore-formers, heat shock is

the most used pretreatment method, in addition to its low cost and simplicity [1-3,6]. However, having a non-sterile operation, the system is constantly exposed to the input of microorganisms through the feeding which could have an adverse effect on hydrogen production. Nevertheless, methods to inhibit H_2-consuming microorganisms may have an effect on the structural and morphological properties of granular sludge, as well as their function and performance [6,11,12]. Therefore, the choice of the inoculum pretreatment method may impact the performance of the reactor by defining the functional members comprising the microbial community. For example, heat treatment can exclude archaea with some certainty since they are not able to sporulate, while cell wash-out accomplish the same function on the basis of differences in microbial duplication times between bacteria and archaea by means of short hydraulic retention times [17-19]. Both methods can eliminate methanogenic activity, but at the same time remove potential hydrogen producers.

The expanded granular sludge bed (EGSB) reactor is one of the leading examples of reactors that use self-immobilized biomass in the form of granules with high cell density and good settling characteristics [20-22]. As a result, there is a large accumulation of biomass in contact with wastewater, enabling high organic loading rates (OLR), low hydraulic retention time (HRT) and good mixing conditions, allowing higher reactor performance [3,21].

The aim of this work was to evaluate the effect of two different inoculum pretreatment strategies, heat treatment and cell wash-out, that shed differences in the structure and identity of the microbial population enriched by each method, producing differences on the hydrogen production performance of two EGSB reactors. The change over the time of the bacterial community and its relation with the operational conditions and reactors performance was elucidated with denaturing gradient gel electrophoresis (DGGE) of 16S *rRNA* genes fingerprints. The results could elucidate which method is more suitable to decrease start-up time and achieve stable performance in hydrogen production systems

2.2. Materials and methods

2.2.1 Inoculum and pretreatments

The original inoculum was methanogenic granular sludge, obtained from a wastewater

treatment plant from a confectionery manufacturer in San Luis Potosí, México. The granular sludge was disaggregated with a 500 µm mesh; volatile suspended solids (VSS) concentration was 42 mg/g sludge, with a VSS/suspedid solids (SS) ratio of 0.71.

The pretreatments applied to the seed sludge were heat shock and cell wash-out. The heat treatment consisted in boiling the disaggregated sludge during 45 minutes. Cell wash-out was accomplished in a continuous stirred-tank reactor (CSTR) with 2 L of working volume and 1 L of head space, inoculated with the disaggregated methanogenic granular sludge (10 g VSS), operated in continuous mode during 10 days at 8 h of hydraulic retention time (HRT) equivalent to the same solids retention time, 20 g glucose/L as substrate, agitation of 250 rpm, 37 °C and pH of 5.7. Methane was not detected during the CSTR operation and the hydrogenogenic biomass developed was recovered and concentrated by centrifugation at 14000 rpm by 15 minutes.

2.2.2 Reactors and substrate

Two acrylic custom-made EGSB reactors (E1 and E2), with 4.3 cm of internal diameter, 96 cm height, a total volume of 1.4 L and working volume of 1.1 L, were used. Glucose was used as substrate (10-25 g/L). The mineral medium used in the EGSB reactors and for the cell wash-out pretreatment had the following composition modified from Davila-Vazquez et al. [23], containing (mg/L): $ZnCl_2$, 75; K_2HPO_4, 125; $MgCl_2 \cdot 6H_2O$, 100; $MnCl_2 \cdot 4H_2O$, 10.77; $FeCl_3 \cdot 6H_2O$, 25.97; $CuCl_2 \cdot 2H_2O$, 3.41; and $NiCl_2 \cdot 6H_2O$, 101.25. In order to keep the operation pH at 5.5 in the EGSB reactors, a phosphate buffer of Na_2HPO_4 and $NH_4H_2PO_4$ was used. Heat shock treated sludge was used as inoculum in E1, while the cell wash-out sludge was used as inoculum in E2. Both EGSB reactors were inoculated with the same amount of biomass at an initial concentration of 10 g VSS/L.

2.2.3 Experimental set-up and monitoring

The EGSB reactors were operated during 104 and 96 days for E1 and E2 respectively. After inoculation, both reactors were filled-up with media containing glucose (20 g/L), keeping a batch mode operation during 12 hours. Afterwards, continuous mode operation started with an initial glucose concentration of 10 g/L, HRT of 10 h and OLR of 24 g COD/L-d. Reactor operation conditions are summarized in Table 2.1. After the EGSBs inoculation, a constant

effluent recirculation was maintained through the batch and continuous operation modes reaching 3 m/h of up-flow velocity. The reactors were operated at room temperature (around 30° C) and 5.5 of pH. Each condition was maintained at least for 10 HRTs.

Table 2.1. Operational conditions for EGSB reactors 1 (E1) and 2 (E2) and number of days elapsed at each operational condition.

Stage	HRT (h)	Glucose (g/L)	OLR (g COD/L-d)	Operation days	
				E1	E2
I	10	10	24	1-16	1-19
II	10	15	36	17-57	20-50
III	10	20	48	58-71	51-63
IV	10	25	60	72-83	64-75
V	8	20	60	84-91	76-83
VI	6	15	60	92-97	84-89
VII	4	10	60	98-104	90-96

2.2.4 Analytical methods and monitoring

Gas production was measured daily using a gas flow-meter based in the water displacement method (SEV, Puebla, Mexico) and calibrated periodically. Standard temperature and pressure conditions were used to report gas volumes (0 °C and 1 atm). H_2 and CO_2 were quantified daily using a gas chromatograph equipped with a thermal conductivity detector (model 6890N, Agilent Technologies, Waldbronn, Germany) as it has been described elsewhere [23,24]. Samples from both EGSBs effluents were collected every day and stored at -20 °C until their analyses. Volatile fatty acids (VFA) were analyzed in filtered effluent samples by capillary electrophoresis. The analytes were quantified by comparison with high purity standards, as previously described [24,27]. Acetone, ethanol, butanol and lactate were analyzed at specific days just to check if they were present by injecting a 1 μL sample in a gas chromatograph 6890N equipped with an auto-sampler 7863 (Agilent, Wilmington, USA) and a capillary column HP-Innowax (30m x 0.25 mm i.d. x 0.25 m film thickness;

Agilent, Wilmington, USA). Helium was used as carrier gas at a flow-rate of 1.5 mL/min. Temperatures for the injector and flame ionization detector (FID) were 220 and 250 °C, respectively. The solvents analysis was performed with a split ratio of 1:0.1 and a temperature program of 35 °C for 2 min, increased to 80 °C (10 °C/min), and was maintained at this temperature for a final time of 15 min. Residual glucose was analyzed by the colorimetric method of DuBois [22]. VSS, SS and COD were analyzed by standard methods [28]. Volumetric hydrogen production rate (VHPR) was calculated in base to the amount of gas produced multiplied by the hydrogen fraction divided by the time and the work volume of the reactor. COD equivalences for residual glucose, VFAs, hydrogen produced and VSS were calculated using the stoichiometric approach, by their half-reactions equations [29]. VSS equivalence to COD was calculated in base of the empiric formula of biomass $C_5H_7O_2N$ [29], equal to 160 g COD/mol of biomass. Hydrogen yield was calculated based on hexose consumed.

2.2.5 DNA extraction and PCR amplification

Five milliliters of sludge for biomass samples were withdrawn at each operational condition and stored at -20 °C for later DNA extraction and analysis. DNA was extracted from 0.1 g of centrifuged biomass, using a commercial kit (PowerSoil DNA isolation kit, Mo Bio Laboratories, Inc) following the manufacturer's protocol. Bacterial specific primers were used for *16s rDNA* amplification. Nested PCR technique was used for amplification, using Taq DNA polymerase (Invitrogen, USA).

The conditions and primers for bacterial nested PCR were previously reported [26], the first round primers were 27F (5'-GTTGATCCTGGCTCAG-3') and 1492R (5'-ACGGYTACCTTGTTACGACTT-3') the reaction conditions were: 94 °C for 3 min; 35 cycles of 94 °C for 60s, 45 °C for 60s, 72 °C for 1 min; and 72 °C for 10 min. The second round primers were 357F-GC (5'GC-clampCCTACGGGAGGCAGCAG-3') and 907R (5'-CCGTCAATTCMTTTGAGTTT-3'); the touchdown reaction conditions were: 96 °C for 4 min; 10 cycles of 94 °C for 30s, 61 °C for 1 min, decreasing 1 °C in each cycle to 56 °C, 72 °C for 1 min; plus 20 cycles keeping constant 56 °C of the annealing temperature; and 72 °C for 7 min. PCR products were visualized in 1% agarose gels stained with ethidium bromide to assess the size and purity of the amplicon.

2.2.6 DGGE assay

Bacterial DGGE was performed and stained as reported by Carrillo-Reyes et al [27]. Relative microbial abundances were estimated on bacterial DGGE gel using band intensities by Quantity One analysis software (Bio-Rad, Hercules, California, USA). Dendrogram was determined by means of the software PAST and using the Ward method. Shannon–Wiener diversity indices (*H*) were calculated on the basis of the intensities of the bands on the gel tracks, as judged by peak height in the densitometric curves, according to the equation: $H = -\sum(Pi\ ln(Pi))$ where, *H* is the diversity index and *Pi* is the importance probability of the bands in a lane $(Pi = n_i /N)$, where n_i is the height of an individual peak and *N* is the sum of all peak heights in the densitometric curves [30].

Selected bands were excised and eluted in 35 µL of deionized water, and then applying a "freeze and thaw" process (-20 °C for 2 h and then 60 °C for 30 min, three times) before storing it for 72 h at 4 °C, allowing DNA to migrate to the liquid. The eluted DNA was reamplified by PCR using primers for bacteria 357F without GC-clamp and 907R. Successfully reamplified PCR products were sent to sequencing to LANBAMA (IPICYT, San Luis Potosí, México). Sequences were analyzed with DNA BioEdit software v7.1.3 (Carlsbad, California, USA), checked for potential chimera artifacts by the Chimera Slayer software version 20101212+dsfg1 (Broad Institute, USA), and then compared to the nonredundant nucleotide database at GenBank using BLAST (http://www.ncbi.nlm.nih.gov/blast/) and Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp).

2.3. Results and Discussions

In the present work two different inoculum pretreatments were applied to determine differences in performance caused by the different operational conditions applied to the two EGSB reactors. Changes in substrate concentration and hydraulic retention time were correlated to the bacterial community structures and their effect on reactor performance. No methane was detected during reactors operation, highlighting the pretreatments efficiency to control the methanogenic activity.

2.3.1 Reactors performance

2.3.1.1 Hydrogen production

The EGSB reactors were operated during more than 96 days. Figure 2.1 shows a box plot ranking data obtained from volumetric hydrogen production rate (VHPR) in E1 and E2 reactors, expressed as quartiles. Remarkable differences between data in same conditions displays low stability in H_2 production rates mainly due to operational conditions and metabolic changes, but trends toward central values can be observed. Hydrogen was produced during the whole experiments and the majority of extreme values were single measurements. The central box lumps together 50% of data, and length denotes its total distribution. The black square represents the average of the data for each operating condition. For E1 (heat shock inoculum), the highest VHPR was obtained in stage IV (60 g glucose/L-d OLR, 10 h HRT, Table 2.1), whereas for reactor E2 (cell wash-out inoculum) the maximum VHPR was obtained during stage II (36 g glucose/L-d OLR, 10 h HRT). The most stable condition for E1 was found at stage I with an OLR and HRT of 24 g glucose/L-d and 10 h, respectively, whereas for E2 was at stage VII at 60 g glucose/L-d and 4 h, respectively. There is a trend towards greater stability when lowering the HRT and higher VHPR even when the same OLR was evaluated in the last four conditions in each reactor.

In both reactors, the average VHPR increased when the influent glucose concentration was increased maintaining an HRT of 10 h (stages I to IV); whereas there was a slight decrease in hydrogen content in the gas produced (40% to 30%) when HRT diminished from 10 h to 4 h (stages IV to VII, data not shown). When the HRT and substrate concentration decreased keeping constant OLR (stages IV to VII), the VHPR decreased in both reactors except for the last operating condition where it increased, doubling the VHPR of the preceding operational condition (Figure 2.1). This increase in VHPR may suggests that a change in metabolic pathways occurred, allowing more hydrogen production.

2.3.1.2 Fermentation products and COD balances

Figure 2.2 shows equivalent chemical oxygen demand (COD) balances for each metabolite identified, hydrogen, VSS in effluent and residual glucose for both reactors. Although the results showed that the main metabolite produced was propionate in both reactors, these

values should be taken with caution because they could be overestimated by the production of lactic acid as it will be discussed later [3,16,20]. The metabolic routes associated to hydrogen production involves acetate and butyrate, that were produced mainly in the same periods where higher VHPR were obtained, stages IV and VII for E1 and E2, respectively.

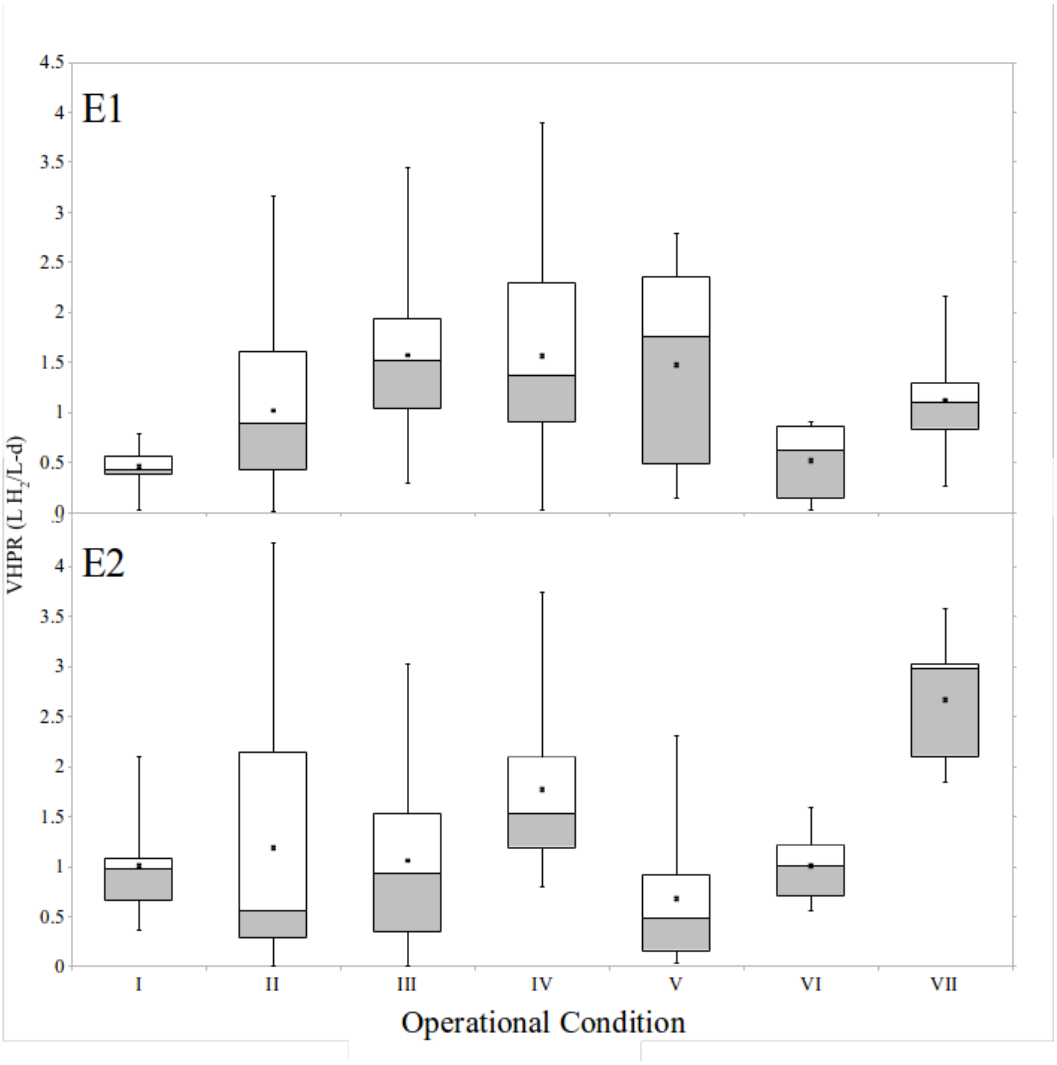

Figure 2.1. Box plot showing the raw data for the volumetric hydrogen production rate (VHPR) distribution lumped together in quartiles for every condition evaluated in EGSB (E1 and E2) reactors. The box size brings 50% of data separated by the median, while whiskers show the maximum and minimum values recorded, either depicting 25% of the data. The black square shows the average value for each operative condition.

Glucose removal (data not shown) in E1 remained close to 100% at the beginning of the operation, but gradually decreased to 90% during the last operation condition (stage VII). A

similar trend was observed in E2, but the lowest glucose removal was 77%, which coincided with the highest glucose concentration of 25 g/L at HRT of 10 h (OLR of 60 g glucose/L-d, stage IV). Afterwards, as both the glucose concentration and HRT decreased, there was an increase in glucose consumption, reaching 100% during the last operation condition (stage VII). The lower glucose removal also coincided with increased production of acetate but also propionate as shown in Figure 2.2. The drop in glucose consumption matches with a period in which there were abrupt changes in metabolite production at the end of stage III and the beginning of stage IV, but later this stage was the most stable in terms of metabolites production. Zhang et al [21] found that other parameters, such as dominant microbial population and substrate influence the distribution in the metabolite composition, and thus on reactor performance.

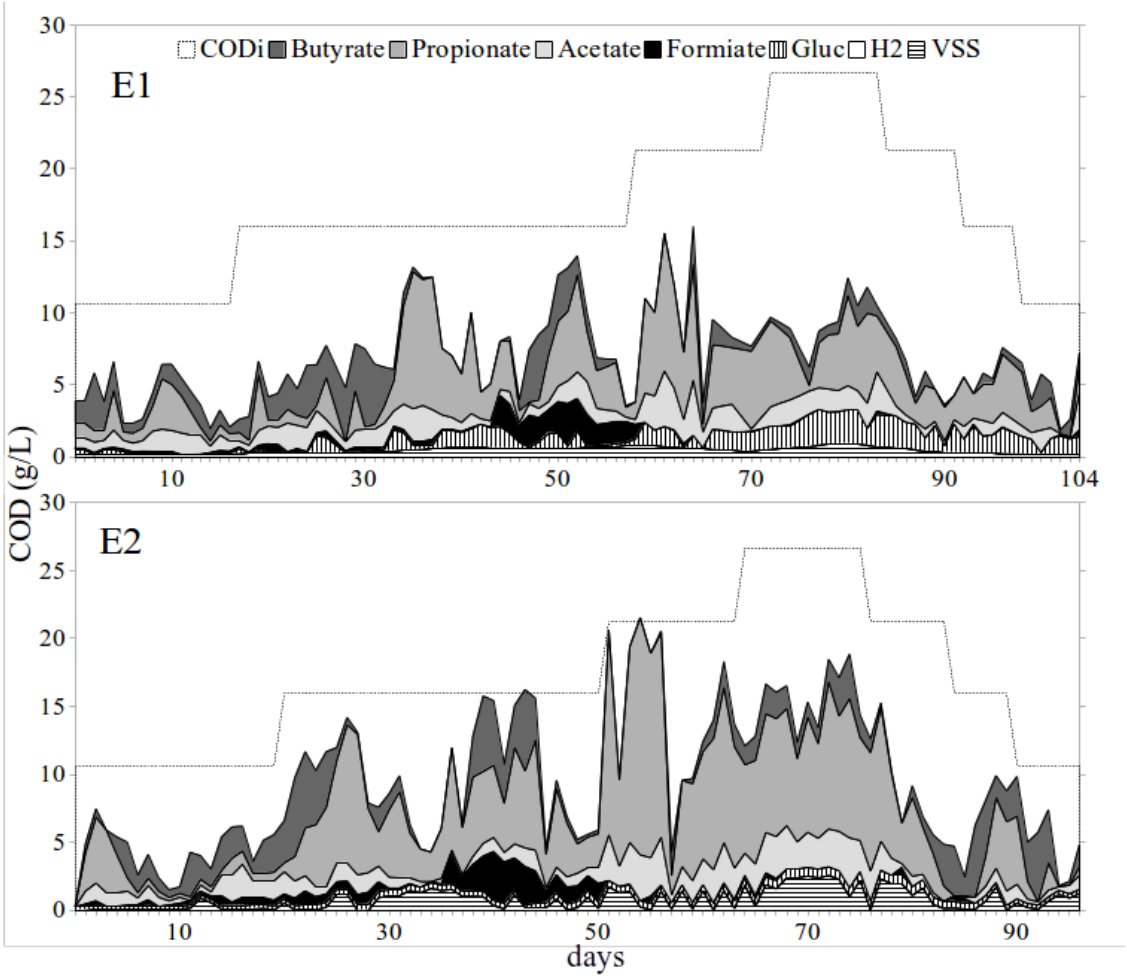

Figure 2.2. Cumulative equivalent COD of the main metabolites produced, volatile suspended solids, as well as the residual glucose, compared to the total COD influent

concentration in both EGSB (E1 and E2) reactors. CODi: initial COD concentration, Gluc: residual glucose, H2: hydrogen, VSS: volatile suspended solids.

Observing Figures 2.1 and 2.2, it can be noted that VHPR increase in both reactors was related with the rise in butyrate concentration as it is observed at stage IV for E1 (OLR 60 g glucose/L-d, HRT 10 h) and at stage VII for E2 (OLR 60 g glucose/L-d, HRT 4 h). An increased production of butyrate is visible at the end of the stage II in both reactors; this may be the reason of the maximum VHPR reached throughout the E2 operation. The different microbial communities and thus metabolic pathways may be related to this behavior. The identified metabolites and hydrogen produced plus residual glucose and VSS obtained from effluent do not balance the amount of COD supplied. This can be explained by the retained biomass in the reactor and other metabolites that were not quantified throughout the experiment. Samples of effluent at specific and independent days during stages II, III, IV and V of the two reactors (6 days for E1 and 5 days for E2) were analyzed by gas chromatography for the presence of lactate and solvents as shown in Table 2.2. Solvents such as acetone, ethanol and butanol are produced in acidic conditions, and matched with those specific days when drops in pH were recorded.

Table 2.2. Solvents, lactate and propionate quantified at specific days. Lactate-propionate ratio shows metabolic shifts in those days.

Reactor	Operation day	Ethanol (mg/L)	Acetone (mg/L)	Butanol (mg/L)	Lactate (mg/L)	Propionate (mg/L)
E1	45	65.9	145.9	-	299.1	-
	49	95.9	-	-	660.7	-
	63	-	36.8	32.3	437.5	1.5
	71	1455.6	-	-	3652.2	-
	76	1134.9	-	-	7024.5	6.3
	86	956.5	-	-	4658.5	17.9
E2	35	132.5	-	-	2445.7	31.2
	54	33.9	33.8	31.5	438.6	2.2
	65	1094.5	-	71.6	5646.8	-
	72	835.3	-	57.4	9431.8	96.5
	78	704.3	-	-	4240.3	7.3

Lactate was found in all the 11 days chosen for analysis at high concentrations whereas propionate was present at low concentrations; due to the methodology used in quantifying the VFA (capillary electrophoresis), where propionate and lactate peaks were overlapped even in low concentrations, it is quite possible that the quantified propionate was actually

lactate. Regarding the hydrogen production in the case of E1, the highest production rate (3.89 L H_2/L-d) was obtained at stage IV while the highest yield (0.54 mol H_2/mol hexose) was obtained at stage II. For E2, highest VHPR (4.16 L H_2/L-d) was obtained at stage II which matches the conditions of the highest molar yield (0.73 mol H_2/mol hexose) obtained during the experiment. Average values show that E2 had better yields and rates except in stages III, IV and V. Only under these three conditions E1 showed better performance.

It is generally accepted that hydrogen yield is directly related to the current microbial community and observed changes in the reactor performance are related to variations in the microbial metabolic function and their environment set by the operating conditions, although reactor configuration seems to have less influence [10]. However, small variations in molar quantities have a large impact on the volume and fraction of the total gas produced. Table 2.3 shows the results obtained in this work compared with those reported in literature from similar reactor configurations. It is important to note that the maximum single point value for VHPR obtained in our experiments is the highest (4.23 L H_2/L-d), also it corresponds to the highest concentration of substrate as well as the highest HRT of the comparison.

Table 2.3. Comparison of maximum hydrogen production under different operational conditions using EGSB reactors. GAC: granular activated carbon.

Working volume	Substrate	H_2 maximum production rate	Growth mode	Inoculum	HRT, OLR, pH, T°
1.3L [25]	Glucose and L-Arabinose (2g/ L)	2.7 L/L-d	Granular	Sludge from a CSTR and granules from a full scale UASB reactor	6h , 8 g/L-d, 7.0, 70°C
1.0 L [35]	Glucose (10g/L)	1.36 L/gVSS-d	Granular and adhesion	Municipal sewage sludge and strain B49	6h , 40 $g_{glucose}$/L-d,-, 35°C
1.0 L [35]	Glucose (10g/L)	1.1 L/g VSS-d	Granular	Municipal sewage sludge	6 h, 40 $g_{glucose}$/L-d,-, 35°C
3.4L [16] with GAC	Starch (4g/L)	1.64 L/L-d	Granular and adhesion	Mixed culture	4 h, 1. 1 g_{hexose}/L-d, 4.4, 30°C
1.1 L (This work)	Glucose (15g/L)	4.23 L/L-d	Granular	Sludge from a CSTR (wash-out cell treatment)	10 h, 36 $g_{glucose}$/L-d, 5.5, 30°C

Comparing E1 and E2 reactors, Figure 2.1 shows that E1 had a slightly lower range of VHPR values but more stability than E2 (operational conditions I, II, III, V, VI and VII),

even though reactor E2 had higher hydrogen production rate values. Heat treatment is highly selective on spore-forming bacteria rather than in specific functional groups [1,6,10], therefore less bacterial diversity is expected. This has been observed for most of the papers reported in the literature where, after heat pretreatment, the inoculum obtained is enriched in Clostridia species. They seem to be responsible for most of the hydrogen produced and therefore its presence and dominance is related to good performance. In cell wash-out pretreatment, a higher diversity is expected, although increased competition for resources in a more diverse community could cause fluctuations in the hydrogen production [10], albeit we observed those oscillations in VHPR in both reactors. Moreover, it is known that the use of non-sterile substrates as feedstock can supply incoming microorganisms that can increase the dynamic of the bacterial community [31].

2.3.2 Microbial community analysis performed by DGGE and sequencing of DNA from predominant bands

Since both reactors were operated under the same conditions, the differences between them must be related to the inoculum pretreatments, which may select different community structures over time, with metabolites profiles reflecting it (Figure 2.2). Both reactors showed DGGE profiles that changed constantly with time according to operational conditions. However there were bands that remained present all the time, although their relative abundances decreased. To understand the effect of the inoculum treatment, the OLR and the HRT on the microbial communities, Cluster analysis was performed with the DGGE profiles of the samples taken from both reactors during conditions I to IV (changes in the OLR) and conditions IV to VII (changes in the HRT), as shown in Figures 2.3A and 2.3B respectively. The analysis showed that the inoculum treatment has an important impact in the communities as most of the samples from the same reactor grouped together during operations at the same OLR (Figure 2.3A). When the reactors were operated at different HRT the effect of the inoculum treatment was not so clear as the distance between the clusters were not so high (Figure 2.3B). But the communities from the different inocula did not present high differences in tems of the bacterial diversity established from the DGGE profiles, measured as Shannon-Wiener diversity index (*H*). The inoculum with heat shock treatment (E1-0) had an H = 2.582, while the inoculum with cell wash-out

pretreatment (E2-0) had an H = 2.378. In order to determine if the differences in the DGGE profiles indicate a difference in the genera composition of the communities, the predominant DGGE bands were excised; their DNA sequences were determined and compared with sequences from the database. As expected, all the microorganisms detected were known as fermenters but with probably different roles in the hydrogen producing reactors (Table 2.4).

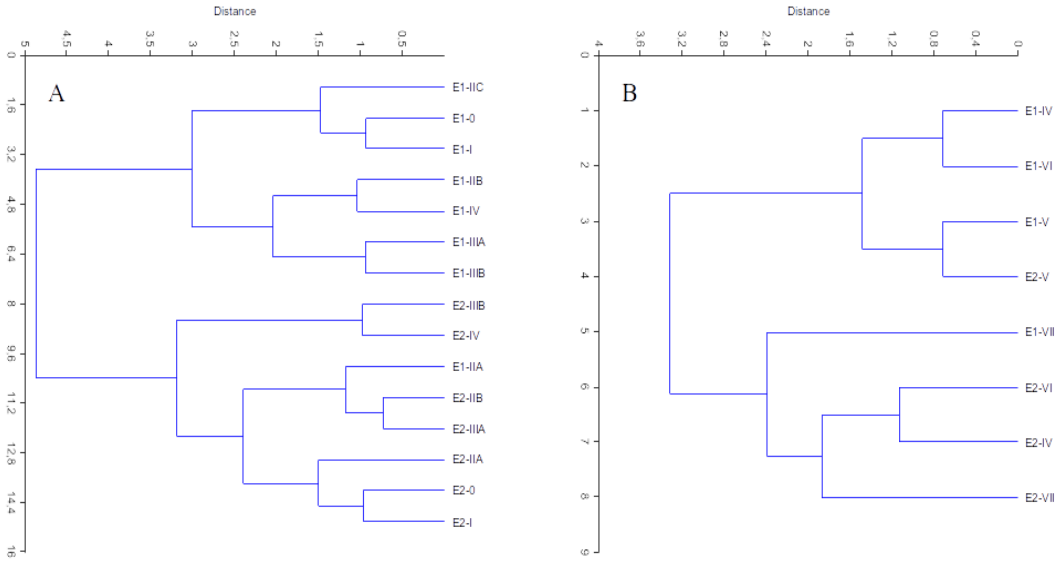

Figure 2.3. Cluster analysis performed with the DGGE profiles of the samples taken from the two EGSB (E1 and E2) reactors during periods I to IV (changes in the OLR, Figure 2.3A) and IV to VII (changes in the HRT, Figure 2.3B). The analysis was performed using the Ward method with the software PAST. E1 corresponding to heat shock inoculum treatment, E2 corresponding to washout cell inoculum treatment. Roman numerals shows the respective stage of operation (see Table 2.1) and capital letters (A, B and C) indicate that more than one sample was taken at the respective stage but at different days. Zero day samples are related to inoculum.

It is known that the H_2 gas production in a fermentation system is mainly due to the activity of the Firmicutes phylum, with Clostridia as the largest producer, while members of Enterobacteriaceae can produce gas in smaller amounts [32]. *Enterobacteriaceae* family can be either helpers or spoilers in fermentation systems. They can remove oxygen quickly and then create a strict anaerobic environment suitable for biohydrogen production and even be producers but, at the same time, they can be considered as substrate competitors that reduce the amount of substrate available, although they produce hydrogen themselves

[1,3,6,26]. Most of the microbial community in the reactors consisted of members affiliated to the genus *Lactobacillus*, that co-existed with hydrogen-producing *Clostridium* and may have an adverse effect on hydrogen production [33], hindering hydrogen production or consumption. In the bands recovered from both reactors there were matches to *Sporolactobacillus* genus. Fang et al. [34] found that significant amount of extracellular polymer substances produced by *Sporolactobacillus* could be helpful on granular formation, but on the other hand it could excrete bacteriocins, which are protein based toxins produced by bacteria to inhibit similar or closely related strains [35], that could cause an adverse effect on hydrogen-producing bacteria by altering the population dynamics. The presence of representatives of the Bacillales order are related with low hydrogen yields, meanly due to the production of lactate [36,37]. However it has been observed that at the beginning of the fermentation the activity of Bacillales significantly reduce oxygen concentration and create an anaerobic environment suitable for gas producers [38].

Table 2.4. Affiliation of band sequence (retrieved from the succesfully secuenced bands of the DGGE) determined using BLAST algorithm.

Band no.[1]	Phylum	Band appareance[2]	bp lenght	Closest match	Query (%)	Indentity (%)	Accession number
1	Firmicutes	E1 (0, IIA, III, VI)	572	*Lactobacillus rhamnosus*	94	92	AB932535
2	Proteobacteria	E1 (0, IIA, IIB, IIIB-VII)	619	*Proteus mirabilis*	89	87	HQ398231
3	Proteobacteria	E 1 (0, IIA, IIB, IIIB, IV)	560	*Enterobacter sp.*	97	92	FJ976546
4	Proteobacteria	E1 (0-IIB)	557	*Klebsiella oxytoca*	96	86	KC593550
5	Firmicutes	E1 (0,I)	567	*Lactobacillus rhamnosus*	96	87	JX556102
6	Proteobacteria	E1 (0-IIA)	564	*Klebsiella oxytoca*	95	94	JF901758
7	Firmicutes	E1 - E2	557	*Lactobacillus casei*	99	99	CP006690
8	Firmicutes	E1 (0-IIB, IV) E2 (I – IV),	572	*Clostridium beijerinckii*	90	98	AB600545
9	Firmicutes	E1 - E2	554	*C. butyricum*	93	95	EU869239

10	Firmicutes	E1 (0, IIA-IIIB)	565	*Lactobacillus casei*	96	90	HM598683
11	Proteobacteria	E1 (IIIB-VI)	611	*Enterococcus faecium*	88	82	CP006620
12	Firmicutes	E1 (IIIA-VI)	644	*Bacillus subtilis*	84	78	DQ055130
13	Proteobacteria	E1 - E2	686	*Enterobacter sp.*	79	81	FJ868805
14	Firmicutes	E1 (0, I, IIB, IV-VII)	595	*Lactobacillus rhamnosus*	93	94	KJ152776
15	Firmicutes	E1 - E2	560	*Sporolactobacillus laevolacticus*	86	97	D16269
16	Firmicutes	E1 - E2	595	*Sporolactobacillus sp.*	92	98	AB681813
17	Firmicutes	E1 - E2	635	*Lactobacillus rhamnosus*	86	99	AB932521
18	Firmicutes	E1 (IV-VII)	577	*Lactobacillus hilgardii*	95	86	KJ128216
19	Firmicutes	E1 (IIA, VII)	573	*Lactobacillus rhamnosus*	95	97	AF243146
20	Firmicutes	E1 (VII)	623	*Lactobacillus rhamnosus*	96	91	AB932535
21	Proteobacteria	E2 (0-IIA)	596	*Enterobacter cancerogenus*	74	94	KF687004
22	Proteobacteria	E2	657	*Klebsiella oxyotoca*	82	94	KC593550
23	Proteobacteria	E2 (IIA-IV)	567	*Erwinia psidii*	78	86	KC201309
24	Firmicutes	E2	594	*Lactobacillus casei*	91	100	JN851813
25	Firmicutes	E2	554	*Lactobacillus paracasei*	98	99	KJ561346
26	Firmicutes	E2 (IIIA, V- VII)	616	*Lactobacillus rhamnosus*	88	87	HQ111082
27	Firmicutes	E2	618	*Lactobacillus rhamnosus*	87	96	KJ459040
28	Firmicutes	E2 (IV-VII)	642	*Lactobacillus paracasei*	85	99	KJ459038
29	Firmicutes	E2 (VI-VII)	614	*Lactobacillus hilgardii*	88	99	KJ128234

[1]Indicates the position of band in gel.

[2]Indicates presence of the band in sample taken either from reactors E1 or E2, Roman numerals shows the respective stage of operation (see Table 2.1) and capital letters (A, B and C) indicate that more than one sample was taken at the respective stage but at different days.

The banding patterns in DGGE and their intensities were used to estimate the relative

abundance of the genera determined by sequencing of DNA bands (Figure 2.4). At this level, eight taxa were identified in E1 and nine in E2, plus a series of bands not successfully amplified that were joined together and represented as "others". Microorganisms affiliated to Clostridia were present most of the time in E1, and the highest abundance detected (stage II-C, 36 g glucose/L-d OLR, 10h HRT, day 52) coincides with an increase in acetate production (Figure 2.2), but not with hydrogen. Stage IV shows that the members of Clostridia had a presence of only 15% of the total intensity of the bands in that stage and yet this period was the one with the highest VHPR (Figure 2.1). Regarding E2, it showed lower presence of microorganism affiliated to Clostridia, but due to the limits of resolution of the method, the absence of band in DGGE does not imply their absence in the system. In this case, the increases and decreases in hydrogen production go hand in hand with changes in the presence of Clostridia. In both reactors the presence of members of *Enterobacteriaceae* was constant and sometimes outnumbered Clostridia. Members of that family are known to produce hydrogen although with relatively minor yields than *Clostridium*. The permanent presence of *Lactobacillus* in both systems may explain the low hydrogen production, although the presence of lactic acid was not confirmed during the whole operation. The role of *Sporolactobacillus* in the performance of the reactors is also unclear, although their presence is reasonable since it is known to be capable to produce endospores and then resist to heat treatment.

Based on observations on the composition of fermentative products, long-term cultures under non-sterile conditions might cause a population shift from hydrogen-producing bacteria to hydrogen-consuming or non-hydrogen-producing bacteria, such as homoacetogens or propionate producers [39]. Samples from reactor E1 displayed more drastic changes in DGGE profile than samples from reactor E2 and lower performance. Pretreatment with heat generally produces good and sustained yields, but gives a less diverse microbial community and this is generally related with system susceptibility to abrupt changes [6,7,11,12]. However, E2 had a slightly more diverse community at the start than at the end of the operation (H_0 = 2.378 and H_{VII} = 2.315, respectively), and showing better performance than E1 (H_0 = 2.582 and H_{VII} = 2.552, respectively). It has been suggested that pretreatment by cell wash-out involved more diversity with focus on function of the microbial community [6,7,12,32], so one can expect more stability than high yields. In fact,

the whole operation of E2 can be seen as a continuation of the cell wash-out pretreatment, so it is tempting to suppose that in a longer time than the used in the experiments, the different microbial communities present in the inoculum selected with different pretreatment could converge into one functionally similar, although with different representatives in their community structure and hence differences in their performance. That would leave the operating conditions as the main responsible for the hydrogen production.

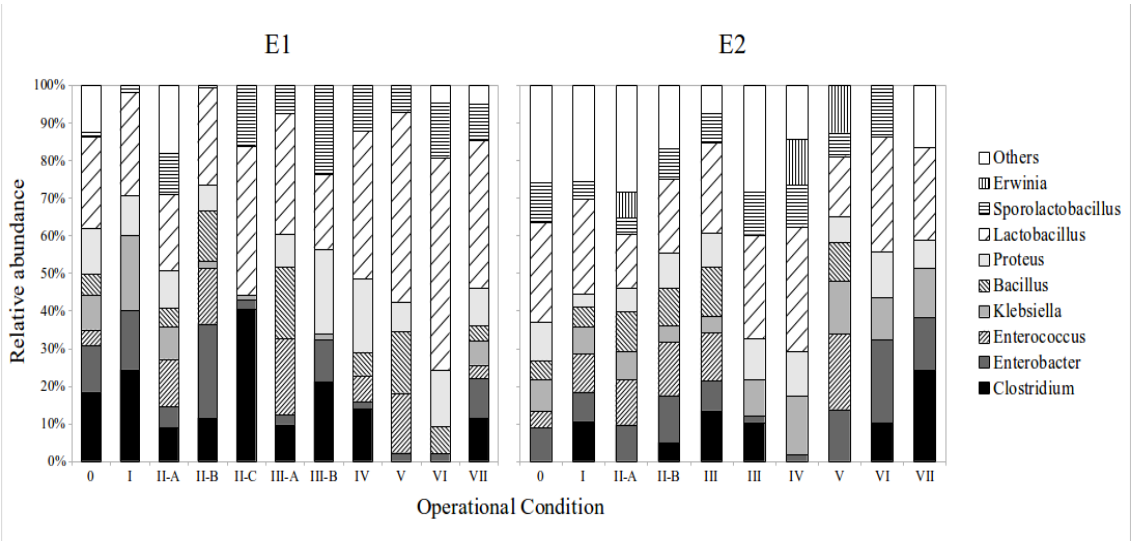

Figure 2.4. Relative abundances obtained from the DGGE bands profiles grouped at genus level in EGSB (E1 and E2) reactors. Roman numerals shows the respective stage of operation (see Table 2.1) and capital letters indicate that more than one sample was taken at the respective stage but at different days.

2.4. Conclusions

EGSB reactors showed differences in their performances and the microbial community selected, with maximum production rates above the ones reported in the literature. Both inoculum pretreatments were effective in preventing methanogenic activity and E2 had a better performance than E1 in terms of VHPR, which implies that the cell washout pretreatment might be more appropriate. Although most of the work in dark fermentation has shown a relationship between increasing hydrogen production and the dominant presence of *Clostridium* genus in the bacterial community, in this case *Enterobacteriaceae* could play a significant role in the production of hydrogen. Besides this, the presence of

Lactobacillus may exert an influence in the physiology of the hydrogen producer community of the reactor. Even if there are differences in the communities of both reactors, it seems that there is a tendency towards a population structure and their diversity that remains constant, regardless of the members. Inoculum treatment seems to have more effect at the beginning of fermentation, after which the operating conditions have a greater impact on performance.

2.5 References

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Chapter 3

Cell wash-out inoculum pretreatment enhances biohydrogen production and performance of anaerobic fluidized-bed reactors

Summary

The effect of two different inoculum pretreatments, thermal and cell wash-out (A1 and A2, respectively) on the performance of anaerobic fluidized bed reactors for hydrogen production was determined. The reactors were operated for 112 days under the same operational conditions using glucose as substrate at increasing organic loading rates and decreasing hydraulic retention times. Both treatments were effective avoiding methanogenesis. Reactor A2 showed better performance and stability than reactor A1 in each one of the different operational conditions. Cell wash-out treatment produced higher hydrogen volumetric production rates and yields than thermal treatment (7 L H_2/L-d, 3.5 mol H_2/mol hexose, respectively). DGGE analysis revealed that the microbial communities developed were affected by the inoculum treatment. Organisms from the genera *Clostridium* and *Lactobacillus* predominated in both reactors, with their relative abundances linked to hydrogen production. Resilience was observed in both reactors after a period of starvation.

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3.1. Introduction

Bioenergy production from organic waste is becoming an essential component in the overall development of sustainable energy sources. Dark fermentation is a promising approach for both the production of energy as biohydrogen and the treatment of agro-industrial waste. Most of research has been focused on the configuration of the reactor, affordable substrates and analysis of microbial populations [1].

Anaerobic fluidized bed reactors (AFBRs) have been proved to be efficient and successful in their scaling and several works report its use for biohydrogen production [2-4]. In AFBRs, biomass colonizes a support forming a biofilm, due to the natural development of the biofilm a portion of it remains as planktonic cells. Contact between biomass and substrate is enhanced by bed fluidization, improving mass transfer and treatment capacities [4], which in turn gives the possibility to apply high organic loading rates (OLR), low hydraulic retention times (HRT) and good mixing conditions, allowing high removal efficiencies or production rates [1,5]. Moreover, the up-flow velocities (V_{up}) commonly used in AFBRs drag out the H_2 produced, avoiding the inhibition by its accumulation and partial pressure [1]. The formation of the biofilm provides a way for the retention of bacteria, which otherwise would not remain within the reactor under certain HRT and environments [6]. In addition the biofilm allows the formation of complex communities, potentially more robust to changes in environmental conditions since it involves the activity of various metabolic pathways occurring in parallel for the same substrate degradation [7]. Those complex communities could be composed of bacteria which have deleterious effects on any desired process, as redirect available electrons to different pathways, unrelated to biohydrogen production. AFBRs had been successfully applied in hydrogen production by dark fermentation with little or no methane production [8-11]. Hydrogen producing reactors are frequently seeded with biomass coming from methanogenic reactors since fermentative microorganisms with the capacity to produce hydrogen are generally present. In order to eliminate the hydrogen consuming methanogens different pretreatments had been tested, such as thermal, chemical or electrical treatments [12,13]. All these methods have been proved to be effective on the inhibition of methanogenic activity, but may also have an effect on the microbial physiology and the reactor performance [5,13].

It has been proposed that the prevalent fermentation pathways depend on the type of seed sludge, because their bacterial community composition is defined by their origin [14]. Comparative works relate discrepancies in hydrogen production with the use of different HRTs and pH [13], which may in fact help to select the bacterial community structure under given conditions [5]. Although other configurations and inocula sources have been tested [15], hydrogen production, performance and microbial diversity in AFBRs operated under similar conditions but seeded with pretreated biomass through two different methods have not been systematically compared. The goal of this work was to evaluate the effect of different operational conditions and inoculum treatments on the performance of hydrogen producing AFBRs. Thermal and cell wash-out pretreatments were applied to methanogenic granular sludge and used to seed two reactors which were operated under the same conditions. Four different OLRs and six different HRTs were tested for each reactor. The performance of both reactors was evaluated and the microbial community dynamics was studied by molecular tools.

The novelty of this work lies in the scrutiny of differences between inocula and population dynamics between the planktonic and biofilm biomass, as well as the effect of incoming biomass through feeding. All this related to performance parameters between reactors.

3.2. Materials and methods

3.2.1 Inoculum and pretreatments

Methanogenic granular sludge was used as inoculum and was obtained from a wastewater treatment plant from a confectionery manufacturer located in San Luis Potosí, México. The granular sludge was disaggregated with a 500 µm mesh; volatile suspended solids (VSS) concentration was 42 mg/g sludge, with a VSS/total suspended solids (TSS) ratio of 0.7. Two pretreatments were applied to the seed sludge: cell wash-out and heat shock. Cell wash-out was accomplished in continuous stirred-tank reactor (CSTR) with 2 L of working volume and 1 L of head space, and inoculated with disaggregated methanogenic granular sludge (10 g VSS). To achieve cell wash-out treatment, the reactor was operated in continuous mode during 10 days at HRT of 8 h; as the reactor was a CSTR the HRT was equivalent to the solids retention time. 20 g/L of cheese whey powder (Darigold Inc., WA,

USA) was used as a complex substrate, to ensure that the pretreatment was due to retention time and not to nutrient limitation. The reactor was stirred at 250 rpm, temperature was maintained at 37 °C and the pH at 5.7. The analysis of the biogas showed that methane was not detected during the operation. At the end of the operation, the biomass developed inside the reactor was recovered and concentrated by centrifugation at 14000 rpm by 15 minutes. The heat shock treatment consisted in boiling the disaggregated methanogenic granular sludge during 45 minutes.

3.2.2 AFBR and substrate

Two acrylic custom-made AFBRs with 4.3 cm of internal diameter, 96 cm height, a total volume of 1.4 L and working volume of 1.1 L, were used. Glucose was used as substrate (10-25 g/L). The mineral medium used in the AFB reactors and for the cell wash-out pretreatment it has been previously described [1]. In order to keep the operation pH at 5.5 in the reactors, a phosphate buffer composed by Na_2HPO_4, 18.81 g/L and $NH_4H_2PO_4$, 99.85 g/L was used. The reactor inoculated with heat-treated sludge was named A1 while the reactor inoculated with cell wash-out treated sludge was named A2. Both AFB reactors were inoculated with the 10 g VSS/L of pretreated biomass.

3.2.3 Experimental set-up and monitoring

Granular activated carbon (GAC) was used as support, with a particle diameter ranging from 500 to 850 µm. Roughly 100 g of GAC was added to each reactor, reaching 12 cm in high, which occupied 174.3 mL of volume in reactor. After inoculation, both reactors were filled-up with media containing glucose (20 g/L), keeping a batch mode operation during 12 hours with recirculation of the liquid to reach 13 m/h of up-flow velocity. Then, the reactor started to be operated under continuous mode with an initial glucose concentration of 10 g/L, HRT of 10 h and OLR of 24 g of chemical oxygen demand (COD)/L-d. Reactor operation conditions are summarized in Table 3.1. After the AFBRs inoculation, a constant effluent recirculation was maintained reaching 13 m/h of up-flow velocity. The reactors were operated at room temperature (30 ± 2°C) and pH 5.5. Each condition assayed was maintained for at least 10 HRTs. In order to observe the resilience of the system, an interval period of four days of inactivity was set after day 96 in the seventh stage, during which

there was not recirculation or fed with fresh medium. Following this, the two previous operational conditions were repeated to compare the performance, after 24 h of batch operation. The reactors were operated during a total period of 112 days.

3.2.4 Analytical methods and monitoring

Gas production was measured daily using a gas-flow meter based in the water displacement method (SEV, Puebla, Mexico) that was calibrated periodically. Standard temperature and pressure conditions were used to report gas volumes (0 °C and 1 atm). H_2, CO_2 and CH_4 were quantified daily by gas chromatography as it has been described elsewhere [16]. Samples from both AFBRs effluents were collected every day and stored at -20 °C until the analysis was performed. Volatile fatty acids (VFA), acetone, ethanol, butanol and lactate concentrations were determined as previously described [17, 18]. Residual glucose was analyzed by the colorimetric method of DuBois [19]. VSS, TSS and COD were analyzed by standard methods [20]. Volumetric hydrogen production rate (VHPR) was calculated in base to the amount of gas produced multiplied by the hydrogen fraction divided by the time and the work volume of the reactor. COD equivalences for residual glucose, VFAs, hydrogen produced and VSS (based on empirical formula of biomass $C_5H_7O_2N$) were calculated using the stoichiometric approach, by their half-reaction equations [21]. Hydrogen yield (HY) was calculated as the moles of hydrogen produced by mole of glucose consumed.

3.2.5 DNA extraction and 16S rRNA gene amplification

At the end of each operational condition, samples (5 mL) were taken from the reactors including planktonic cells and GAC with the attached biofilm. The biofilm samples were separated from the GAC by washing it with PBS buffer (10 mM Na_2HPO_4 and NaH_2PO_4, plus NaCl 140 mM, pH 7.5), detaching the cells from the support by sonication for 5 minutes, then separating the cells from the granulated carbon by centrifugation as described elsewhere [22]. Another samples were also taken from the feeding pipe (sample EXT). As both reactors were fed with medium from the same container, the entrance of external

microorganisms is the same. EXT sample was prepared from three homogenized samples obtained from the feed line, in a section of about 1 m prior to the branching point to feed pumps of each reactor. These samples were collected at 30, 60 and 100 days of operation. However, due to the constant cleaning of the tubing to prevent clogging, biofilm formation previous to sampling did not exceed three days. All samples were stored at -20 °C for later DNA extraction and analysis. DNA was extracted from 0.1 g of centrifuged biomass, using a commercial kit (PowerSoil DNA isolation kit, Mo Bio Laboratories, Inc) following the manufacturer's protocol. Bacterial specific primers were used for 16S rRNA amplification. Nested PCR technique was used for amplification, using Taq DNA polymerase (Invitrogen, USA). PCR conditions and primers used for each round had been reported previously [18]. PCR products were visualized in 1% agarose gels stained with ethidium bromide to assess the size and purity of the amplicon.

3.2.6 DGGE assay

Bacterial DGGE was performed and stained as reported by Carrillo-Reyes [22]. Relative microbial abundances were estimated on bacterial DGGE gel using band intensities by Quantity One analysis software (Bio-Rad, Hercules, California, USA). A matrix was constructed with the intensity of each band and their position on the gel. Dendrogram was determined by means of Vegan and Cluster packages and using R language. Shannon–Wiener diversity indices (*H*) were calculated on the basis of the intensities of the bands on the gel profiles, as judged by peak height in the densitometric curves, according to the equation: $H = -\sum(Pi\ ln(Pi))$ where, *H* is the diversity index and Pi is the importance probability of the bands in a lane $(Pi = ni/N)$, where ni is the height of an individual peak and N is the sum of all peak heights in the densitometric curves [23]. Species richness (*S*) is notated as number of different bands for each corresponding lane. Non-metric multidimensional scaling (NMDS) analysis was performed using the PAST software using the Bray-Curtis similarity measurement [24].

Selected bands were excised from the DGGE and the DNA was recovered as it has been described [18]. The 16S rRNA gene fragment from the eluted DNA was reamplified by PCR using primers for bacteria 357F without GC-clamp and 907R [22]. PCR products that

were successfully obtained were sent to sequencing to LANBAMA (IPICYT, San Luis Potosí, México). Sequences were analyzed with DNA BioEdit software v7.1.3 (Carlsbad, California, USA), checked for potential chimera artifacts by the Chimera Slayer software version 20101212+dsfg1 (Broad Institute, USA), and then compared to the non redundant nucleotide database at GenBank using BLAST (http://www.ncbi.nlm.nih.gov/blast/) and Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp). Sequences were deposited in the GenBank database (NCBI) under the accessing numbers KU755531 to KU755549.

3.3. Results and discussion

3.3.1 Reactors performance

The anaerobic fluidized bed reactors were operated during eleven stages as shown in Table 3.1. In both reactors each stage lasted the same number of days. The reactor inoculated with heat-treated sludge was named as A1, while the reactor inoculated with cell wash-out treated sludge was named as A2.

During all the operating time methane production was not detected in the biogas produced in both reactors, demonstrating the effectiveness of both pretreatments to discard methanogens from the original granular sludge. For better appreciation of the distribution and trend in both VHPR and HY, the values obtained for both reactors were grouped in quartiles to construct box-plots, as shown in Figure 3.1. Each box comprises 50% of the data from the second quartile (25 to 50% of upstream data distribution), to the third quartile (50 to 75% of the data), and the line dividing each box represents the median. A clear trend was observed for both reactors, the average VHPR and HY increased when OLR reached 60 g COD/L-d (stage V). When the reactors were operated at this OLR using lower HRT (stages VI to VII) the average production of hydrogen slightly decline, but when the HRT was set to values lower than 2 h the values drastically dropped (stages VIII to IX). Nevertheless, the performance of reactor A2 was in general better than the obtained for reactor A1, giving values 40% greater than for reactor A1 regarding to the average values, except for stage II (Table 3.1).

Table 3.1. Operational conditions for the anaerobic fluidized bed reactors inoculated with heat shock and cell wash-out pretreated biomass (A1 and A2, respectively), number of days of each period in such conditions and average and maximum values of volumetric hydrogen production rate (VHPR) and hydrogen molar yield (HY) for each stage condition. The stages that were repeated after the four days of reactors inactivity on day 96 are marked with apostrophe (').

Stage	HRT (h)	OLR (g COD/L-d)	Operation days	VHPR (L H_2/L-d) average / maximum		HY (mol H_2/mol hexose) average / maximum	
				A1	A2	A1	A2
I	10	24	1 – 11	0.8 / 1.3	1.8 / 3.5	0.3 / 0.4	0.5 / 1.1
II	10	36	12 - 22	0.7 / 1.9	0.8 / 1.7	0.2 / 0.5	0.2 / 0.5
III	10	48	23 - 57	0.2 / 0.7	0.5 / 2.1	0.05 / 0.2	0.1 / 0.5
IV	10	60	58 - 70	0.3 / 1.5	0.9 / 2.9	0.1 / 0.6	0.3 / 0.8
V	8	60	71 - 80	2.0 / 4.0	2.9 / 5.3	0.6 / 1.4	0.8 / 2.4
VI	6	60	81 - 88	2.9 / 4.5	4.5 / 6.9	0.9 / 1.4	1.5 / 2.7
VII	4	60	89 - 97	2.4 / 3.4	3.7 / 5.0	0.9 / 1.7	1.6 / 3.5
VI'	6	60	98 - 102	3.7 / 7.4	5.3 / 7.7	0.9 / 1.5	1.2 / 1.7
VII'	4	60	103 - 106	2.1 / 2.3	3.0 / 4.1	0.7 / 0.9	0.9 / 1.2
VIII	2	60	107 - 110	0.2 / 0.3	0.96 / 1.0	0.2 / 0.5	0.8 / 1.3
IX	1	60	111 - 112	0.08 / 0.1	0.3 / 0.3	0.1 / 0.2	0.37 / 0.38

The most stable periods (less scattering data) for both reactors were in general those in which lower amount of hydrogen was produced (stages VIII and IX). On the contrary, a high dispersion of the data was observed when the VHPR and HY reached higher values. Notably, the decrease in the glucose concentration of the influent and decrease in HRT from stages VII to IX (except for VI') had an immediate and negative impact on average volumetric hydrogen production rate, especially for reactor A1, but appeared not to have a drastic effect on the molar yield, especially for reactor A2 (Figure 3.1, Table 3.1).

It has been shown since short HRTs induce a greater flow of substrate to the reactor, increasing the substrate conversion rate [13], which could also promote the growth of hydrogenogenic bacteria because of their lower specific growth rates. However, coupled with increased OLRs, low HRTs could result in high food to biomass ratios, which could lead to an imbalance in competition between H2- and non H2-producing bacteria [25,26] that could explain the spread of data observed from stages V to VII'. The repeating periods

grew out of an interest in observing whether or not the production was recovering after a disturbance simulated by a stop on feeding.

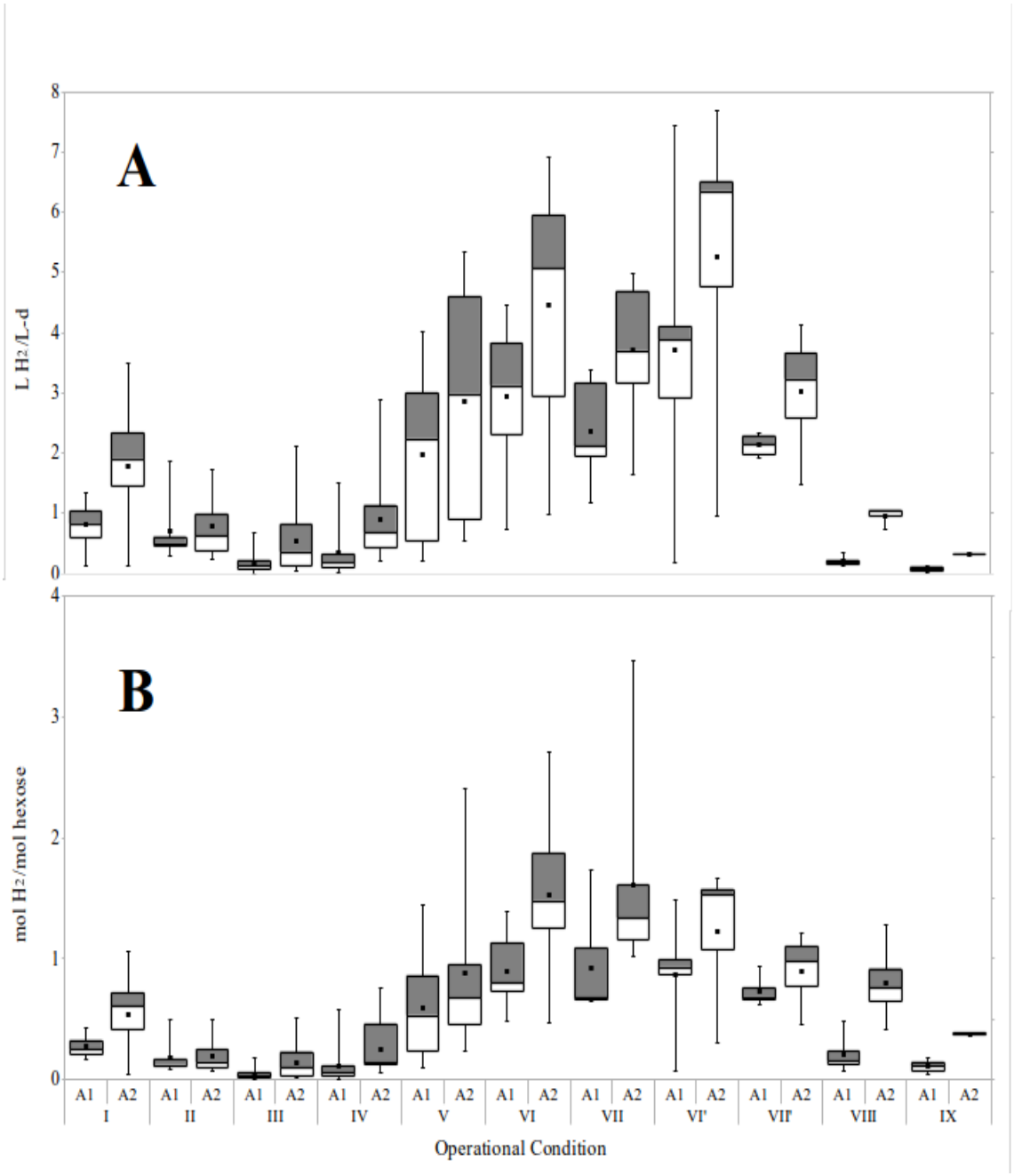

Figure 3.1. Box-plots constructed with the values of volumetric hydrogen production rate (VHPR) (A) and hydrogen molar yield (HY) (B) obtained for anaerobic fluidized bed reactors inoculated with heat shock and cell wash-out pretreated biomass (A1 and A2, respectively) during each stage assayed.

The results show that production not only can be recovered but may even be improved. However, it can hardly be due only to repetition of operating parameters. Changes in microbial community adapted to the previous repetition of these periods may explain the observed improve as it will be discussed later.

It is noteworthy that during the stage VI' both reactors reached maximum VHPR values higher than those previously registered for the same condition (stage VI, Figure 3.1, Table 3.1), with increases of 26% and 18% in average values for reactors A1 and A2, respectively. However, this was not repeated in subsequent stages. This is probably because the biomass was already adapted to the previous conditions of stage VII, so that the increase in substrate concentration in VI' was directly reflected as increased VHPR (57% and 42% in average VHPR, for A1 and A2 respectively) although the dispersion of data also increased (coefficient of variation (C.V.) values from 32% to 70% in A1 and 30% to 49% in A2). The change in conditions for the following stage (VII') resulted in an immediate decrease in hydrogen production. For reactor A1 this decrease was quickly stabilized, while for A2 a gradual increase was recorded, albeit at slightly lower levels than in the same operational condition during the previous stage VII (Figure 3.1). By lowering the HRT keeping the same OLR an immediate decrease in the hydrogen production occurred in both reactors, but this decrease was less evident in A2. In the final stage (IX) the VHPR performance declined in both reactors to values near to 0.4 L H_2/L-d.

Reactor A2 showed better performance than reactor A1 in each one of the different operational conditions as shown in Table 3.1. It is noteworthy that although the VHPR begins to decline in the period VII, the molar yield keeps increased, so the reactor A2 continued showing improved performance. As seen by comparing repeated periods, the values reached in the period VI' in either cases are similar to the values obtained in the VI period, in both VHPR and HY. However, it was not the same for the period VII, where lower values were achieved in the subsequent repetition. During periods VIII and IX, the decline in the performance of both parameters was remarkable.

Regarding the VHPR, the values reported in the literature in similar biofilm based reactors were higher than the values obtained in this work (Table 3.2). However, when comparing OLRs employed, all of them handled greater loads than those employed in this experiment,

except one [27]. With respect to molar yields, our results were higher than those reported in other studies, although in this case, the OLR used in two reports were lower than the used in this study [9,27].

Table 3.2. Comparison of maximum values in the hydrogen production (HY and VHPR) under different operational strategies using anaerobic fluidized bed reactors.

Reactor work volume (L)	Sustrate (g/L)	HY (mol H_2 /mol hexose) VHPR (L H_2 /L-d)	Inoculum	Operation conditions			
				HRT (h)	OLR (g COD/L-d)	pH	T (°C)
[2]1.4	Glucose (30)	1.16, 56.6	Activated and digested sludge	1	720	4.0	37°C
[3]1.4	Glucose (10)	1.7, 182 (biofilm); 158 (granular)	Municipal sewage sludge	0.25	960	5.5	37°C
[8]10	Sucrose (20)	2.67, 22.2	Municipal sewage sludge	2	480	5.8-6.8	35°C
[9]4.2	Glucose (2, 10)	2.49, 35	UASB sludge	2 1	24 240	4.0 3.6	30 30
[10]4.2	Glucose (4)	2.59, 29	UASB sludge	2-1	48	5.5	30
[11]4.2	Glucose (5)	2.55, 53	UASB sludge	2-1	N.A	N.A.	25°C
[27]4.2	Glucose (2)	2.29, 27.6	UASB sludge	2	24	3.8	30°C
1.12 (this work)	Glucose (15, 10)	3.5, 7.7	UASB sludge	6 4	60 60	5.5 5.5	30°C 30°C

N.A. not available.

3.3.2 Fermentation products and COD balances

For both reactors the COD balance of the main fermentation products and metabolites presented a very similar behavior (Figure 3.2). In the first four stages lactate was predominant, after which butyrate became predominant, followed by acetate. The

increment in these two metabolites during the following stages was correlated with the increase in both VHPR and HY obtained for the same periods. Higher hydrogen production was observed one or two days after the concentration of VSS increased in the effluent, mainly in A2. Biofilm biomass increments was observed throughout the reactors operation, with growth exacerbated at times that coincide with the decrease in glucose consumption and hydrogen produced. Formation, accumulation, and cyclic release of biomass from the biofilm were observed for both reactors leading to technical problems that were more evident for reactor A2. Lee et al. [26] and Peixoto et al. [28] observed that excessive biomass growth could reduce bed porosity, restricting space for planktonic cells and thereby reducing hydrogen production. Although these works were based on packed beds, in the AFBRs tested also we observe lapses of excessive growth in biofilm. This can also create micro environments in which accumulation of fermentation products and hydrogen is promoted, thus inhibiting hydrogenase activity [13]. The elimination of excess biofilm may hinder mass transfer, or the transfer of active biomass present in biofilm released into a planktonic biomass, which in turn could have formed a new biofilm with higher activity than before. The formation of clusters could have an adverse effect in mass transfer decreasing the glucose consumption and increasing it again after the excess of biomass was removed from the reactor [13,26,28].

Glucose consumption ranged from 17.2% to 99.9% in both AFBR (Figure 3.2), and was usually enhanced with decreasing influent substrate concentration and increase of HRT. In both reactors the glucose consumption was above 90% in the first operating condition and down to 70% in the stage II. During the third stage glucose consumption remained at 50% with a slight and steady increase to 60% for both reactors, which coincides with the period of lower production and hydrogen yield. In the following conditions, glucose consumption dropped to values of 20% with sharp increases, which does not coincide with increases in hydrogen production recorded for those periods. Inside the reactor, the biofilm formed on the GAC was aggregated forming clumps, which rose from the bottom of reactor, losing part of the biofilm in the effluent. Increase in glucose consumption after loss of biomass excess was observed for both reactors throughout the experiment, with remarkable biomass production towards the end of stage III and subsequent periods.

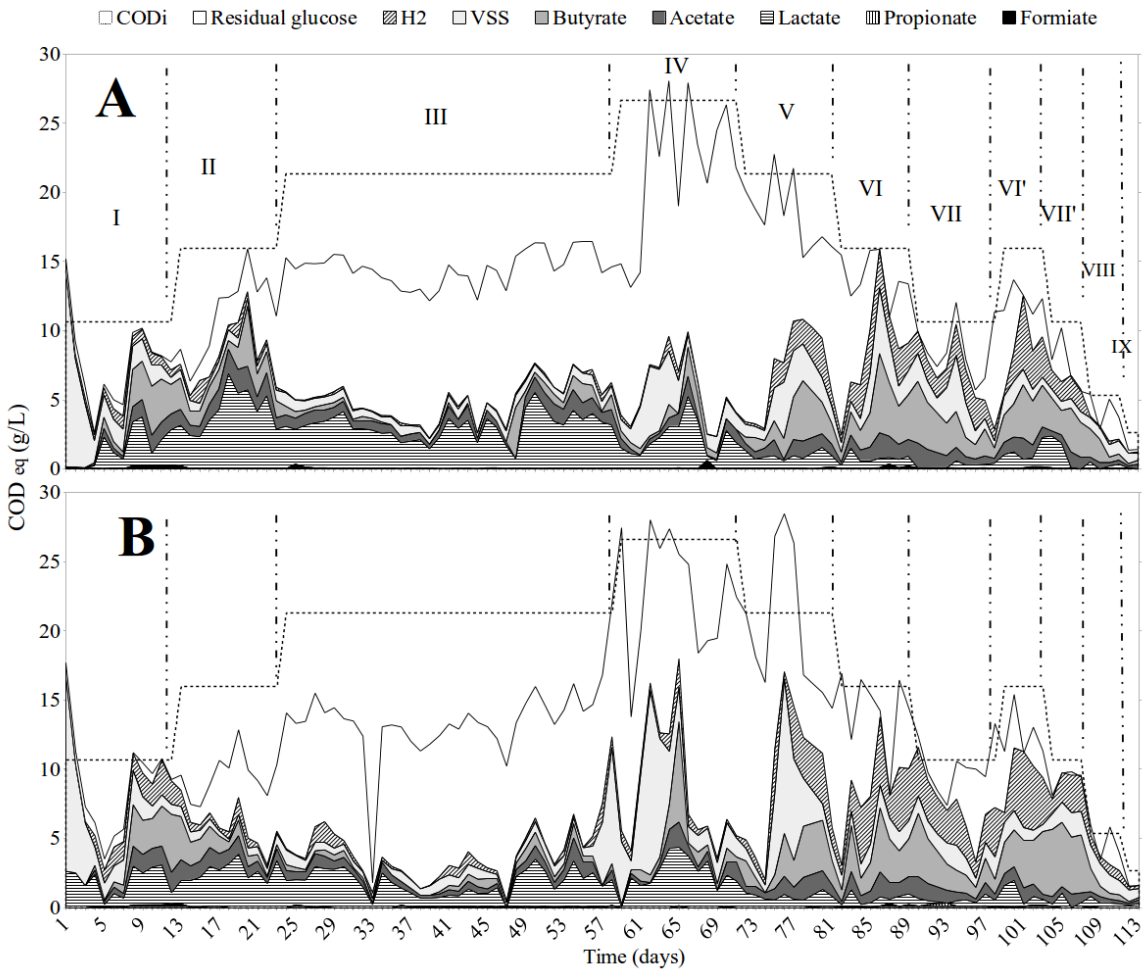

Figure 3.2. COD balances of the metabolites and biomass as well as residual glucose quantified in the effluent of reactor A1 (A) and A2 (B). The equivalent COD of H_2 produced was included in balance.

After the inactivity period, constant changes were observed in substrate conversion. In reactor A1, at stage IX glucose consumption raised to 90%, despite the loss of biofilm continuously observed in both reactors. By contrast, in reactor A2 a swift stabilization was observed in glucose consumption, up to values close to 100%. For example, in A1 the average VHPR oscillated fom 2.3 to 3.7 and then to 2.1 L H_2 /L-d when switching stage VII to VI ' and then to VII' respectively. The C.V. value was raised from 32% to 70%, and then dropped to 9% for each stage. In A2, average VHPR changed from 3.7 to 5.3 and to 3 L H_2 /L-d, and C.V. values from 29% to 49%, and 39% for the same stages.

The decrease in HRT from 4 h to 1 h could have a negative impact on the size of the hydrogenogenic microbial population in the reactors due to several factors, namely, the increase of food to biomass ratio, changes in metabolic pathways to compounds that promote biomass retention (as extracellular polymers), incomplete conversion of the substrate and cell wash-out because of the high dilution rate. As discussed earlier, a recovery is expected in production to previous levels after inactivity, but this was raised. Possibly, lack of glucose and acidification of the medium favored the survival of those capable of sporulation and/or using AGVs as carbon source, having a positive effect on the expression of favorable metabolic pathways in hydrogen production.

3.3.3 Microbial community analysis performed by DGGE and sequencing of DNA from predominant bands

As both reactors were operated under the same conditions, the differences between the biomass enriched in each reactor were presumably due to the pretreatment applied to the inoculum, which in combination with the continuous operation may select different community structures over time, with metabolite profiles reflecting it (Figure 3.2). The original methanogenic granular sludge was sampled before the treatments and, in order to check the influence of the microbial biomass entering to the system with the feeding solution, the thin biofilm formed inside the feed tubing was also analyzed (sample EXT). A DGGE was performed in which samples from both AFBRs were run together with the samples from treated inocula and samples from conditions of lowest and highest performance of H_2 production, from which representative bands were excised, DNA recovered, re-amplified and sequenced (Figure 3.3 and Table 3.3).

NMDS analysis based on this DGGE gel profiles showed a separation between the samples taken from the two reactors when comparing the samples taken from the suspended cells or from the biofilm, indicating that the treatment of the inoculum defines the community developed in each reactor. For reactor A1, biofilm samples were separated from the planktonic samples, showing a strong effect of the adhesion to the support; this effect was not so strong for reactor A2 as the samples were closer positioned in the graph (Figure 3.4).

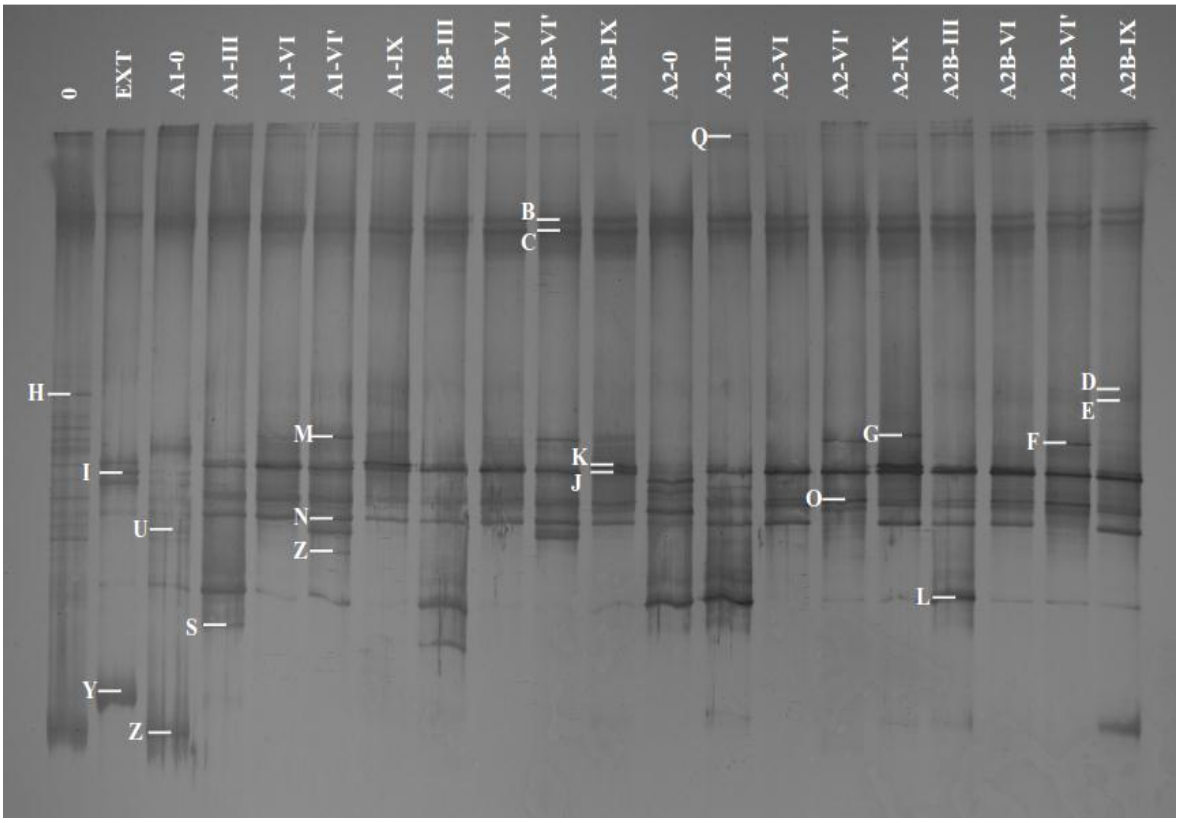

Figure 3.3. DGGE gel performed with the samples taken during the stages with low and high performances from both reactors showing the position of excised bands. In this tags, sample 0 corresponds to the original methanogenic sludge, sample A1-0 and A2-0 represents the inoculum from heat-shock and cell wash-out pretreatments respectively. The numbers after hyphen indicate the proper stage. Capital letter "B" indicates that the sample was taken from biofilm, meanwhile the others were planktonic samples. Apostrophe (') denotes the repeated stages. Sample shown as "EXT" indicates the biomass obtained from feed tubing. Bars indicates the bands that were successfully amplified and sequenced. Each band was designated by a capital letter. At the top of the picture each lane is tagged with the corresponding period from which the sample was taken.

Members of the genus *Clostridium* were present in all the samples tested, increasing their relative abundance in those periods where higher hydrogen production was obtained (Figure 3.5 and Table 3.3). Differences in abundances were observed between biofilm (capital letter "B") and planktonic samples for the same stages. Although nine bands were relative to Clostridia, only three were present throughout the experiment.

Table 3.3. Comparison of the sequences retrieved from the DGGE bands and sequences from database using the BLAST tool from NCBI.

Band no.[1]	Band appareance[2]	Sequence lenght	Accession number	Phylum	Closest match	Match accesion number	Indentity (%)
B	All samples	442	KU755531	Firmicutes	*Clostridium sardinense*	X734461	91
C	All samples	505	KU755532	Firmicutes	*Clostridium tyrobutyricum*	NR_044718	87
D	All samples	402	KU755533	Firmicutes	*Clostridium pasteurianum*	CP009268	99
E	All samples	511	KU755534	Firmicutes	*Clostridium pasteurianum*	CP009267	98
F	All samples	563	KU755535	Firmicutes	*Clostridium tyrobutyricum*	KR011768	96
G	All samples	564	KU755536	Firmicutes	*Clostridium pasteurianum*	NR_103938	99
H	All samples	402	KU755537	Bacteroidetes	*Uncultured bacterium clone*	KC860740	97
I	0, EXT, A1-VI', A1B-VI', A0-II	466	KU755538	Firmicutes	*Lactococcus lactis*	KM207840	99
J	All samples	563	KU755539	Firmicutes	*Clostridium pasteurianum*	EF140983	99
K	All samples	566	KU755540	Firmicutes	*Clostridium pasteurianum*	NR_103938	99
L	All samples	592	KU755541	Firmicutes	*Lactobacillus paracasei*	CP013921	98
M	EXT, A1-III,VI,IX, A1B-VI,IX, A2-0, A2-VI',IX, A2B-IX	563	KU755542	Firmicutes	*Clostridium tyrobutyricum*	KR018740	99
N	0, EXT, A0-I,III,VI,VI', A2-VI', A2B-VI'	583	KU755543	Firmicutes	*Sporolactobacillus laevolacticus*	LC064803	96
O	0, EXT, A1-0, III, VI, VI', A2-VI', A2B-VI'	490	KU755544	Firmicutes	*Lactobacillus satsumensis*	LN870302	95
Q	0, A2-VI	381	KU755545	Firmicutes	*Uncultured bacterium*	FN667298	80

S	0, A1-III, A1B-III	587	KU755546	Firmicutes	*clone Lactobacillus rhamnosus*	KU366370	98
U	All samples	558	KU755547	Firmicutes	*Uncultured bacterium clone*	JX271027	99
Y	EXT, A1-III, A2-III	468	KU755548	Proteobacteria	*Klebsiella oxyotoca*	LC049175	96
Z	0, EXT, A1-0, A1-III, A1B-VI', A2-III, A2-III'	425	KU755549	Proteobacteria	*Bacillus megaterium*	KF053067	80

[1]Indicates the position of band in gel (Fig 3.3).
[2]Indicates presence of the band in sample taken either from reactors A1 or A2, Roman numerals show the respective stage of operation (see Table 3.1) and capital letter B indicates that the sample was taken from biofilm. EXT designate the sample taken from feed tubing.

Lactic acid bacteria (LAB), known to interfere with hydrogen production, as well as for the production of bacteriocins capable of inhibiting the growth of competitors [29], were also present in all samples, with main presence during the low production condition in A1 (columns A1-III and A1B-III). Both groups, Clostridia and Lactobacilli, were present in sample 0 corresponding to the original methanogenic sludge, and were still present in samples 0-1 and 0-2 representing the inoculum heat-shock and cell wash-out pretreatments respectively, although in different proportions. Were even present in the sample obtained from the feed tubing (shown as EXT in Figures 3 and S1). This is consistent with previous reports in which natural fermentation of wastewater is feasible for H2 production by dark fermentation [15]. The relative abundances of microorganisms represented by DGGE bands from which the sequences could not be determined were grouped as “others” in Figure 3.5.

In order to observe the differences in the relative abundance of the organisms from the *Clostridium* genus and the lactic acid bacteria (LAB) detected in the two reactors, box-plots were constructed with the relative abundance of these two important groups of microorganisms in the samples taken from the biofilm (capital letter “B”) and from the planktonic cells for each reactor (Figure 3.6). The abundance of lactic acid bacteria (LAB) were calculated as the sum of the relative abundance calculated from the intensities of the

bands presenting sequences related to the sequences from *Lactobacillus*, *Sporolactobacillus* and *Lactococcus* in each sample.

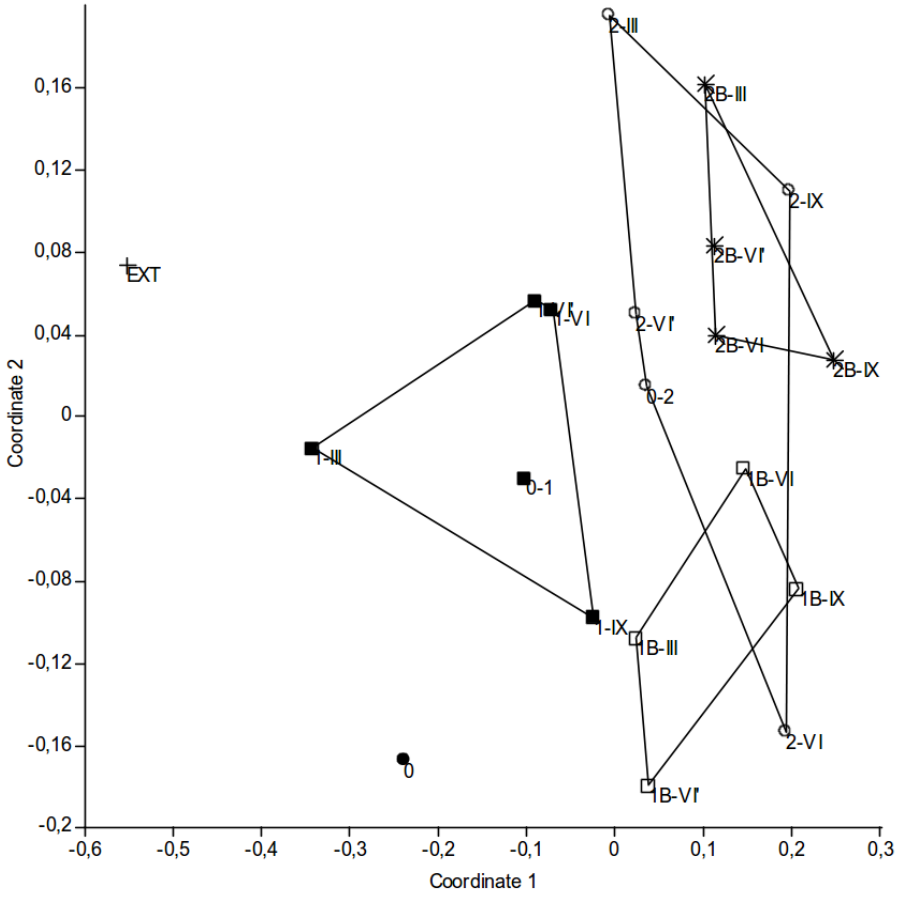

Figure 3.4. Non-metric multidimensional scaling (NMDS) analysis performed using the Bray-Curtis similarity measure based on the DGGE gel profiles of representative samples taken from both reactors. Filled squares: samples from reactor A1 taken from the planktonic biomass, empty squares: samples from reactor A1 taken from the biofilm, open circles: samples reactor A2 taken from the planktonic biomass, aster: samples from reactor A2 taken from the biofilm, filled dot: sample taken from the inoculum previous to the treatment, cross, sample taken from the inflow pipe.

In general, the samples taken from reactor A2 presented higher relative abundance of *Clostridium* especially in biofilms and this is related with the better performance observed. Samples taken from the biofilm attached to the GAC presented higher abundance of *Clostridium* and lower abundance of LAB than the samples taken from the suspended cells.

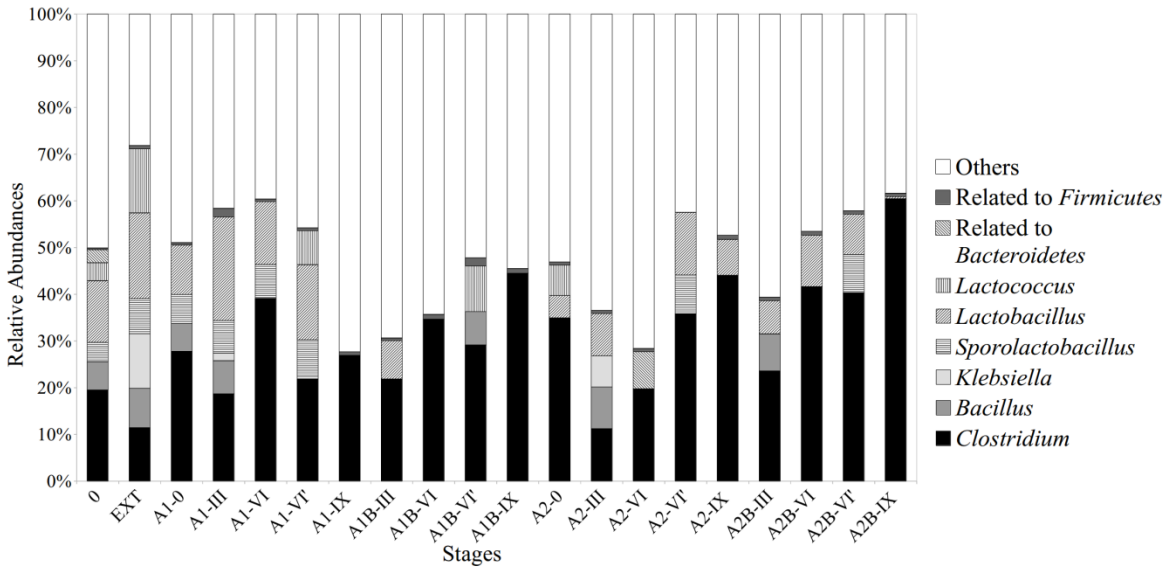

Figure 3.5. Relative abundance of the different microorganisms represented by DGGE bands detected in the samples taken from both reactors. Sample 0 corresponds to the original methanogenic sludge, samples 0-1 and 0-2 represents the inoculum heat-shock and cell wash-out pretreatments respectively. Stages are according to Table 3.1.

This is an important finding as indicate a positive selection of the inert support for hydrogen production (Figure 3.4). The predominance of Clostridium, in high hydrogen production performance stages, as well as LABs in periods of poor performance, is a similar trend observed by Etchebehere et al., [30] studding a set of samples taken from 20 different reactors. The authors observed that the samples taken from reactors during high hydrogen production presented greater abundance of *Clostridium* or other microorganisms reported as high hydrogen producers.

The presence of LAB can be traced back to the inoculum (Figure 3.5). Methanogenic granular sludge was obtained from a wastewater treatment plant of a confectionary

manufacturer, whose main products are based on chocolate and milk. This explains its presence in the original inoculum, but the reason for their survival after pretreatment is not so clear. Probably, the amount of solids in the sludge subject to heat-shock pretreatment, coupled with the boiling time employed (45 min), allowed the formation of micro environments, through which survived LAB and other non-sporulating bacteria.

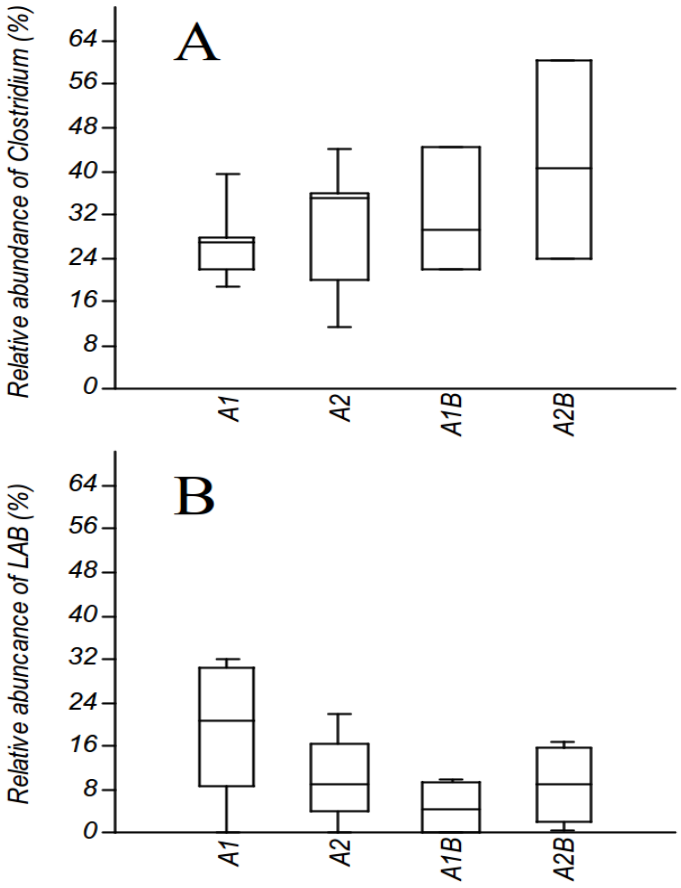

Figure 3.6. Box-plots showing the distribution of the relative abundance values of organisms from the genus *Clostridium* (A) and lactic acid bacteria (B) in the samples taken from the suspended biomass and from the biofilm attached to the support from reactors A1 and A2.

From all LAB identified only *Sporolactobacillus* is known as endospore forming microorganism [31], a feature that gives it its name, for others it is necessary to resort to other explanations. In LAB, small heat shock proteins (Hsp) have been found to interact with the cell membrane, to modulate the lipid physical state under stress conditions, and found to accumulate in dormant bacterial cells (non-growing state) through which

individuals may survive for an extended period of time under long-term severe stress conditions, like heat-shock [32]. Together, these circumstances might favor the persistence of LABs through the heat pretreatment used in the inoculum of reactor A1. For reactor A2, pretreatment by cell wash-out was performed using cheese whey as a substrate, so easily LABs were able to survive through kinetic control. Although the presence of LAB is reported throughout the literature in relation to dark fermentation, and their presence is considered inevitable part of the microbial community, little research has been done on how to control their presence. It is not yet clear whether their presence negatively affects the performance in hydrogen production, or if their presence actually provides an advantage in stabilizing the process [30]. Although a clear relationship was observed in the relative abundance of the identified organisms and the reactors performances, it has to be taken into account that non-identified fraction of the community could also played a key role, since sample A2-VI had just 20% of relative abundance of organisms identified as Clostridia (Figure 3.5), and this sample corresponds to the operational condition with higher biohydrogen production.

Differences observed between planktonic and biofilm communities were as expected, but this is not in agreement with previous observation that reported higher production of hydrogen in the suspended biomass [26,33], but these authors used packed bed reactors in which the suspended biomass are trapped within the support and has less possibilities of cell wash-out. This might suggest that the type of media also has a role in the selection of microbial community generated.

Two more DGGE were performed to compare all the samples taken from each reactor in the different stages of operation. Cluster analysis showed that the communities from both reactors (planktonic cells and biofilm adhered to the support) changed during time and according to operating conditions (Figure 3.7). For the reactor A1 (inoculated with heat treated biomass), the clustering of communities suggests a long-term influence of the incoming biomass with the influent represented as EXT (Figure 3.7-A). Changes in the population structure appear to be chaotic rather than gradual, as no temporal pattern was observed in the clustering, particularly for planktonic biomass. The opposite occurs in reactor A2, the sample taken from the pretreated inoculum (A2-0) is located immediately next to the samples in sequence of the operational conditions like most of the other

samples, indicating that the community dynamics had gradual changes adjusting immediately to each operational condition in both biofilm and planktonic biomass (Figure 3.7-B). Differences between biofilm and planktonic biomass decreased in the last operating conditions, suggesting that the biofilm may exert an effect of resistance to changes in population structure due to operational changes and/or cell wash-out under HRT. Although both reactors samples profiles of biofilm and planktonic biomass were grouped separately, the biofilms performed the role of reservoir of the community, perhaps promoting population dynamics with moderate but constant changes, which would achieve stability levels faster in A2 than A1. Abrupt changes in community structure in reactor A1 may involve more complex dynamics, with short stability periods over time.

Based on the DGGE profiles, the Shannon-Wienner diversity index (*H*) and richness (*S*) were determined (Table 3.4). The results obtained showed that methanogenic sludge was more diverse in their bacterial community, and that the communities become simpler after each pretreatment and later during the operational conditions applied to the reactors. It is remarkable that the communities that persisted after both pretreatments presented similar *H* and *S* values. The same was observed for the communities developed during the stages with best performances (VI and VI' for both reactors). Although the inoculum pre-treatments were based in different strategies of selection, the communities that survived presented similar diversity and richness but not the same taxonomic composition, pointing out that the microbial community identity is defined at greater extent by the type of selected pretreatment (Figure 3.7, Table 3.4).

The selective pressure of the operational conditions tested explained changes in the assemblage of the microbial community of each reactor. Low OLR and high HRT showed more diverse communities (high *H* and *S*), indicating the proliferation of non-specialist species in the conversion of glucose in H_2, but, when the OLR was increased to 60 g COD/L-d decreasing HRT to 6 h, the community becomes more specialized (lower *H* and *S*).

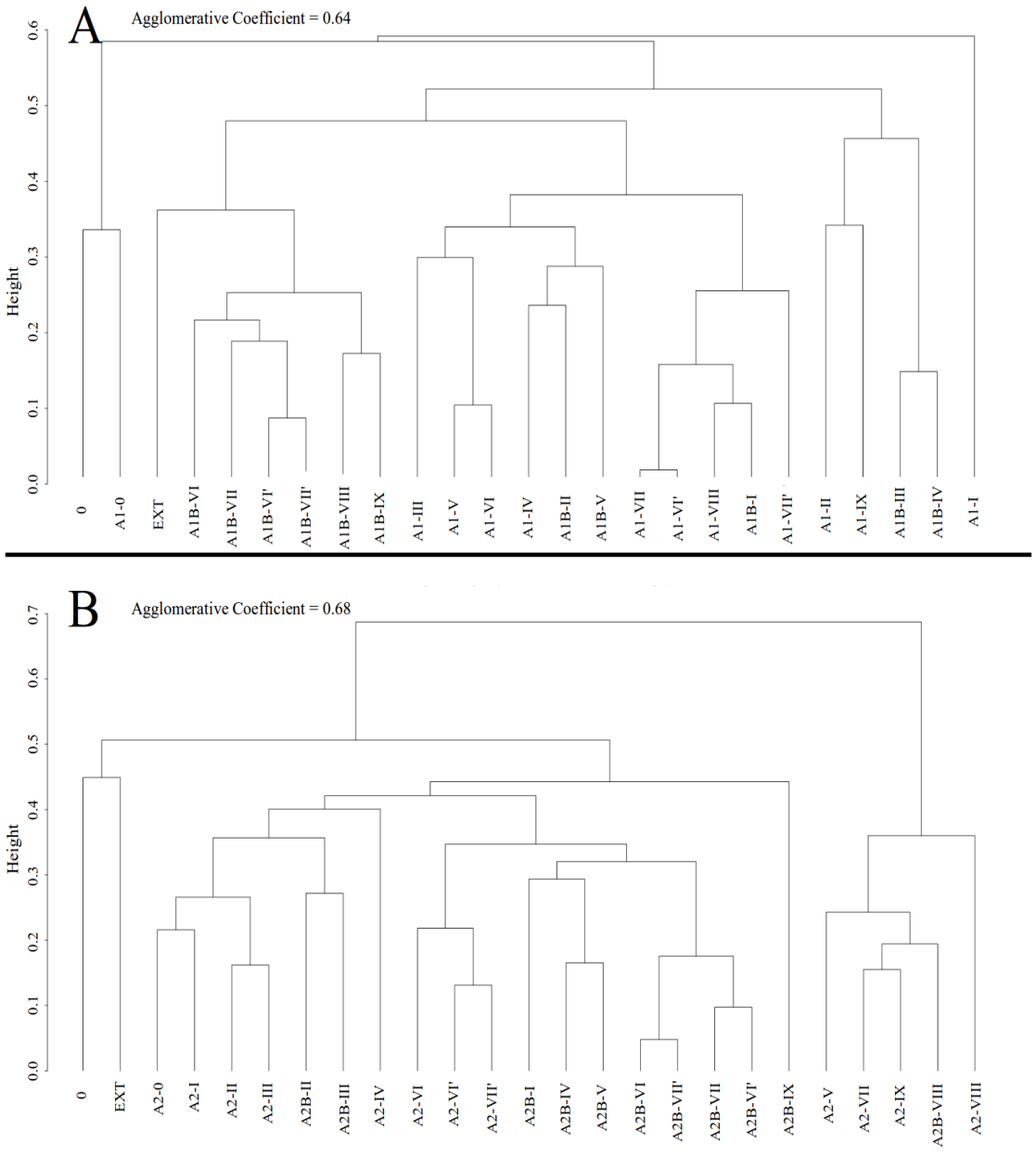

Figure 3.7. Cluster analysis of DGGE gel profiles obtained from samples taken during all operational conditions assayed for both reactors. Sample 0 corresponds to the original methanogenic sludge, sample A1-0 and A2-0 represents the heat-shock (A) and cell wash-out (B) pretreatments respectively. The number after hyphen indicates the proper stage.

Table 3.4. Shanon Weiver diversity index (*H*) and species richness (*S*) of the microbial communities developed in each reactor during each stage of operation. A1 and A2 represent samples taken from the suspended biomass; A1B and A2B represent samples taken from the biofilm attached to the granulated activated carbon. In this table, 0 (zero) means the inoculum after the corresponding pretreatment. Original methanogenic granular sludge had values of 3.25 and 29 for *H* and *S*, respectively. Sample EXT had values of 1.66 and 17 for *H* and *S*, respectively.

Stage	*Diversity index (H)*				*Species richness (S)*			
	A1	A2	A1B	A2B	A1	A2	A1B	A2B
0	2.34	2.34	-	-	21	24	-	-
I	2.05	2.00	1.90	2.03	15	19	16	20
II	1.76	2.15	1.75	1.94	14	23	17	19
III	2.05	2.21	2.44	1.83	18	22	25	19
IV	1.75	1.84	2.14	2.12	18	19	20	21
V	1.90	1.73	1.78	1.92	15	18	18	19
VI	1.67	1.67	1.52	1.25	16	18	18	15
VII	1.91	1.75	1.78	1.64	16	18	16	19
VI'	1.58	1.64	1.65	1.57	17	16	18	17
VII'	2.05	1.76	2.03	1.81	18	19	18	18
VIII	1.74	1.48	2.14	1.56	17	16	17	16
IX	1.83	1.59	1.36	1.65	19	18	15	18

Both reactors show increased performance in the H_2 production, thereby deducting that a selection of specialist species in substrate conversion to H_2 as metabolism preferred for obtaining energy occurred. Although not identified, many bands (represented all together as "other" in Figure 3.5) have relative abundances between 5% and 15% by themselves, especially in period VII. Some of these unidentified bands with high relative abundance could have a direct effect on the performance of each reactor. Richness, diversity and performance values in these periods (VI' and IX) indicate the specialization of the surviving microbial community. Biofilm samples showed slightly lower *H* values than planktonic cells samples taken at the same stages. The fluidization process could be limiting the microbial consortia retained, while favoring the presence of *Clostridium* and other producing hydrogen, as shown in Figure 3.5 and 3.6 in samples from biofilms.

The cell wash-out pretreatment, based on kinetic control, was the most convenient, and this is in agreement with previous results of our work group using granules. The principal differences between this work and the previous one with EGSB reactors [18] is the kind of

self-aggregated biomass. Although the same pretreatments were used, the reactor configuration necessarily exerted a selective pressure upon the microbial community which has been previously molded by respective pretreatment. Biofilm developed in AFBR seems to be more appropriated to enrich the community with Clostridia than granules, judging by the respective relative abundances. This has a direct impact on hydrogen production rate and molar yield, which were superior in this work.

3.4 Conclusions

Cell wash-out is a more suitable treatment for biohydrogen production than heat pretreatment, avoiding the cost of energy for heating. Both pretreatments were effective in preventing methanogenic activity. HY and VHPR were increased with the decrease of HRT, achieving maximum values of 3.5 mol H2/mol hexose and 7.7 L H2/L-d at 4 h and 6 h, respectively. Pretreatments promoted different microbial communities in both reactors, selected by both pretreatments and each operational condition, with the continuous presence of *Clostridium* genus members. Both reactors showed resilience after a starvation period, showing similar values to previous performances.

3.5 References

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Chapter 4

Interaction of pure strains with different metabolic pathways during hydrogen production in batch and continuous reactors

Summary

Although high hydrogen yields are possible to achieve during dark fermentation in high rate reactors, the process is unstable and the production of hydrogen presents high fluctuations. To understand potential causes of this behavior, we studied the interaction of four strains belonging to the genera *Clostridium*, *Lactobacillus*, *Megasphaera* and *Rahnella*, previously isolated from a hydrogen-producing reactor. Kinetic and metabolic characteristics of the isolates were investigated in batch cultures either in monocultures and cocultures. The changes in yield and hydrogen production rate, as a result of interactions were analyzed in co-cultures in continuous reactors. The results showed that even if *Rahnella* is a low-hydrogen producer, it has a fast growth rate and competes with *Clostridium* for substrate, whilst *Megasphaera* is able to use volatile fatty acids to produce hydrogen. Although *Lactobacillus* may hinder in hydrogen production, members of this genus could play a pivot role in the community balance.

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4.1 Introduction

Biohydrogen is an attractive biofuel and an alternative for recovering energy from organic residues [1–3]. Several works have been performed to optimize the conditions to achieve high hydrogen yield and production [1,4,5]. Although biohydrogen production by dark fermentation process is feasible, the production of hydrogen presented high fluctuations [3,6,7]. These fluctuations can be due to the hydrogen consumption by homoacetogens or methanogens, or by the interaction between fermenters, with different capacity to produce hydrogen, and also by competitors.

In a previous work studying the microbial community of 20 different hydrogen-producing reactors, three physiological groups of microorganisms were observed: high-yield hydrogen producers (*Clostridium, Kosmotoga, Enterobacter*), fermenters with low-hydrogen yield (mostly from Veillonelaceae), and competitors (*Lactobacillus*) [7]. Although these three groups of microorganisms are frequently detected in hydrogen producing reactors, little is still known about the complex interactions that occur in natural communities and how these interactions can be managed to perform a stable process. One possible way to study these interactions is to build a customized consortium composed by diverse members, each one contributing with its unique and essential metabolic capacity [8]. In simple mixtures composed by pure cultures, the metabolic changes are easier to detect because of the reduced diversity of the community, and can reveal important information about the conditions that promote high yields and production rates [9,10]. Furthermore, most of the research works using pure cultures were generally focused on maximizing production and hydrogen yield and not in the design of co-cultures emulating the structure of a hydrogenogenic community, such as those present in dark fermentation reactors.

In order to understand the differences in performance, dynamics and stability of the hydrogen producing process we studied the interactions of four strains previously isolated from a hydrogen producing bioreactor and that are representative of the physiological groups previously commented**.** The strains belonged to genera with different capacity to produce hydrogen (*Clostridium, Rahnella* and *Megasphaera)* and one genus frequently described as competitor (*Lactobacillus*). The kinetic and metabolic characteristics of the isolates were determined in batch cultures under sterile conditions. The changes in the yield and hydrogen production as a result of the interactions between the members of the

communities were analyzed in customized consortiums composed by different combinations of the strains. Continuous-mode reactors were inoculated with chosen bacterial mixtures under non-sterile conditions, in order to verify their performance and resilience. The analysis of the microbial communities was monitored by T-RFLP (Terminal Restriction Fragment Length Polymorphism) and denaturing gradient gel electrophoresis (DGGE), which made possible to determine the diversity of the species, qualitative and semi-quantitatively [11].

4.2 Materials and methods

4.2.1 Strains and media

Four strains were used in the experiments. The strains were previously isolated from a continuous stirred-tank reactor (CSTR) that produced biohydrogen using cheese whey. The identification of the strains was done by 16S rRNA gene sequences, and are depicted as *Clostridium tyrobutyrivum* strain RT3, *Megasphaera cereviseae* strain PR2AD2, *Rhanella aqualitis* strain R4 and *Lactobacillus casei* strain M15**.** These strains are represented as C, M, R and L respectively.

A minimum mineral phosphate buffer media was used for the cultivation and maintenance of the four strains, an contained (mg/L): $ZnCl_2$, 75; K_2HPO_4, 125; $MgCl_2 \cdot 6H_2O$, 100; $MnCl_2 \cdot 4H_2O$, 10.77; $FeCl_3 \cdot 6H_2O$, 25.97; $CuCl_2 \cdot 2H_2O$, 3.41; and $NiCl_2 \cdot 6H_2O$, 101.25 [12].

4.2.2 Batch tests

The growth kinetics of the monocultures was performed in triplicate and in quintuplicate for the co-cultures. The co-cultures were as follows: C+L+M+R, C+L+M, C+M+R, C+L and C+R. Hach tubes of 10 mL were used as experimental units, with a working volume of 8 mL. To facilitate the process of fermentation and the bacterial kinetic study, glucose was used as a model substrate, at a concentration of 5 g/L. Prior to sterilization, the tubes with the medium were bubbled with N_2 to reach anaerobic conditions. The assays were initiated with cultures previously grown until the end of the logarithmic phase, and then brought to fresh medium until an optical density (OD) between 0.02 and 0.05 determined spectrophotometrically at OD 600 nm, both mono- and co-cultures. For the co-cultures, each strain was previously grown at at 0.4 OD in logarithmic phase and co-inoculated in

equal proportion relative to the other strains. The cultures were incubated at 30 °C with agitation at 150 rpm. The pH was initially adjusted to 7.0.

4.2.3 Continuous reactors

Two continuous assays were conducted. The first one with a mixture of the four strains, and the second one without the *Lactobacillus* strain. A CSTR was used with a total volume of 5 L and a working volume of 3 L. Again, each strain was previously grown at 0.4 OD in logarithmic phase in equal proportion relative to the other strains. A volume of 100 mL of each strain was used in order to prepare the inoculum. The volatile suspended solids (VSS) concentration in the reactor was 0.2 g/L in reactor. The reactors were maintained at 30 °C, 150 rpm and the pH was initially adjusted to 5.5. Glucose, 12.5 g/L, was used as substrate and the hydraulic retention time (HRT) and the organic load rate (OLR) were 10 h and 30 g COD/L-d, respectively. In order to take advantage of the log phase inoculum, and to avoid drastic changes in the ratios of the different strains due to the differences in their duplication times, reactor operation was started directly in continuous mode during 32 days, under non-sterile conditions.

4.2.4 Analytical determinations

Optical density was determined in a spectrophotometer (Aquamate, ThermoSpectronic, USA) at 600 nm. Gas production was measured using the liquid displacement technique with an inverted burette for batch tests, and with a gas flow meter calibrated periodically for continuous assays (Milli Gascounter, Ritter Apparatebau GmbH & Co., Bochum Germany). The production of hydrogen gas in the headspace was quantified by gas chromatography (GC 2010 Shimadzu Corporation, Kyoto, Japan), using argon as the carrier gas under conditions previously reported [3]. Standard conditions of pressure and temperature to report gas volumes were considered (0 ºC and 101.325 kPa). Modified Gompertz equation was used (Eq. 4.1) to adjust the kinetics of hydrogen production and obtaining the Hmax, Rmax and λ parameters using Matlab v. R2015a. This equation has been used to model gas production previously [12]:

$$H(t) = H_{max} * \exp\left\{-\exp\left[\frac{2.71828 * R_{max}}{H_{max}}(\lambda - t) + 1\right]\right\} \quad (4.1)$$

H(t) in mL is the total volume of hydrogen produced in the culture at time *t* given; *Hmax* (mL) is the maximum amount of hydrogen produced; *Rmax* (mL/h) is the maximum rate of hydrogen produced; λ (h) is the lag time before potential hydrogen production. The liquid samples were taken with a syringe in the batch tests (about 0.2 mL), and directly from the effluent in the case of the bioreactor, and frozen at -20 °C until analysis. Volatile fatty acids (VFAs) were analyzed by capillary electrophoresis as described by Castelló et al. [3]. Glucose was quantified using a commercial kit (Wiener Lab., Argentina), following the manufacturer's instructions. VSS were analyzed by standard methods [14]. The volumetric hydrogen production rate (VHPR) was calculated from the amount of gas produced in a given period of time, and then multiplied by the fraction of hydrogen in the headspace, dividing this by the time elapsed and the work volume of the reactor. The balance of equivalent electrons for residual glucose, produced metabolites, hydrogen and VSS was obtained by the stoichiometric approach, using half redox reaction equations of each one of them [15]. VSS electron equivalents were calculated based on the empirical formula of biomass: $C_5H_7O_2N$ [15]. Hydrogen yield was calculated based on the glucose consumed. The specific growth rate (μ) was determined according to $Lnx = \mu t + Lnx_i$ plotting the logarithm of the biomass concentration of the exponential phase of the growth curve (*Ln x*) versus time (*t*), a straight line is generated, whose slope is μ. The cell doubling time was obtained by $t_d = Ln2/\mu$.

4.2.5 DNA extraction and community analysis

Samples from the suspended biomass in CSTR were collected every day in both reactors, and stored at -20°C, but only those of interest were selected for further analysis. DNA was extracted from samples taken from the reactor biomass using an UltraClean SoilDNAExtraction Kit (MO BIO Laboratories Inc. Carlsbad, CA, USA) according to the manufacturer's protocol. The composition of the microbial community was determined using T-RFLP and DGGE analysis of the 16S rRNA gene. For T-RFLP analysis, the PCR reaction (using primers 27 forward and 1492 reverse), purification, digestion with

restriction enzyme MspI, fragment separation and analysis were performed as described elsewhere [3]. In case of DGGE, nested PCR was used, with conditions and primers previously reported [16], and performed and stained as reported by Carrillo-Reyes et al. [17]. DNA quantity and quality were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Relative microbial abundances were estimated for T-RFLPs using hights of representative peaks using Peak Scanner Software v2.0 (Applied BioSystems, USA), and for bacterial DGGE gel using band intensities by Quantity One analysis software (Bio-Rad, Hercules, California, USA).

4.3. Results and discussions

4.3.1 Batch assays

Fermentations lasted 165 h for mono-cultures and 42 h for co-cultures, once consumed the entire substrate. Monocultures had different adaptation times in the medium employed. *Rahnella* (R) grew faster, followed by *Clostridium* (C) and *Megasphaera* (M). *Lactobacillus* (L) failed to grow on the medium used probably due to lack of cofactors (mainly complex B) necessary for their metabolism. Although an increase of the OD was observed after 140 h of incubation, this might be due to cryptic growth, cell lysis and product release cofactors in the cytoplasm (Figure 4.1). In the co-cultures, most of them had a similar growth, with a lag phase near to 12 h, except for mixtures without *Lactobacillus* (*Clostridum+Megasphaera+Rahnella* and *Clostridium+Rahnella*, denoted as C+M+R and C+R, respectively). Interestingly, the lag phase of the two microbial mixtures was similar to the monoculture of *Rahnella*, suggesting that this strain dominates rapidly due to its growth rate. The accumulated production of hydrogen was also variable between the pure cultures and their mixtures. *Clostridium* and *Megasphaera* showed a hydrogen production directly associated to growth, meanwhile *Rahnella* and the co-cultures showed a hydrogen production partially associated to growth (Figure 4.2).

In Table 4.1 the kinetics profiles of hydrogen production, adjusted with the modified Gompertz equation are shown. The trend is well suited for pure strains, but does not reflect the stepped H_2 production that was observed with the co-cultures. This is important because it masks a hydrogen production that could not be linked to the consumption of glucose as substrate, as it seems to be the case of *Megasphaera* as monoculture and co-cultures in

which was present, but allows kinetic calculations for each assay. Maximum production occurred in the monoculture *Megasphaera*, followed by the mono-cultures of *Clostridium* and *Rahnella*, with H_{max} of 5.04, 4.40 and 3.43 ml of hydrogen, respectively (Table 4.1). *Megasphaera* is the strain having the highest lag phase and production rate, with 74.5h and 0.094 mL/h, respectively. The higher hydrogen production rate was showed in the co-culture of *Clostridium* and *Lactobacillus*, with *Rmax* of 0.3647 ml/h, but it is also among the least total hydrogen produced, with only 0.54 mL.

Table 4.1. Gompertz parameters and kinetic data with their respective standard deviations, obtained in batch tests by each culture condition tested. *Lactobacillus* (L) culture failed to growth on the medium used as monoculture. *Hmax* (mL); λ (h); *Rmax* (mL/h); *HY* (mol hydrogen /mol hexose consumed); μ (h^{-1}); and t_d (h).

	Pure cultures			Co-cultures				
	Clostridium (C)	*Megasphaera* (M)	*Rahnella* (R)	C+L+M+R	C+M+R	C+L+M	C+L	C+R
H_{max}	4.40 ± 0.41	5.04 ± 0.23	3.43 ± 0.63	1.62 ± 0.41	1.19 ± 0.08	1.24 ± 0.05	0.54 ± 0.11	0.22 ± 0.05
λ	44.70 ± 6.40	74.51 ± 12.01	41.30 ± 1.70	9.80 ± 0.25	21.53 ± 2.04	11.99 ± 0.76	10.56 ± 0.80	13.57 ± 2.82
R_{max}	0.17 ± 0.03	0.09 ± 0.02	0.27 ± 0.08	0.11 ± 0.01	0.18 ± 0.01	0.08 ± 0.02	0.36 ± 0.04	0.04 ± 0.01
R^2	0.99 ± 0.01	0.98 ± 0.01	0.99 ± 0.01	0.98 ± 0.01	0.96 ± 0.02	0.98 ± 0.02	0.99 ± 0.01	0.99 ± 0.01
HY	0.97 ± 0.14	1.09 ± 0.15	0.76 ± 0.11	0.27 ± 0.04	0.19 ± 0.03	0.24 ± 0.03	0.18 ± 0.03	0.05 ± 0.01
μ	0.08 ± 0.01	0.16 ± 0.02	0.10 ± 0.01	0.35 ± 0.05	0.32 ± 0.05	0.24 ± 0.03	0.16 ± 0.02	0.34 ± 0.04
td (h)	8.81 ± 1.25	4.46 ± 0.63	7.09 ± 1.00	1.98 ± 0.28	2.18 ± 0.31	2.84 ± 0.40	4.34 ± 0.61	2.04 ± 0.29

Pachapur et al. [18] observed the use of organic acids as substrates in the production of hydrogen. This was observed in every one of the co-cultures that included *Megasphaera* in the mixture, coinciding with the decrease in the optical density (decay death phase) and with an abrupt increase in hydrogen production (Figure 4.3). The presence of valeric and isovaleric acids was associated almost exclusively with the growth of *Megasphaera*. *Clostridium* also showed a decrease in the fatty acids produced, specifically formic and propionic acids, between 100 and 120 h, although this had no effect on the optical density or hydrogen production. Lactic acid was detected in appreciable quantities in co-cultures inoculated with *Lactobacillus*, indicating that this strain was able to establish a favorable

syntrophic relationship with the other members of the co-culture and grow in the medium.

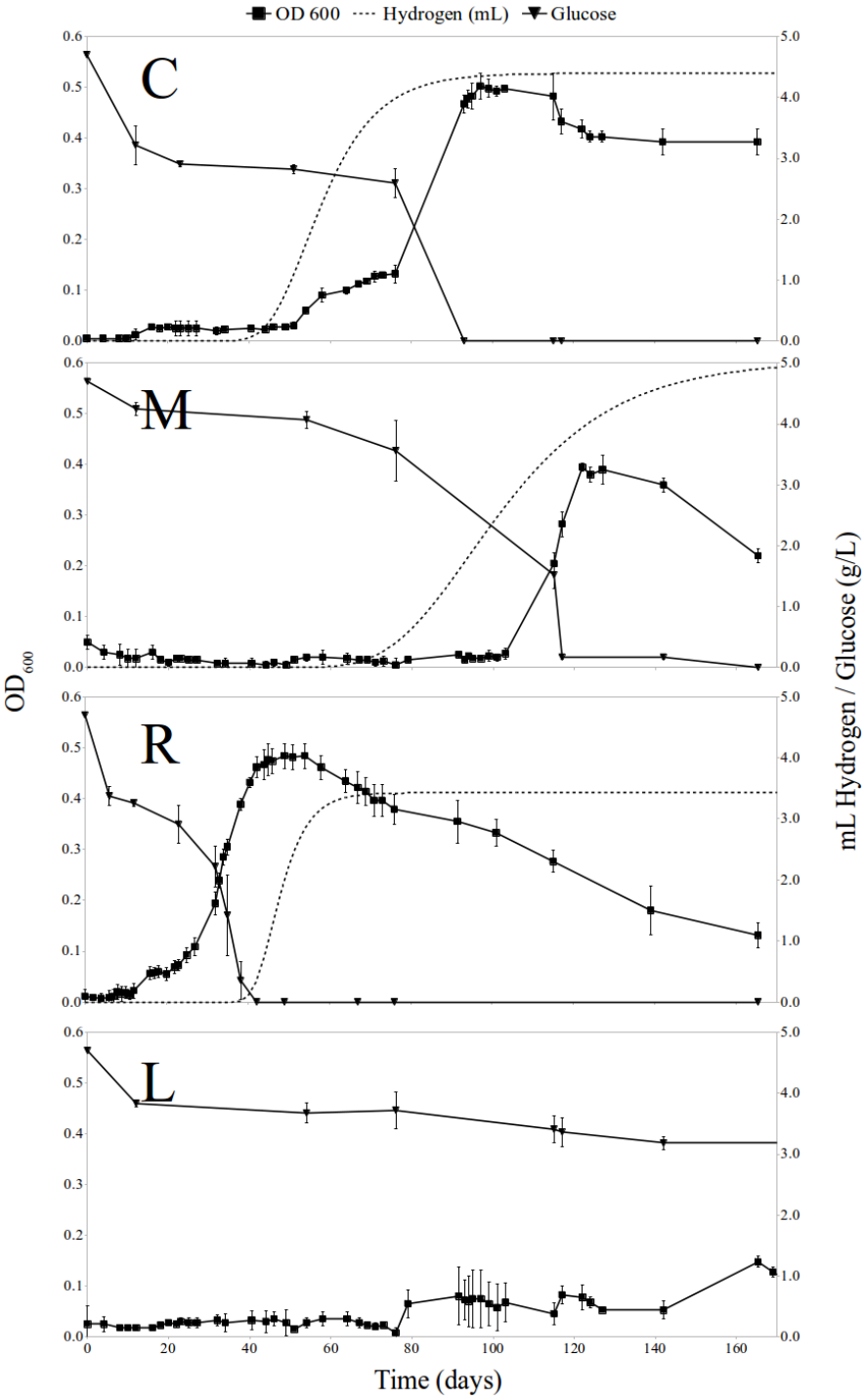

Figure 4.1. Growth curves, hydrogen production and glucose consumption in pure strains as mono-cultures. C) *Clostridium*, M) *Megasphaera*, R) *Rahnella*, L) *Lactobacillus*. (■) OD_{600}, (**---**) hydrogen produced adjusted by Gompertz in mL, (▼) Glucose concentration in g/L.

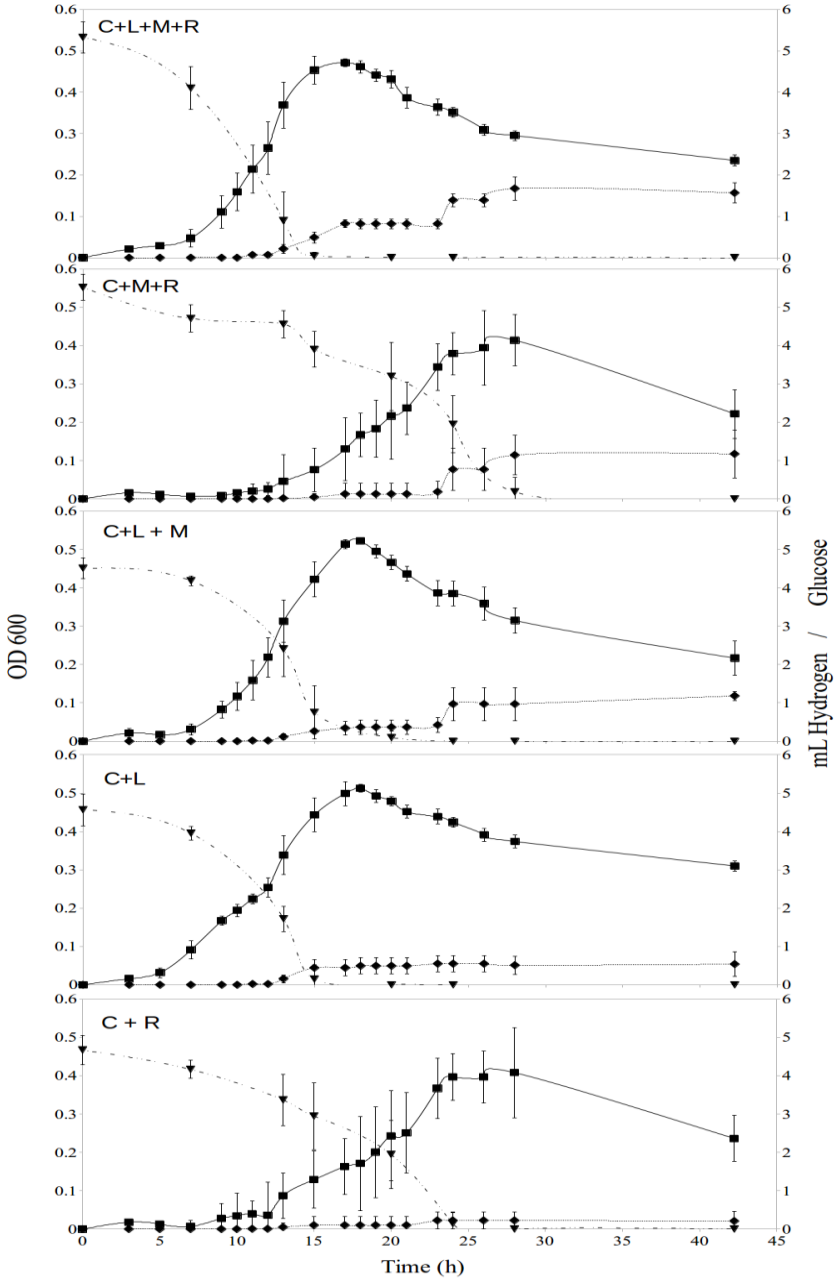

Figure 4.2. Growth curves, hydrogen production and glucose consumption in co-cultures of the pure strains. Each capital letter denotes the strain that is part of each mixture: C, *Clostridium*; M, *Megasphaera*; R, *Rahnella*; L, *Lactobacillus*. (■) OD_{600}, (♦) hydrogen produced in mL, (▼) Glucose concentration in g/L.

The co-cultures that included *Lactobacillus* were similar to each other in terms of optical density and glucose consumption (Figure 4.2). Presence of members of the genus

Lactobacillus in reactors for biological hydrogen production is generally related with in performance and stability issues. The production of bacteriocins by lactic acid bacteria it has also been studied to evaluate their for its effect on the hydrogenogenic community [19]. However, Callon et al. [20] showed that when lactic acid bacteria are associated with other Gram positive bacteria (like *Clostridium* and *Megasphaera),* synergy may occur in antimicrobial activity. Although that study was focused on *Leuconostoc* (that also belongs to *Firmicutes* phylum) it appears to also have an effect against Enterobacteria members. Being *Rahnella* a member of *Enterobacteriaceae* family, this antimicrobial activity could have a relationship with the behavior observed in the co-cultures in which *Lactobacillus* was not added, suggesting that its presence could have an effect on growth control of the other members of the community as *Rahnella* by providing stability, although having a negative effect on the potential hydrogen production.

4.3.2 Co-cultures in CSTR

After the analysis of the results from the batch test, it was decided to test the co-cultures composed by *Clostridum + Lactobacillus + Megasphaera + Rahnella* and *Clostridum + Megasphaera + Rahnella* (*C+L+M+R* and *C+M+R*, respectively) in continuous reactors, denoted as CSTR1 and CSTR2 respectively, that were operated under non-sterile conditions. Figure 4.4 shows the performance of the volumetric hydrogen production rate and the hydrogen yield in both reactors. In the CSTR1, during the 32 days of operation a stable period was not reached, but it was possible to observe a cyclical behavior in hydrogen production and yield. Starting from day 9, H_2 production gradually increased for three consecutive days up to 2 L H_2/L-d, descending the next day. Two days later, a further increase with subsequent decrease three days later. This performance was repeated again with the same number of days between increases and decreases. In the CSTR2, the hydrogen production was low during start up, but after day 17 was maintained above 2.5 L H_2/L-d.

The maximum values in the hydrogen production reached 2.93 L H_2/L-d and 3.8 L H_2/L-d, with yields of 0.3 and 0.35 mol H_2/mol glucose for CSTR1 and CSTR2, respectively.

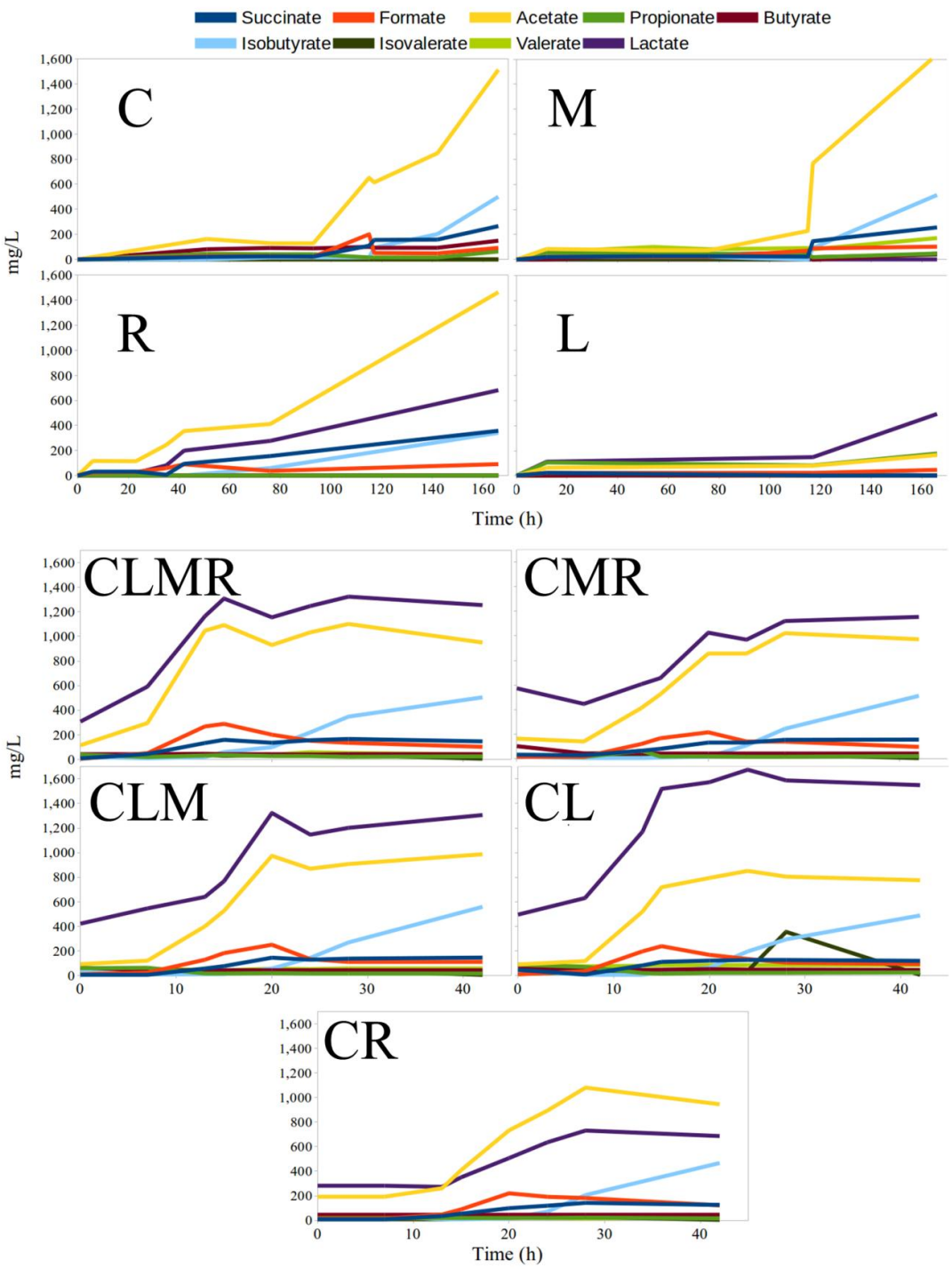

Figure 4.3. Metabolites produced by the strains in mono- and co-cultures under assayed conditions. Different sampling times were employed for each one of mono-cultures, according to their growth curve (Figure 4.1). For co-cultures, all the sampling times were the same, and metabolites produced during the inoculum preparation are shown at time zero (0 h). C) *Clostridium*, M) *Megasphaera*, R) *Rahnella*, L) *Lactobacillus*, *C+L+M+R*, *C+M+R*, *C+L+M*, *C+L*, and *C+R* are co-cultures, and the capital letters denotes the strains that compose the mixture.

Towards the end of the assay, in CSTR2 a biofilm was formed in the walls of the reactor, which could have had an effect on the planktonic biomass and therefore in its performance. Electron equivalents balance for the metabolites produced during continuous reactors operations were determined and are presented in Figure 4.5. In CSTR1 lactate was the predominant, followed by butyrate and acetate, while for CSTR2 that similar trend was reversed after the second half, coinciding with the hydrogen production. Higher hydrogen production and hydrogen yields are directly related to increases in butyrate and acetate in the first half, while during the second half was related with the increase of acetate, followed by butyrate.

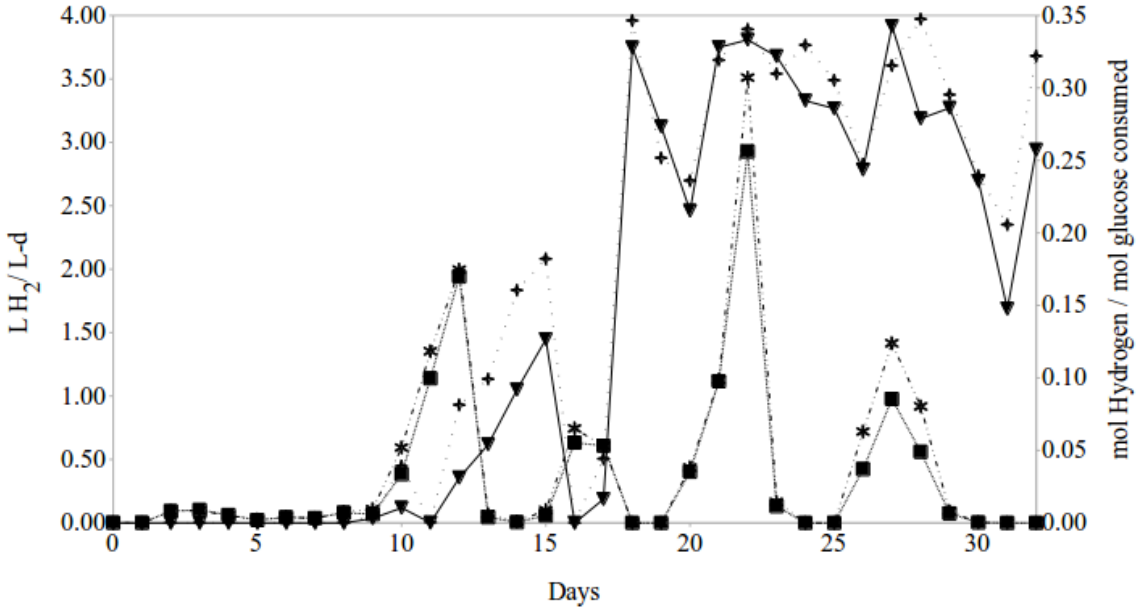

Figure 4.4. Hydrogen production and hydrogen yield performance in CSTR. (■)VHPR and (*****) HY are for CSTR1, and (▼) VHPR and (+) HY are for CSTR2.

In CSTR1 the increase in the amount of the produced lactate does not appear to be related directly with the drop in hydrogen production. By contrast, in CSTR2, lactate is detected since the beginning and could have had an inhibitory effect. Although *Rahnella* presented mixed fermentation, in batch tests it showed that the quantities of lactic acid produced are small compared to other metabolites, and do not explain the measured concentrations in the co-culture without *Lactobacillus*. Lactic acid bacteria, however, are present ubiquitously in

the non-sterilized media used for fermentation culture, facilitating their appearance if simple sugars are used as substrate [19].

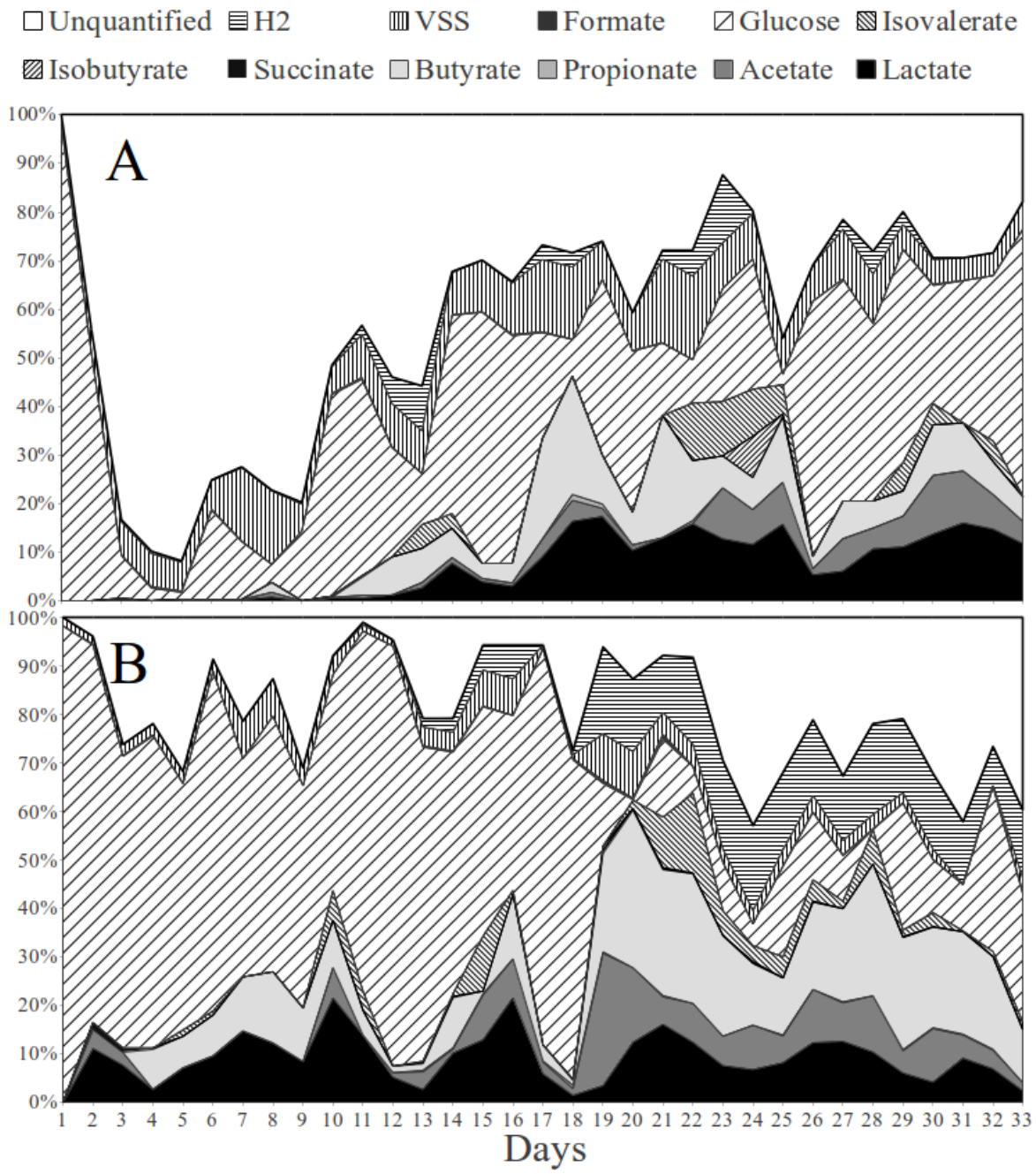

Figure 4.5 Equivalent electron balances of the metabolites and biomass as well as residual glucose quantified in the effluent of CSTR1 (A) and CSTR2 (B). The equivalent electrons of H_2 produced were included in balance.

The presence of isovalerate (valerate was not found in continuous experiments) seems to be related to periods of increased glucose consumption, which indicate increased activity of *Megasphaera*, possibly due to increased competition for the substrate, although there were periods in both reactors in which isovalerate was present with a high percentage of residual glucose, as shown in Figure 4.5. It was observed a high proportion of unquantified electrons in CSTR1, unlike CSTR2. In both cases, glucose consumed was not the largest source of electrons quantified in effluent, but a large proportion could not been adjudicated in CSTR1. Possibilities to explain this is are not targeted metabolites (as compounds of the Krebs cycle others than succinate), small structural molecules as peptides or aminoacids. Even an unobserved accumulation of extra polymeric substances at the interior of reactors could explain it.

4.3.3 Bacterial community analysis

In order to relate the performance of both reactors and the profile of metabolites with the identity of the strains used as inoculum, biomass samples were taken during periods of the points of highest and lowest hydrogen production in order to be analyzed by T-RFLP (Figure 4.6). In addition to the samples retrieved from the reactor operation, a sample of the biomass growing in the influent line (tubbing) was included in the analysis of relative abundance, that sample was considered to be representative for both reactors of the incoming biomass present in the non-sterilized media to the system, and a sample of the biofilm that grew in the wall of CSTR2 during the second half of its operation.

It was expected to see how the four pure strains that composed the inoculum would had been forming a consortium, developing population dynamics in which they would obtain advantages by the inoculum size over the incoming bacteria to the system in a non-sterile environment. However, T-RFLPs profiles shown that within the first 24 h the four initial strains of the inoculum were reduced to 12%, of which only *Lactobacillus* and *Rahnella* were detected in CSTR1. At the 5th day, *Clostridiu*m began to increase in relative abundance, with a maximum in the 9th day. The four strains were present in enough abundance since the 5th day, with a 95% as maximum in abundance in the 14th day, although just *Lactobacillus* was present in the samples. In CSTR2, *Clostridium* and *Rahnella* counted as much as 55%, but *Megasphaera* was not detected after 24 h from

inoculation, but at the following days the strains did not exceed 10% and they were not always detected. Interestingly, it was during the second half of CSTR2 operation in which the hydrogen performance was obtained, but the abundance of Clostridia stayed below 10%, with *Rahnella* about 10%. Even when it was not inoculated, a peak with the same nucleotide length as the correspondent for the *Lactobacillus* strain was found in CSTR2 on day 17.

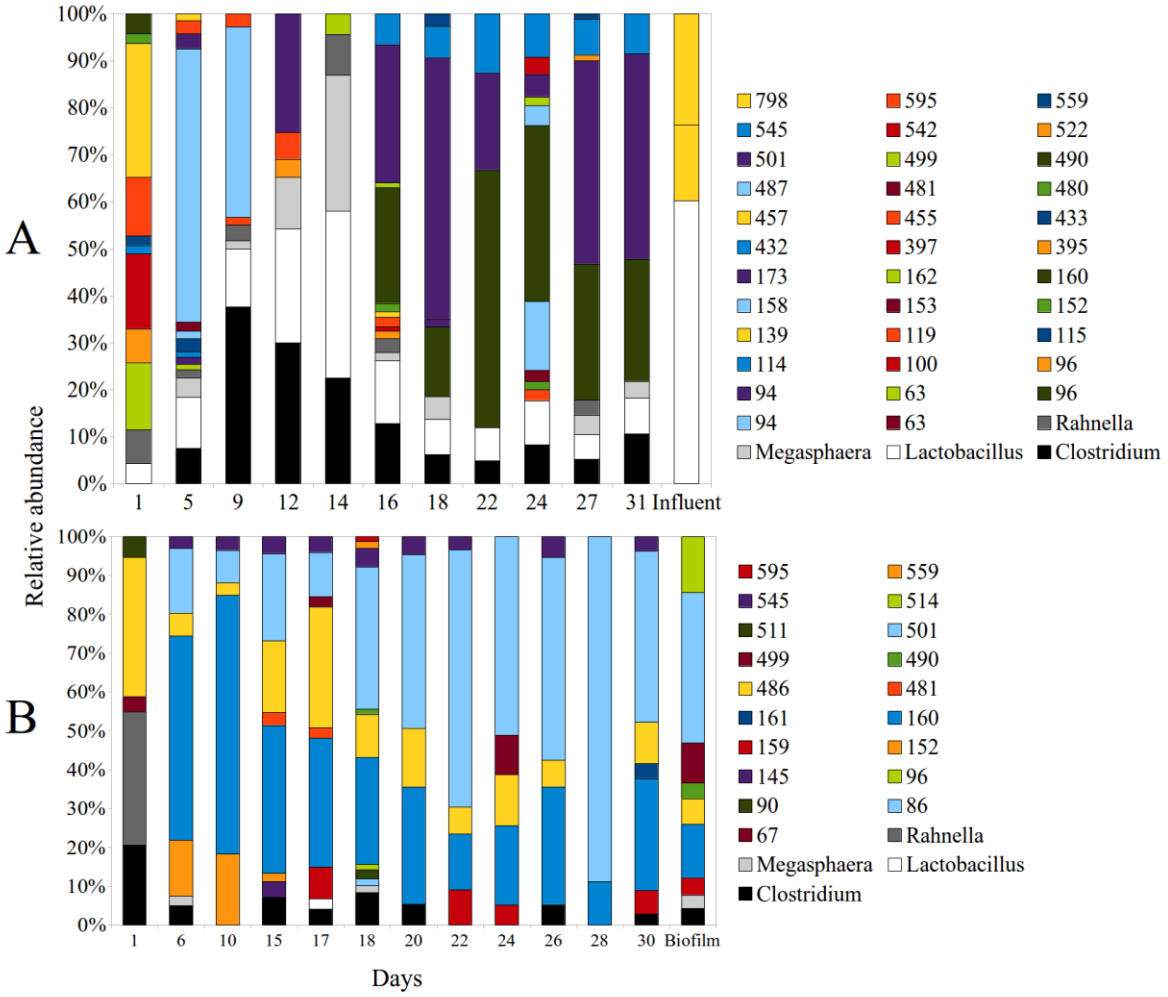

Figure 4.6. Relative abundance of T-RFLP profiles during tests on CSTR. "Influent" denotes the sample obtained inside the feeding pipe, considered representative of the incoming biomass to the system. "Biofilm" represents the sample obtained from the biofilm developed in the CSTR2 wall during the second half of operation.

The sample from influent showed that a peak with similar length that the peak from *Lactobacillus* was accessing to the system by the feed tubing, or at least a related strain with number of base pairs after enzymatic digestion. Although the identity of a good

percentage of relative abundances in samples remained unknown, it is possible to infer their participation in the performance of each reactor. For example, on day 22 in CSTR1, the higher production rate and hydrogen yield was obtained, but as shown in the corresponding T-RFLP profile, this does not appear to be due to Clostridia. Unidentified hydrogenogenic microorganisms may have contributed with most of the hydrogen produced. Similarly in CSTR2, days with low hydrogen production showed low abundance in the initial three strains.

While in CSTR1, the presence of *Lactobacillus* could explain the delay in the production of hydrogen, in CSTR2 the prevalence of Clostridia even in periods of no production is contradictory. Beckers et al. [21] reported that during the co-culture of *C. butyricum* and *Citrobacter freundii*, analysis of metabolites during fermentation indicated that *C. freundii* grew faster than *C. butyricum* at the beginning, but at the end dominated *C. butyricum*, resulting a decrease in the final production of hydrogen. During the initial stages, *C. freundii* consumed most of glucose causing inhibition of *C. butyricum* and then, due to the absence of suitable conditions, sporulation of *C. butyricum* started. The possible presence of Clostridia in form of spores and not as active cells in both reactors could explain their presence at the time of DNA extraction, although the hydraulic retention time used and levels of not consumed glucose do not support this theory. However, it is clear that the initial presence of *Lactobacillus* in the inoculum has a negative effect on production and yield of hydrogen in CSTR1, and seems to be its characteristic behavior in continuous mode.

Although the profile of metabolites in CSTR2 shows activity of *Megasphaera*, this strain does not show appreciable abundances by the method of T-RFLP, although this does not mean its absence in the system. In order to confirm the results obtained by T-RFLP and correlate performances and community structure, DGGEs with the same DNA samples were performed. Profiles of relative abundance and the sequence of DNA of the predominant bands were obtained (Figure 4.7). Contrary to what was obtained with T-RFLPs, with DGGE it was possible to observe the four strains since the beginning, but subsequent samples did not shown the presence of *Clostridium*.

The sequence of the DNA of three bands retrieved from samples taken from CSTR1 were obtained, the comparison with the database (NCBI) showed that these sequences were closely related with sequences from the *Clostridium* genus (Table 4.2). Like the pure strain used as reference to construct the inoculum, two sequences of these bands were related to the sequence from *C. tyrobutyricum*, while the other sequence was related to the sequence of *C. acetobutylicum*. The latter is well known for being good hydrogen-producing bacteria. In the case of CSTR2, four bands were identified, the sequence of three of them were related to sequences from different Clostridia (*C. tyrobutyricum, C. acetoutylicum* and *C. beijerinckii*) and one sequence was related with the sequence from *Sporolactobacillus*, in turn know for being a bacteriocin producer, that can hinder hydrogen production [5]. The presence of high hydrogen producers and competitors, as well as the dynamics involved in the community explain the differences in performance between reactors.

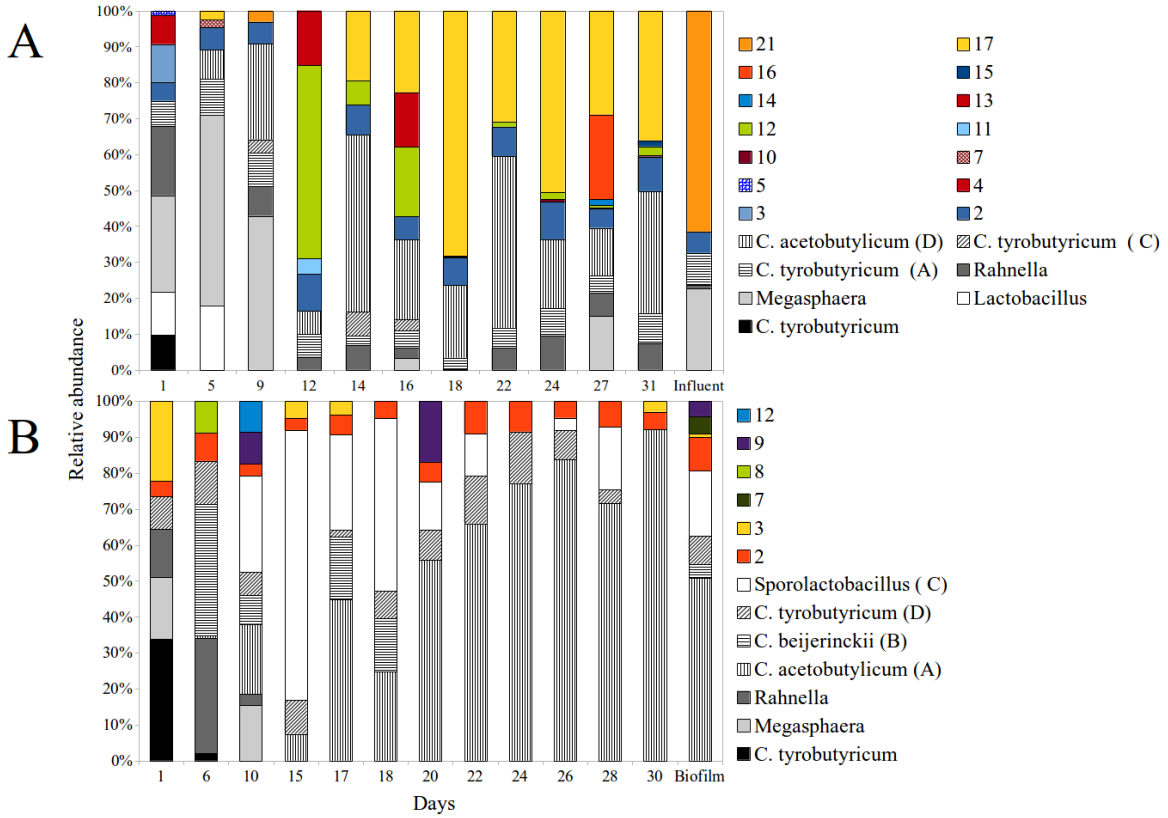

Figure 4.7. Relative abundances obtained from both reactors using the positions and relative brightness from each band in respective DGGE profile. Bands that were successfully identified are shown with the capital letter assigned in their respective gel. A) CSTR 1. B) CSTR 2.

The establishment of a bacterial community different to that one inoculated, implies the

competition for resources and subsequent displacement of the original population. But even in a fast growing frame in which bacteria takes place, changes observed after 24 h seems to be a short period of time. Kinnunen et al. [22] establishes the idea that a resident community, particularly when is subjected to an "invader" (a foreign individual or group), from an ecological perspective, nothing distinguishes the community member from the invader because none have had a prior common existence. However, in this particular case the displacement of the original inoculum seems to be the microbiome previously existing in our lab. Perhaps a higher amount of inoculum, relative to working volume could have prevented or delayed the establishment of a new structure in the community. Despite of this, the presence or absence of *Lactobacillus,* in both batch and continuous modes, seem to have an effect in the community. While in batch mode appears to play a stabilizer role (lower standard deviation, Figure 4.2), in continuous mode could create conditions in which other non-hydrogen producers competitors would thrive. The dispersal rate, defined as the number of entering cells per unit of time, depends in a greater extend on the environment [22].

Table 4.2. Comparison of the sequences retrieved from the DGGE bands and sequences from database using the BLAST tool from NCBI.

Band no. [1]	Sequence lenght	Phylum	Closest match	Match accesión number	Indentity (%)
1-A	471	Firmicutes	*Clostridium tyrobutyrivum*	CP016280	93
1-C	346	Firmicutes	*Clostridium tyrobutyrivum*	CP014170	81
1-D	563	Firmicutes	*Clostridium acetobutylicum*	KJ951058	94
1-F	564	Firmicutes	*Clostridium tyrobutyrivum*	KP754673	98
2-A	565	Firmicutes	*Clostridium acetobutylicum*	KJ951058	99
2-B	570	Firmicutes	*Clostridium beijerinckii*	KX378860	99
2-C	591	Firmicutes	*Sporolactobacillus laevolatus*	LC064803	91

[1]Indicates the position of band in gel. In first column, number (1) denotes bands identified for CSTR1, and (2) denotes bands identified for CSTR2.

Actually, much of the current microbial communities in laboratories with anaerobic reactors have been selected by subsequent experiments with similar hydraulic retention times. The low richness and diversity of the inoculum may have eased their displacement.

In Table 4.3 the data obtained by Elsharnouby et al. [9] are shown, in relation to reports in the literature using mono and co-cultures in hydrogen production. As mentioned above, most of the work reported focused on microbial mix that maximizes yields and/or production rates, while in this work the focus was to emulate a simplified community association, such as those operations that have been found using complex inoculum. Despite this, the production rate achieved is close to several works reported, although the molar yield is within the lowest ones.

Table 4.3. Operational and hydrogen production performance parameters, reports with pure strains. ND - Not determined. Obtained and modified from Elsharnouby et al., (2013).

Strain	Reactor	T (°C)	Substrate	Substrate concentration (g/L)	pH	HY (mol H_2 /mol hexose)	VHPR (L H_2 /L-d)
Clostridium beijerinckii RZF-1108	Batch	35	Glucose	9	7	1.97	0.5
	Batch		Glucose	10	6.5	1.96	2.54
Clostridium butyricum CGS5	Batch	37	Sucrose	17.8	5.5	1.39	3.9
C. butyricum / E. Coli	Batch	37	Glucose	3	6.5	1.65	0.52
Klebsiella pneumoniae ECU-15	Batch	37	Glucose	10	6	2.07	10.08
Klebsiella pneumoniae DSM2026	Batch	37	Glycerol	20	6.5	0.53	12.2
Clostridium tyrobutyricum FYa102	Batch	35	Glucose	3	7.2	1.47	1.6
	CSTR		Glucose	12	6	1.06	10.3
Clostridium acetobutyricum M121	Batch	37	Glucose	3	7.2	1.8	1.42

Escherichia coli S6	Batch	30	Glucose	5	6.8	0.49	0.34
Escherichia coli WDHL	Batch	37	Glucose	15	6	0.3	0.45
	Batch		Glucose			1.12	0.32
	Batch		Lactose			1.02	0.37
	Batch		Glucose + Galactose	7.5, 7.5		1.02	0.59
Clostridium tyrobutyricum + Megasphaera cerevisie + Rahnella aqualitis	CSTR	30	Glucose	12.5	5.5	0.35 (this work)	3.5 (this work)

4.4. Conclusions

In this work it was possible to demonstrate the effect of substrate competition and differences in growth rates on the hydrogen production using constructed consortiums composed by strains with different fermentative metabolic pathways. In batch, *Clostridium* was the leading producer of hydrogen in dark fermentation processes, its performance was linked to the reciprocal interaction with other microorganisms. The presence of *Megasphaera*, or the functional group that represents, have a positive effect on the production of hydrogen by decreasing the amount of accumulated organic acids when carbohydrate concentration is low. On the other hand, the function of *Lactobacillus* in batch and continuous assays seems to be contradictory. In batch may have been acting like a stabilizer in the interactions between strains, but seems to play a pivot role in the establishment of competitors in continuous mode. The consortium inoculated in the continuous reactors did not dominate the reactor. The selection of bacteria was not affected by the inoculum and the same operational conditions lead a stable or unstable performance.

4.5 References

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Chapter 5

Perspectives, conclusions and final remarks

5.1 General discussion

Biohydrogen is a promising way to recover and obtain energy from biomass that is considered waste. This is possible thanks to specialized bacteria under anaerobic conditions. Methanogenic sludge is recognized as an excellent source of hydrogenogenic bacteria, since their presence is essential for methane production [1]. However, it is necessary to take measures to remove any methanogenic activity of biomass [1–4]. In this work, the effect of inoculum pretreatments on the microbial community, and hence in their physiological performance in hydrogen production were evaluated. Although it is a simple sugar, glucose was used instead of a complex substrate, in order to avoid complications with measurements, results and their interpretation.

In Chapter 2, two inoculum pretreatments were assayed using EGSB reactors: heat shock and cell wash-out. Maximum molar yield (0.92 mol H_2/mol hexose) and volumetric hydrogen production rate (4.23 L H_2/L-d) were obtained with organic loading rates of 36 g hexose/L-d at HRT of 10 h with cell wash-out pretreated sludge, with *Enterobacteriaceae* family members as the mean components of the bacterial community followed by *Clostridium* (Table 2.3 and Figure 2.4) [5]. Using AFB reactors in Chapter 3, Cell wash-out treatment produced maximum hydrogen volumetric production rates and yields than thermal treatment (7 L H_2/L-d, 3.5 mol H_2/mol hexose, respectively) at 60 g glucose/L-d at HRT of 6 h, but in this case with *Clostridium* as the mean producer (Table 3.1 and Figure 3.5).

Figures 2.1 and 3.1 show the trend in hydrogen production in EGSB and AFB reactors, respectively. In EGSB reactors the hydrogen production gradually increase in the reactor inoculated with heat-shock pretreated sludge (E1), until stage IV (60 g hexose/L-d and HRT of 10 h), with a subsequent decrease in production and further rise in the last operational condition (Figure 2.1). Similar trends were obtained with reactor E2 (wash-out

pretreated inoculum), although with higher values. The relative abundance of the members forming the communities of the EGSB reactors (Figure 2.4) showed that their performances were correlated with the dynamics of the bacterial community, since hydrogen-producers mainly *Clostridium*, followed by *Enterobacter, Enterococcus, Klebsiella, Bacillus* and *Proteus* as well as *Enterobacteriaceae* family members shared similar abundances.

Something slightly different occurred with AFB reactors, whose hydrogen production fallen immediately after the first operative condition (stage I, Table 3.1) to increase back from stage IV until stage VI (60 g hexose/L-d and HRT of 6 h) and down again until stage IX. Similarly, both heat-shock and wash-out pretreatments (A1 and A2, respectively) showed the same trend, but with higher values in the case of reactor A2. The analyses of the relative abundances (Figure 3.5) showed that *Clostridium* was present during the whole experiment in both reactors, but with less presence in reactor A1 than reactor A2. Lactic acid bacteria (only *Lactobacillus, Sporolactobacillus* and *Lactococcus* were identified) had higher abundance in reactor A1, mainly as planktonic biomass, while it remained without major changes between planktonic and biofilm biomass in reactor A2. The lower hydrogen production of reactor A1 can then be explained by lactic acid bacteria, but unlike the data obtained in EGSB reactors, a considerable proportion of bacteria could not be identified, which could clarify the performance of both reactors.

In this case, the possibility of evaluating a starvation period by cutting off the feeding supply allowed to observe the resilience of the system, reaching performances similar to those obtained previously with the same operational conditions (Table 3.1 and Figure 3.1, stages VI, VII, VI' and VII'). The effect of the starvation period, both AFB reactors came closer to similar values of richness and diversity, and conceivably similar abundances in ecological functional groups, judging by the relative abundances of the bands obtained by DGGE and the similar performance of both reactors (Table 3.4 and Figures 3.1 and 3.5), which is highly possible due to the shared history of both inoculum. In fact, repeated stages (VI' and VII') produced slightly more hydrogen than the previous (stages VI and VII, Table 3.1 and Figure 3.1), and the increased abundance of *Clostridium*, mainly in biofilm samples and specially in reactor A2, due to the selective pressure triggered by this period, in which the ability to sporulate could be advantageous.

Both EGSB and AFB reactors were inoculated with pretreated biomass from the same

granular methanogenic sludge. Figure 3.5 shows the relative abundance of this original sludge denoted as zero sample (0) and the relative abundances resulting from each of the pretreatments (A1-0 for heat-shock and A2-0 for wash-out). In principle, the pretreated inoculum for both EGSB and AFB reactors should have the same composition since they come from the same sludge and equal methods were applied. Comparing heat-shock pretreated inoculum for both reactors (zero for E1 in Figure 2.4 and A1-0 in Figure 3.5) similarities are clear. However, it was not the same with wash-out pretreatment, since in EGSB this inoculum was obtained in a CSTR using glucose as substrate, while for AFB the substrate was cheese whey. At first sight, the use of a complex substrate could enrich the inoculum with *Clostridium*, but at the same time it had to select more strongly to lactic acid bacteria, what did not happen. The bacterial load that already included the methanogenic sludge plus the time occupied by the microbial community during the cell wash-out process allowed to establish interaction dynamics that should consolidate it as a consortium established through greater diversity in its structure. And this approach is equally valid both for EGSB and for AFB reactors wash-out pretreatment inoculum regardless of the substrate used.

These results show the convenience of using cell wash-out as a pretreatment to obtain a conditioned hydrogenogenic microbial community for better reactor performance, since hydrogen-producers were suitably adapted to competitors, but at the same time the difference in the biomass capacity that was able to develop in each reactor configuration. It is clear that formation of granules or biofilms substantially enhanced biomass retention, and reactor volumetric hydrogen production rate was highly related to the biomass retention. Although the literature indicates that biofilms-based reactors gives lower work volume due to the space occupied by the support compared with a granulation-based system, and therefore tends to deliver lower performance in the production of hydrogen than that obtained by granulated biomass [6–11], we obtained better results using AFB than EGSB reactors. However, the rapid hydrogen-producing culture growth and higher OLR conditions limit the application of biofilm anaerobic biohydrogen processes since excessive production of fermentative biomass would result in wash-out of support carriers in a fluidized bed reactor or system upset in a fixed-bed reactor [6]. Nevertheless, granulated biomass may not be suitable for operation under HRTs shorter than 4 h as was used with

AFB reactors, because granules may disaggregate due to higher hydrodynamic forces, and also is not suitable for substrate containing high solid content [12]. In Chapter 3, we still were able to operate the AFB reactor at HRT of 1 h, during which biomass was washed-out. The role and relationships between different ecological functional groups was studied in Chapter 4, using mono- and co-cultures of representative strains isolated from compost. This allowed observing not only the mono- and co-culture kinetic parameters, but also the profiles of the produced metabolites in various stages of growth, which was a tool to understand the physiology of the system. Although *Clostridium* is known for it excellent performance in hydrogen production, *Megasphaera* was able to produce more hydrogen, but requiring considerably more time and interestingly, after glucose was consumed, using the fatty acids as a substrate. Although in all the experiments the same culture medium proposed by Davila-Vazquez was used [13], and given the fact that lactic acid bacteria was an important component in the samples of the developed communities in the reactors presented in Chapters 2 and 3, *Lactobacillus* as a pure strain was unable to grow using this same medium (Figure 4.1). Many lactic acid bacteria seem to be unable to produce some cofactors that are needed for growth [14,15], therefore it is necessary to provide them in the culture medium. In complex communities, this is satisfied by syntrophic relationships, such as those established in reactors. The only feasible explanation to the observed growth of *Lactobacillus* in the co-cultures is that the other strains produced those cofactors and made them available in the culture medium for their use. It is unclear when the presence of *Lactobacillus* is noxious and when it is innocuous to the performance in hydrogen production, since it is possible to find it appreciably both in high and low hydrogen production conditions [15,16].

Although T-RFLP delivers data with higher quality data, DGGE allowed to correlate population dynamics with performance data, as well as to identify some members of the bacterial community. The interpretation of microbial ecology and evolution via 16S rRNA sequences has been complicated in recent years by the fact that many bacteria harbor multiple, heterogeneous rRNA operons [17,18]. Bacterial genomes can contain between 1 and 15 of such operons and that 16S rRNA sequences can differ up to several percent between operons, which creates a significant problem for culture-independent analysis of microbial communities since it can lead to a severe overestimation of microbial diversity

based on 16S rRNA approaches [17]. This could be avoided or restricted using another molecular markers together with 16S rRNA [19–22].

5.2 Conclusions and final remarks

The results obtained in this thesis showed that the pretreatment of the inoculum chosen for the inhibition of the methanogenic activity in the microbial community has an impact on the performance of the microbial community for the production of hydrogen. Cell wash-out pretreatment is capable of generating a more competent hydrogenogenic microbial community than that obtained by heat-shock pretreatment. Although the reactor configuration also has a direct and immediate effect in the hydrogen performance, it is the structure and composition of the microbiota that makes up the biomass which determines the potential for hydrogen production under suitable conditions, as it is shown by the results presented in Chapter 2 and Chapter 3. However, the reactor configuration and how the biomass is immobilized seemed to determine the operating conditions in which better productions can be obtained.

The starvation period improved the production of hydrogen in both reactors by repeating the previous operating conditions, therefore an in-depth study of the hydrogenogeic communities could lead to its use as a way to recover the low productions in conjunction with other methods.

The long-term effects of the incoming biomass from the feeding system were suggested in Chapter 2 and observed in Chapter 3, although the operational conditions employed could allow the settlement of external biomass. While lactic acid bacteria were found to occupy an important part of the relative abundance in the communities sampled, did not appear to have a dominant effect on the reactor performance, and this seems to have a correlation with the simplicity of the culture medium [16]. The presence of lactic acid bacteria could be traced directly to the original methanogenic inoculum, meanly by the characteristics of the treated wastewater in the plant from which was obtained, as discussed in Chapter 3, and the incoming biomass to the system as a consequence of a non-sterile feeding. However, a noxious effect of *Lactobacillus* on performance was observed in the CSTRs described in Chapter 4. Whilst in batch experiments *Lactobacillus* seems to have a stabilizer effect

(Figure 4.2), an increase in its relative abundances in CSTRs were correlated to diminished hydrogen production. It is clear that the simplicity and quantity of the strain mixture prepared as inoculum for CSTRs was not enough to confront the microbial load entering to the reactor from the non-sterile culture medium. This could be solved using a more complex mixture of isolated strains and/or greater amount of inoculum, however, there are still large gaps in the understanding and application of an ecological theory at the level of microbial communities [23–26]. More batch and continuous experiments under sterile conditions using co-cultures increasingly complex and exclusively composed of known strains and very well described will help to get a closer representation of these bacterial communities. New tools, methods and technologies, like technologies based on RNA as metagenomics and metatranscriptomics [20-22], together with statistical analytical tools [22] will help to identify and understand the role of ecological functional groups that allow manipulation of the hydrogen production process.

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