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Producción de hidrógeno en reactores de biomasa fija, implicaciones de microorganismos hidrogenotróficos

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Resumen

Producción de hidrógeno en reactores de biomasa fija, implicaciones de microorganismos hidrogenotrópicos

Palabras clave: hidrógeno, metano, UASB, lecho empacado, homoacetogenesis

El hidrógeno es considerado el combustible del futuro por su mayor contenido energético comparándolo con cualquier otro combustible. Su producción por vía biológica permite valorizar residuos o aguas residuales, como el suero de leche, pudiendo producirlo de manera renovable además de ser carbono neutral. En la producción fermentativa de hidrógeno, las bacterias anaerobias utilizan los compuestos orgánicos para producir energía metabólica y componentes estructurales, produciendo H₂ y una mezcla de ácidos grasos de cadena corta, así como algunos alcoholes. El uso de suero de leche como sustrato para la producción de hidrógeno ha sido estudiado en reactores de biomasa suspendida, obteniendo velocidades de producción muy promisorias. Con el objetivo de aumentar dicha producción, favorecida por una mayor concentración de biomasa, en el presente trabajo se exploró el uso de reactores de biomasa fija (UASB y lecho empacado). Una limitante de los sistemas granulares como los reactores UASB es el largo tiempo de arranque necesario para establecer una comunidad productora de hidrógeno. En este sentido se evaluaron diferentes estrategias de arranque y estructuras del inóculo. Una disminución gradual del tiempo de residencia hidráulica desde 24 a 6 horas, con una velocidad de carga orgánica (VCO) constante de 20 g DQO/L-d fue la estrategia que produjo una biomasa hidrogenogénica mas activa. La producción inesperada de metano a la par del hidrógeno llevó a la evaluación de diferentes estrategias para controlar la metanogénesis. Un aumento en la VCO de 20 a 30 g DQO/L-d incrementó hasta 172% la producción de hidrógeno, disminuyendo la producción de metano en 75%. La única estrategia que inhibió completamente la metanogénesis fue aplicar un segundo tratamiento térmico a la biomasa. Sin embargo, esta última estrategia también seleccionó a consumidores de hidrógeno, como los homoacetogénicos. El análisis de las comunidades en los reactores UASB con técnicas moleculares como PCR-DGGE y clonación mostraron una proliferación de organismos relacionados con los productores de hidrógeno Clostridium tyrobutyricum, Citrobacter freundii y Enterobacter aerogenes, así como los homoacetogénicos Blautia hydrogenotrophica, Oscillibacter valericigenes y Clostridium ljungdahlii. Mientras que los responsables de la producción de metano fueron hidrogenótrofos de los géneros Methanobrevibacter y Methanobacterium. Posteriormente se evaluó la producción de hidrógeno en reactores de lecho empacado, inoculados con la fermentación natural del suero de leche. Las velocidades de producción alcanzadas en los reactores de lecho empacado fueron similares a las alcanzadas en los UASB durante su arranque, demostrando la factibilidad de usar la fermentación natural del suero de leche como inóculo. La relevancia de los homoacetogenos se evaluó en ensayos en lote, probando que durante el tiempo de operación del reactor la actividad homoacetogénica fue desplazada por la producción de metano. En el presente trabajo se elucidó la relevancia del consumo de hidrógeno por metanogénesis y homoacetogénesis en reactores de biomasa fija, y se observó que la magnitud de dicho consumo fue similar para ambas actividades. Además se establecieron los parámetros operacionales adecuados para controlar la metanogénesis. Futuras investigaciones son necesarias para determinar los factores que controlan la homoacetogenesis. Este trabajo contribuye dando una aproximación a la utilización del suero de leche como fuente de energía renovable resolviendo un potencial problema ambiental.
Abstract

Hydrogen production in biomass fixed reactors, implications of hydrogenotrophic microorganisms

Keywords: hydrogen, methane, UASB, packed-bed, homoacetogenesis

Hydrogen is considered the fuel of the future due to the highest energy content compared with any fuel. The biological hydrogen production allows valorizing waste or wastewaters, like the cheese whey, being a renewable and carbon neutral fuel. In the fermentative hydrogen production, anaerobic bacteria use organic substrates to generate metabolic energy and structural components, producing $\text{H}_2$ and a mix of short chain fatty acids and alcohols. The cheese whey has been used largely to produce hydrogen in suspended biomass reactors, achieving promising rates. The present work explored the use of biomass fixed reactors (UASB and packed-bed) with the aim of increasing the production rate, favored by the higher biomass concentration. A limitation in granular systems, like the UASB is the long time required to develop a hydrogen producing community. In this sense, different start-up strategies and inoculum structures were evaluated. A gradual decrease of the hydraulic retention time from 24 to 6 hours, with a constant organic loading rate (OLR) of 20 g COD/L-d, produced the more active biomass. The unexpected methane production addressed the evaluation of operational strategies to control the methanogens. An increment of the OLR from 20 to 30 g COD/L-d increased the hydrogen production rate 172%, whereas the methane production was reduced 75%. The only strategy that completely inhibited the methane production was a second heat treatment to the biomass. However, the latter strategy also selected homoacetogens, which are hydrogen consuming organisms. The community analysis of the biomass withdrawn from UASB reactors with molecular techniques, such as PCR-DGGE and cloning, showed a proliferation of organisms related to hydrogen producers such as Clostridium tyrobutyricum, Citrobacter freundii and Enterobacter aerogenes, as well as the homoacetogens Blautia hydrogenotrophica, Oscillibacter valericigenes and Clostridium ljungdahlii. The archaeal community analysis showed that methane was produced by hydrogenotrophs from genera Methanobrevibacter and Methanobacterium. Afterwards, the hydrogen production in packed-bed reactors inoculated with natural fermented cheese whey, was evaluated. The hydrogen production rates achieved in these reactors were similar to those obtained in the UASB reactors, during the start-up, showing the suitability of using natural fermented cheese whey as inoculum. The relevance of homoacetogens was evaluated in batch assays, proving that during the reactor operation time the homoacetogenic activity was replaced by the methane production. The present work elucidated the relevance of the hydrogen consumption in biomass fixed reactors by methanogens or homoacetogens, observing that the magnitude of these activities are similar. Moreover, the adequate operational parameters to control methanogenesis were established. Further research is needed to determine elucidate the factors that control the homoacetogenesis. This work contributes to give an approach to the use the cheese whey as a renewable energy source, solving a potential environmental problem.
Chapter 1

Biological hydrogen production, process conditions

Summary

The fermentative hydrogen production is considered an alternative for conventional fuels as it is carbon neutral, has the highest energy content (122 kJ/g) and can be produced through an organic waste valorization. Among possible substrates cheese whey, a readily biodegradable by-product, is a potential substrate for hydrogen production. Several factors contribute to a stable hydrogen production in bioreactors, such as inoculum, pH, hydraulic retention time, substrate concentration, temperature, etc. Fixed biomass reactors offer some advantages over suspended biomass reactors, supporting higher organic loading rates and producing higher volumetric rates of hydrogen. Nevertheless, fixed biomass reactors may favor the presence of hydrogen consuming organisms that decrease the hydrogen yields achieved. In the present chapter, the main operational conditions controlling the fermentative hydrogen production are reviewed.
1.1 Introduction

Actual environmental challenges derived from the use of fossil fuels, such as greenhouse gases emission, acid rain and the future depletion of those fuels, drive a search of clean and renewable energy sources (Saxena et al., 2009). In this regard, the energy from biomass is considered a clean option, since it is carbon neutral and there is a wide accessibility of agricultural, wood and municipal wastes and wastewaters (Davila-Vazquez et al., 2008). Therefore, technologies for methane and hydrogen production from biological processes, as well as ethanol production by fermentation are important alternatives for the energy production from biomass (Davila-Vazquez et al., 2008).

Among the possible fuels from biomass, hydrogen (H₂) is considered the most promising due to its technical, economic and environmental benefits. H₂ has the highest energy content compared with the other existing fuels (122 kJ/g), can be used directly in a combustion internal engine or in a fuel cell to produce electricity. Moreover, unlike the conventional produced H₂ from fossil fuels, the biological produced H₂ is a clean and renewable fuel (Das and Veziroglu, 2008).

Biological hydrogen is produced through an anaerobic digestion process, also known as dark fermentation. By-products of this process are organic acids, which can be valuable metabolites for other energy producer process, such as methanogenesis. The use of organic wastes or wastewater to produce hydrogen have a double purpose, the generation of clean energy and waste valorization, saving the environmental costs related with the treatment or waste disposition (Das and Veziroglu, 2008; Redwood et al., 2009).

1.2 Substrates used for biohydrogen production

Different factors are considered to select a suitable substrate for the fermentative hydrogen production, such as the substrate cost, availability, carbohydrates content and biodegradability. Different studies have used model substrates like glucose, sucrose and starch due to their easy biodegradability and their presence in wastewaters (Das and Veziroglu, 2008; Davila-Vazquez et al., 2008).
Other substrates used have been effluents from the food and beverage industry, and agricultural wastes, showing the viability to use more complex organic compounds to produce hydrogen (Buitrón and Carvajal, 2010; Castello et al., 2009; Wang et al., 2006). The use of these wastes may require a pretreatment for the hydrolysis of complex substrates, to remove undesirable components and for nutritional balance (Kapdan and Kargi, 2006).

1.2.1 Dairy industry effluents

Among the dairy industry effluents, the most common by-product generated during the cheese manufacturing is the cheese whey. The estimated world production of whey is around 160 million tonnes per year (Guimaraes et al., 2010). Cheese whey has a high organic content (60-80 g COD/L) largely due to lactose; moreover it contains proteins, nutrients (N and P), some vitamins and inorganic salts. A valorization process of this effluent is usually a common alternative, but the cost associated is not sustainable for small and medium factories, for those factories the methane and hydrogen production may be a suitable alternative. Otherwise, the environmental release of this effluent can cause an excess of oxygen consumption, eutrophication and toxicity, among other detrimental effects (Prazeres et al., 2012).

Biological processes have been used for the treatment of cheese whey, generating valuable products, such as methane, ethanol, lactic acid, and hydrogen (Prazeres et al., 2012). The conventional anaerobic digestion of cheese whey has some drawbacks, such as the high content of carbohydrate that causes a rapid acidification inhibiting the methanogenic phase (Vidal et al., 2000), therefore alkalinity supplementation has been proposed (Prazeres et al., 2012). Another potential problem can be biomass flotation due to the fat content (Mockaititis et al., 2006).

The fermentative hydrogen production using cheese whey has gained attention as an alternative to overcome the drawbacks of anaerobic digestion. In this sense, successful hydrogen producing systems have been developed (Azbar et al., 2009; Davila-Vazquez et al., 2009).
1.3 Fermentative hydrogen production

During the fermentative hydrogen production, bacteria oxidize the organic wastes to obtain structural components and metabolic energy for their growth. This oxidation produces electrons that need to be “disposed” in order to keep the electric neutrality in the cell. Bacteria use protons (H\(^+\)) as final electron acceptor, being these reduced to molecular hydrogen (H\(_2\)). At first, electrons are transferred to internal electron carriers (NADH\(_2\) and ferredoxin), which have to be oxidized to allow continuous fermentation. Besides electrons disposal through proton reduction to hydrogen, certain amount of electrons are used in new biomass production and in other reduced compounds such as butyrate, ethanol, lactate, propionate, and others (Das and Veziroglu, 2008; Lee and Rittmann, 2009).

Therefore, the main objective of the fermentative hydrogen production is to direct the largest number of electrons to protons. If only acetate and hydrogen are produced from the oxidation of one mol of hexose, 4 mol of H\(_2\) and 2 mol of acetate would be obtained. In practice, the yield of a mesophilic acid fermentation is around 2 mol H\(_2\)/mol hexose (Hawkes et al., 2007; Lee and Rittmann, 2009). The latter yield is explained by the bacterial need to keep the energy and to balance the flux of electrons during the fermentation. The fermentative bacteria synthesize ATP trough glycolysis (2 mol ATP/mol of glucose) and from acetate and butyrate production (1 mol ATP/mol of acid). Regarding to energy conservation, the bacteria have to favor the acetate formation. However, its production from Acetyl-CoA does not involve oxidation-reduction. Therefore, reduced compounds or H\(_2\) have to be generated to dispose the electron produced during glycolysis and pyruvate decarboxylation (Hawkes et al., 2007; Lee and Rittmann, 2009). Hence, the final products of the fermentation are a mix of acetate/butyrate or acetate/ethanol, depending on the operational conditions and the microorganisms present (Guo et al., 2008; Yu and Mu, 2006).
1.4 Process conditions for the continuous hydrogen production

The capacity to produce hydrogen through oxidation of organic compounds has been applied in reactors to produce this fuel in a continuous way. The main goals are to increase both the yield of hydrogen obtained and the volumetric hydrogen production rate (VHPR) in order to evaluate their potential application in fuel cells (Das and Veziroglu, 2008; Davila-Vazquez et al., 2008). The efficiency of the applied reactors depends on different operational factors such as the source of the inoculum and pretreatment, pH, hydraulic retention time (HRT), organic loading rate (OLR), temperature, among others (Buitrón and Carvajal, 2010; Chen et al., 2009; Das and Veziroglu, 2008; Davila-Vazquez et al., 2009).

1.4.1 Inoculum

Hydrogen producing bacteria can be facultative or strict anaerobes, in the last years several studies have shown the major abundance of species from genus *Clostridium* in the inocula. Other reported genera are *Micrococi*, *Enterobacteria*, *Thermoanaerobacterium*, *Thermobacteroides*, *Ruminococcus*, *Anaerotruncus*, *Megasphaera*, *Pectinatus* (Castello et al., 2009; Davila-Vazquez et al., 2008; Ueno et al., 2001).

Even though some works have used pure cultures as inoculum (Collet et al., 2004; Fritsch et al., 2008; O-Thong et al., 2008), the use of mixed cultures is closer to a real application, less expensive and easier to operate and maintain (Lee et al., 2009). Several sources of mixed cultures inocula have been evaluated (soil, sediments, compost, anaerobic and aerobic sludge, etc.). Using anaerobic sludge as inoculum requires a pretreatment in order to eliminate methanogens. Common pretreatments are based on the sporulation capacity of some hydrogen producing bacteria, undergoing the sludge to harsh conditions like heat, acid or basic medium, and aeration, among others (Hu and Chen, 2007).

1.4.2 pH

A slightly acidic pH contributes to inhibit the methanogens growth, can increase the VHPR and enhance the stability of the hydrogen producing reactors. Different reviews report the
optimum pH between 4.5 and 6.5 (Das and Veziroglu, 2008; Davila-Vazquez et al., 2008; Hawkes et al., 2007). Besides the control of methanogenesis, the pH is determinant in the reactor performance affecting the metabolic pathway. For instance, Guo et al. (2008) found an acetate/ethanol fermentation at pH 4.2 – 4.4, whereas the VHPR decreased at pH 5, changing the metabolic pathway to acetate, butyrate and propionate.

1.4.3 Hydraulic retention time and substrate concentration

Both the hydraulic retention time (HRT) and substrate concentration determine the organic loading rate (OLR); the optimum HRT range is between 0.5 and 12 hours, whereas the substrate concentration is around 20 g/L (Davila-Vazquez et al., 2008). By increasing the OLR, raising the substrate concentration or diminishing the HRT, it is expected an increment in the hydrogen production rate, due to the increase in the organic availability, until certain limit is reached as is described below.

The increase in the substrate concentration is limited by undissociated by-products inhibition (acetic, butyric and propionic acid); Van Ginkel and Logan (2005) reported 19 mM of undissociated acids as the threshold concentration for a significant H2 yield reduction and a switch to solventogenesis, which is the solvents production, such as acetone, butanol and ethanol. In the same sense, Castro-Villalobos et al. (2012) reported a complete biomass growth inhibition at 30 mM of undissociated acids.

The HRT reduction to increase the OLR is limited by the maximum specific growth rate ($\mu_{max}$), in order to avoid the biomass wash-out. This growth rate is specific for each culture, an anaerobic sludge inoculum had a $\mu_{max}$ of 0.172 h$^{-1}$, corresponding to a minimal HRT of 5.9 h (Chen et al., 2001). Due to the uncoupled solids retention time to the HRT in biomass fixed systems, lower HRT can be applied. For instance, Gavala et al. (2006) compared an UASB (up-flow anaerobic sludge blanket) reactor and a CSTR (completely stirred tank reactor) for hydrogen production, finding that the optimal HRTs were 2 and 6 h, respectively.
1.4.4 Temperature

Temperature is an operational parameter affecting the bacterial growth rate and the metabolic activity. Fermentative hydrogen production can be developed at mesophilic, thermophilic and hyperthermophilic conditions (Davila-Vazquez et al., 2008). Comparing mesophilic and thermophilic conditions (35 and 55°C) in two CSTRs, Gavala et al. (2006) found that the thermophilic reactor had higher hydrogen yield and specific hydrogen production. These results are explained by the lower biomass generation due to higher maintenance energy demands at 55 °C. Higher \( H_2 \) productions at thermophilic conditions are attributed to thermodynamic considerations; another advantage is pathogens and methanogens removal, however high temperatures contribute to proteins denaturalization and increases the energy cost (Davila-Vazquez et al., 2008).

1.5 Fixed biomass reactors

The reactor configuration influences \( H_2 \) production, largely related to the biomass concentration. The biomass suspended CSTR is the most reported process in \( H_2 \) production. Some advantages of this configuration are the good mass transfer and diffusion due to the flocs size and porosity. One of the highest hydrogen production rates in a CSTR, 1.39 L/L-h, was achieved by Davila-Vazquez et al. (2009), applying an OLR of 138.6 g lactose/L-d with a volatile solids concentration of 5 g/L. Nevertheless, the CSTR has the disadvantage of the potential biomass wash-out at low HRT, as was discussed in section 1.4.3 (Hawkes et al., 2007).

The fixed biomass reactors are used in a lesser extent for hydrogen production; these reactors can retain high biomass concentrations due to granule sedimentation (7-9 g volatile solids/L) or to the biomass attached in a support (20 g volatile solids/L) (Castello et al., 2009; Guo et al., 2008; Zhang et al., 2007). Therefore, higher OLR can be applied, at lower HRT than in CSTR without the problem of biomass wash-out (Hawkes et al., 2007). For instance, Zhang et al. (2007) applied an OLR of 500 g/L-d, at a HRT of 0.5 h in an anaerobic fluidized bed reactor, obtaining a hydrogen production rate of 2.36 L/L-h. Lee et
al. (2003) applied an OLR of 961 g/L-d at the same HRT obtaining a hydrogen production rate of 7.4 L/L-h, in a packed-bed reactor.

Other advantages of fixed biomass reactors are the resistance to inhibitory compounds, more stability compared to suspended growth systems and the possibility to obtain an effluent with a low solids concentration (Gavala et al., 2006). A significant example of reactor stability is the work of Yu and Mu (2006), whom operated an UASB reactor during 3 years proving different substrate concentrations and HRT with stable hydrogen productions, reaching the higher yield (1.44 mol H₂/mol glucose) at 7.1 g COD/L-d.

However, in fixed biomass reactors the solid retention time cannot be controlled; therefore, slow-growing hydrogen consuming microorganisms such as methanogens cannot be easily washed-out.

1.6 Hydrogen consuming microorganisms

Besides the metabolic pathways that generate reduced compounds instead of hydrogen, another cause of low hydrogen yields is the presence of microorganisms capable to consume H₂. Typical hydrogen consumers in fermentative systems are methanogens and homoacetogens (Calli et al., 2008; Castello et al., 2009). The methanogenic relevance in hydrogenic systems is clear due to the stoichiometric relation of 4 mol of H₂ consumed per mol of CH₄ produced in hydrogenotrophic methanogenesis. However, there are contradictory results that question the relevance of hydrogen consumption by homoacetogenesis. In this sense, whereas Kramer and Bagley (2008) established that homoacetogenic activity was of minor concern, Calli et al. (2008) attributed the instability of fermentative hydrogen producing systems to homoacetogenic activity.

A condition that favors the establishment of hydrogen consuming microorganisms is a solids retention time longer than the duplication time of hydrogen consumers (Dinamarca and Bakke, 2009); as occurs in biomass fixed reactors. In this regard, Koskinen et al. (2007) suggested that the instability in a fluidized-bed reactor was due to the enrichment and efficient adhesion of H₂ consumers in the carrier. Moreover, methanogenic occurrence is a concern in reactors inoculated with anaerobic sludge, even though it was pretreated or

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not (Castello et al., 2009; Wang et al., 2007). Therefore, the presence of hydrogen consuming microorganisms in fixed biomass reactors is a major concern for the appropriate operation of hydrogenogenic reactors.

1.7 Scope and structure of the thesis

Fermentative hydrogen production using cheese whey has gained attention in the last years. Most of the studies have been developed in CSTR (Azbar et al., 2009; Davila-Vazquez et al., 2009; Venetsaneas et al., 2009; Yang et al., 2007), and in lesser extent in biomass fixed reactors such as UASB (Castello et al., 2009); where only Davila-Vazquez et al. (2009) achieved significant hydrogen production at 138 g lactose/L-d. Therefore, the aim of this thesis was to evaluate the fermentative hydrogen production in fixed biomass reactors, using cheese whey as substrate. Due to the high solids retention time in these reactors, it was evaluated the relevance of hydrogen consumption by methanogens and homoacetogens, assessing strategies to eliminate the methanogenic activity.

In Chapter 2, the start-up strategies to enhance the hydrogen production in two UASB reactors were evaluated. Different operational parameters and inoculum structures (granules and disaggregated granules) were assayed and the bacterial community was evaluated using PCR-DGGE.

Once methane presence was detected in UASB reactors, different operational strategies were tested to decrease the methane production as shown in Chapter 3. In Chapter 4 the changes in the microbial communities developed during the application of decreasing methane production strategies were determined using molecular biology techniques.

In Chapter 5 hydrogen production in two anaerobic packed bed reactors was evaluated using as inoculum a natural fermented cheese whey powder solution; the implication of hydrogen consuming microorganisms was also evaluated using batch assays and the microbial communities were studied by PCR-DGGE. The global results obtained in this thesis are discussed in Chapter 6, accompanied by final recommendations.
1.8 References


Chapter 2

Different start-up strategies to enhance biohydrogen production from cheese whey in UASB reactors

Summary

The effect of different operational strategies and inoculum structure (granules and disaggregated granules) during the start-up of four up-flow anaerobic sludge bed hydrogenogenic reactors was investigated. The more stable hydrogen production rates (VHPR) obtained were 0.38 and 0.36 L H$_2$/L-d, in reactors operated with a constant organic loading (OLR) rate with both inoculum structures, whereas in reactors operated with an increasing OLR methane started to be produced earliest in time. Specific hydrogenogenic activity results proved that the disaggregated inoculum produced a more active biomass than the granular one, but not granule formation was evident. The methane hydrogenotrophic activity was the main limitation of the systems evaluated. In the reactors inoculated with disaggregated sludge the start-up strategy did not influence the bacterial DGGE fingerprint, in contrast to the reactors started-up with granular sludge; members of the *Clostridium* genus were always present. The results demonstrated that operational conditions during the start-up period are crucial for the production of hydrogenogenic biomass.
2.1 Introduction

Present concerns about limited fossil fuel reserves and climate change drive a search for renewable fuels. Hydrogen (H\textsubscript{2}) is considered a sustainable alternative for fossil fuels, due to its highest energy content compared to other fuels and that it can be combusted or used directly in fuel cells to produce electricity and only water as by-product. Indeed, renewable energy sources e.g. biomass or wastewater can be utilized for hydrogen generation through a fermentative process using microorganisms, making this process promising for energy production (Davila-Vazquez et al., 2008b; Hallenbeck, 2009); due to the biological origin, the term biohydrogen is used. Dairy industry effluents are a potential substrate for biohydrogen production, due to their high biodegradable carbohydrate content (Kapdan and Kargi, 2006). Around the world, the cheese industry produces over 160 million tonnes of cheese whey per year (Guimaraes et al., 2010), a by-product that contains around 70% of lactose (on a dry basis), and has been used successfully for biohydrogen production (Castello et al., 2009; Davila-Vazquez et al., 2009).

Recent research approaches are aimed to develop continuous biohydrogen producing systems, using different reactor technologies, being the continuous stirred tank reactor (CSTR) the most frequently reported (Guo et al., 2008; Hawkes et al., 2007). Although the stirring condition can improve the mass transfer efficiency, the CSTR has the potential problem of cell washout due to the suspended nature of the biomass within the reactor (Guo et al., 2008).

The critical parameter related to the increase of biohydrogen productivity is the organic loading rate (OLR). Several reports show that a high OLR may increase the hydrogen production rate; for instance, Guo et al. (2008) found than an increase from 12 to 120 kg COD/m\textsuperscript{3}-d in the OLR produced a gain of about 7 times in the hydrogen production rate, reaching a value of 0.71 L/L-h. Biological reactors can withstand high OLR as long as they can retain high biomass concentrations like those using granular sludge (Chang and Lin, 2004). Such systems, like the up-flow anaerobic sludge blanket (UASB) reactors, have been used for biohydrogen production (Mu and Yu, 2006; Zhao et al., 2008). However the main constrain of using an UASB reactor for hydrogen production is to obtain granular sludge with a high biohydrogen production rate. Although the formation and stabilization
of hydrogen producing granules takes a long period of time, usually several months (Chang and Lin, 2004; Mu and Yu, 2006), some successful strategies have been reported such as acid incubation of a previous enriched hydrogenogenic biomass in a CSTR (Zhang et al., 2007) and the use of sterilized granules as biomass support at hyper-thermophilic conditions (Kotsopoulos et al., 2006). The drawbacks of these procedures are the time required to create hydrogenogenic granules in a CSTR and the energy requirements for keeping the temperature at 70°C, respectively.

Alternatively, it is proposed the use of strategies based on controlling either the hydraulic retention time (HRT) or the OLR during the reactor start-up to promote the selection of hydrogenogenic microorganisms within the granule; for example, gradual OLR increases can promote a slow growth of microorganisms creating a dense granule, and low HRT can wash out microorganisms with low adhesion capabilities. These strategies were successfully proven to overcome the long start-up period for the development of methanogenic granules and biofilms (Cresson et al., 2008; Liu et al., 2002). Nonetheless, according to our knowledge, similar strategies have not been applied for the development of hydrogenogenic biomass.

The source of the inoculum is another important factor in the performance of the fermentation system, determining the hydrogen yield, the microbial community structure and the metabolic pathway (Akutsu et al., 2008; Baghchehsarae et al., 2010). Moreover, the structure of the inoculum can play a key role in the formation of hydrogenogenic granules. In this regard, anaerobic granular sludge has been used like support for the development of hydrogen producing granules (Kotsopoulos et al., 2006), but the use of disaggregated granules as inoculum has not been studied to establish a hydrogenogenic community.

Therefore, in this work different strategies (changing operational parameters) and different inoculum structure (granules and disaggregated granules) were studied to evaluate their effect on the reactor start-up period and the volumetric hydrogen production rate (VHPR) in UASB reactors using cheese whey powder (CWP) solution as a synthetic dairy wastewater. The performance of the reactors was evaluated and specific hydrogenogenic activity (SHA) batch assays were conducted regularly in order to characterize the
development of the hydrogenogenic biomass, changes in the bacterial communities were
analyzed using polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–
DGGE).

2.2 Materials and methods

2.2.1 Seed sludge

The inoculum was anaerobic granular sludge from a full-scale up-flow anaerobic sludge
bed (UASB) reactor treating wastewater from a confectionery factory in San Luis Potosí,
México. Two different heat treatments were applied to the inoculum to inactivate
methanogenic microflora as follows: i) the granular sludge was boiled for 45 minutes and
then disaggregated in an anaerobic chamber (95% nitrogen and 5% hydrogen) by pressing
it out repeatedly through a 21G x 1” syringe needle; ii) due to an expected resistance of
methanogens due to the protective environment in the granular structure (Hu and Chen,
2007), the granular sludge was boiled three times during 1 hour, with 22 hours between
each treatment. Figure 2.1 shows the structure of each inoculum. The reactors were seeded
with 20 g volatile suspended solids (VSS)/L of either granules or disaggregated granules.

![Photographs of the disaggregated (a) and granular (b) inoculum.](image)

**Figure 2.1.** Photographs of the disaggregated (a) and granular (b) inoculum.
2.2.2 Medium composition

CWP was purchased from Grande Custom Ingredients Group (Wisconsin, USA). The soluble lactose content of CWP was 75.5% with 11.5% protein (w/w). An initial stock solution of 100 g CWP/L was centrifuged 10 minutes at 7000 g to remove insoluble material. After that, soluble chemical oxygen demand (COD) of the solution was determined. The medium used to feed the reactors and for the specific hydrogenogenic activity (SHA) tests, contained a known amount of CWP-COD and was supplemented with (mg/L): NH₄H₂PO₄, 4500; K₂HPO₄, 125; MgCl₂·6H₂O, 100; ZnCl₂, 75; FeSO₄·6H₂O, 25; MnSO₄·7H₂O, 15; Na₂MoO₄·2H₂O, 12.5; CuSO₄·5H₂O, 5; CoCl₂·6H₂O, 3. Additional Na₂HPO₄ was added to increase the buffer capacity of the medium in order to control the pH at the desired value.

2.2.3 Reactor operation

Four different UASB reactors with the same configuration and working volume (1.3 L) were used for the study. Two different start-up strategies (A and B) and two different inocula structure (disaggregated granules and granules) were used. Reactors were named as R1, R2, R3 and R4 and were operated as follows: R1 and R2 were inoculated with disaggregated granules and start-up strategies A and B, were applied respectively; whereas R3 and R4 were inoculated with granular sludge and start-up strategies A and B, were applied respectively (Table 2.1). After inoculation the reactors were filled-up with medium containing 8 g COD/L and left in batch mode for 12 h with effluent recirculation to obtain an up-flow velocity of 1.5 m/h. Then the reactors were switched to continuous mode and operated for 84 days maintaining the recirculation flow.

During the first 50 days of continuous operation the following start-up strategies A or B were applied.

**Start-up strategy A.** The reactors were started with an initial OLR of 5 g COD/L-d which was increased geometrically, achieving a final OLR of 20 g COD/L-d after 50 days; the OLR was calculated each day with following formula

\[ \text{OLR}_t = \alpha \text{OLR}_{t-1} \]  

(2.1)
where $\text{OLR}_i$ and $\text{OLR}_{i-1}$ are the OLR on days $i$ and on day $i-1$, respectively, and $\alpha$ is a constant parameter calculated from the following equation:

$$
\alpha = \sqrt{\frac{\text{OLR}_n}{\text{OLR}_i}}.
$$

(2.2)

where $n$ is the duration of the OLR increase (in days), $\text{OLR}_i$ and $\text{OLR}_n$ are the OLR at the first and last day of the start-up phase, respectively.

Table 2.1. Different start-up strategies and inoculum used in the reactors.

<table>
<thead>
<tr>
<th>Start-up strategy</th>
<th>Reactor</th>
<th>Inoculum</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>Disaggregated</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Granular</td>
</tr>
<tr>
<td>B</td>
<td>2</td>
<td>Disaggregated</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Granular</td>
</tr>
</tbody>
</table>

The HRT was kept constant at 6 h, above the HRT limit of 5.8 h, equivalent to the maximum specific growth rate of 0.172/h reported for hydrogen-producing cultures (Chen et al., 2001); this strategy was modified from the one proposed by Cresson et al. (2008).

**Start-up strategy B.** The reactors were operated at a constant OLR of 20 g COD/L-d, whereas the HRT was gradually decreased, in a period of 50 days, from 24 h to 6 h (24, 18, 16, 14, 12, 10, 8, 6 h). The reactors were operated during 6 or 7 days at each HRT.

At the end of the start-up period, from day 51 to day 84, the reactors were operated at the same conditions (OLR of 20 g COD/L-d and HRT of 6 h) to promote a steady state. The reactors OLR and HRT during all the experiment are depicted in Figure 2.2. The reactors were maintained at ambient temperature (22-25 °C) and at a pH value of 5.9, unless otherwise indicated.

The performance of the reactors was followed by analyzing the effluent three times a week for COD, volatile fatty acids (VFA), ethanol and residual lactose; VSS concentration was assayed once a week. Gas production was measured using a liquid-displacement device filled with water. All gas volumes are reported as measured (0.81 atm and 25°C).
2.2.4 Batch activity assays

Specific hydrogenogenic activity (SHA). SHA was determined in the seed sludge (granules and disaggregated granules) and in the biomass withdrawn from the reactors sampling ports on days 33 and 82. Measurements were performed in 120 mL glass serum bottles with 80 mL of working volume containing 4.5 g VSS/L of biomass and sealed with rubber septa according to the optimized method described by Davila-Vazquez et al. (2008a), at a COD concentration of 25 g/L and an initial pH value of 7.5. The gas composition of the headspace was determined as described in the analytical methods. The SHA was calculated dividing the maximum hydrogen production rate by the VSS concentration.

Specific hydrogenotrophic methanogenic activity (SHMA). SHMA was measured in seed sludge of reactors R3 and R4 and in biomass withdrawn from the sampling ports of the same reactors on days 33 and 82. Glass serum bottles (160 mL) with 80 mL of working volume were used for the assay. The headspace was displaced with a mixture of H₂/CO₂ (80/20%). The pressure of the headspace was measured periodically during 5 days, to calculate the methane production stoichiometrically (Equation 2.3). All batch activity assays were carried out in duplicate at 35°C with horizontal agitation at 120 rpm.

\[ 4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \] (2.3)

2.2.5 Electron balance

The flow of electron equivalents (e⁻ eq) during reactor operation was determined through an electron balance according to equation (2.4), modified from the proposed by Lee and Rittmann (2009):

\[ e^- (COD_i) = e^- (metabolites) + e^- (gas) + e^- (residual lactose) + e^- (N.D.) \] (2.4)

where \( e^- (COD_i) = e^- eq \) corresponding to the COD of the feed solution (an equivalence of 8 g COD/ e⁻ eq was considered); \( e^- (metabolites) = e^- eq \) of each metabolite produced; \( e^- (gas) = e^- eq \) of H₂ and CH₄ produced; \( e^- (residual lactose) = e^- eq \) of the residual lactose in the effluent; \( e^- (N.D.) = e^- eq \) of the not determined products, resulting from the difference of the \( e^- (COD_i) \) and the other equation terms. The electron flow is reported as fraction, being \( e^- (COD_i) \) the total e⁻ eq available, and the electron sinks, \( e^- (metabolites, gas, residual \)
lactose and N.D.) are a fraction of this value; the electron equivalents directed to biomass production were not considered.

2.2.6 Analytical methods

Hydrogen, CH$_4$ and CO$_2$ content in the gas were determined by gas chromatography (GC, 6890N Network GC System, Agilent Technologies, Waldbronn, Germany) equipped with a thermal conductivity detector and a Hayesep D column (Alltech, Deerfield, Illinois, USA) with the following dimensions: 10’ x 1/8”x 0.085”. Temperatures of the injection port, oven and detector were 250, 60 and 250ºC, respectively. Nitrogen was used as carrier gas with a flow-rate of 12 mL/min. Pressure of the headspace in the hydrogenotrophic methanogenic assays was measured with a manometer (Model 407910, Extech Instruments Corporation, Massachusetts, USA). Lactose, volatile fatty acids (VFA: acetate, propionate butyrate, lactate and formate) and ethanol were analyzed as previously described (Davila-Vazquez et al., 2008a). COD and VSS were analyzed according to Standard Methods (APHA/AWWA/WFE, 2005).

2.2.7 Bacterial community analyses by 16S rRNA genes using DGGE

2.2.7.1 DNA extraction

Ten milliliters of sludge were withdrawn from each reactor at day 33 and 84 of continuous operation and kept at -20°C, DNA was extracted from these samples and the samples from the different inoculum (granules and disaggregated granules) based on a previously published protocol (Wisotzkey et al., 1990). In brief, the samples were slowly thawed and 300 µL were centrifuged at 7000g for 10 min. The pellet was washed with PBS buffer (10 mM NaH$_2$PO$_4$ and Na$_2$HPO$_4$, plus NaCl 140 mM, pH 7.5) and ethanol solution (1:1), a second wash with PBS was done and the pellet was incubated in 500 µL of extraction buffer (10 mM Tris/HCl, pH 8) and 20 µL of lysozyme (20 mg/mL) during 30 minutes at 37°C and 250 rpm. Afterwards, the following reagents were added: NaCl 5M (600 µL), 2-2% (w/v) polyvinilpirrolidone-cetyltrimetylammonium bromide (200 µL), PK buffer [100 mM Tris/HCl pH 7.6, 5 mM EDTA pH 8, 50 mM NaCl] (600 µL) and 20 µL of proteinase
K (20 mg/mL), the mixture was incubated during 2 hours at 55°C and 250 rpm. Then, 15 µL of RNase (0.5 mg/mL) were added and samples were incubated during 30 minutes at 37°C and 250 rpm. For nucleic acids purification, one volume of chloroform–isoamylic alcohol (24:1) was added, mixed and centrifuged for 3 min at 7000g. This purification step was repeated two times using the supernatant. To precipitate nucleic acids, one volume of isopropanol at -20°C and 0.3 volumes of ammonium acetate (5M) were added to the supernatant and incubated overnight at -20°C before centrifugation at 4°C for 40 min at 7000g. The pellet was first washed with 70% ethanol and centrifuged at 7000g for 40 min. The DNA pellet was dried at room temperature and resuspended in 15-50 µL of sterile deionized water. DNA integrity was observed in 1% agarose gels, stained with ethidium bromide.

2.2.7.2 PCR amplification

Amplification of the 16S rRNA gene from the purified DNA was carried out by PCR using Taq DNA polymerase (Dongsheng, China). The PCR primers used were the forward primer 341F-GC

\[ 5’-CGCCCGCCGCGCGGCGGCGGCGGCGGGGCACGGGGGCTACGGGAGGCAGCAG-3’ \]

and the reverse primer 907R (5’-CCGTCAATTCMTTTGAGTTT-3’) for bacterial identification of complex microbial populations (Schäfer and Muyzer, 2001); the forward primer contains a 40 nucleotide GC-clamp at the 5’ end to prevent the complete dissociation of the two DNA strands during the DGGE. Reaction conditions were as follows: initial DNA denaturation at 96 °C for 4 min, followed by 10 cycles of denaturation at 94°C for 30 s and annealing for 1 min decreasing 1°C in each cycle the temperature from 61°C-56°C, followed by an extension at 72°C for 1 min. Once the temperature reached 56°C, 20 more cycles were performed; final extension lasted 7 min at 72°C. PCR products were visualized in 1.5% agarose gels, stained with ethidium bromide to assess the size, purity and concentration of DNA.
2.2.7.3 DGGE analysis

DGGE analysis was performed with Dcode Universal Mutation Detection System (Biorad, Hercules, California, USA). The PCR products were separated in 8% polyacrylamide gels (1mm thick) in 0.5 X TAE buffer (20mM Tris–acetate, 10 mM sodium acetate, 0.5mM EDTA, pH 8.0) with a linear denaturing gradient (urea–formamide) from 30 to 60%. Electrophoresis was carried out at 60°C at a constant voltage of 70 V during 20 h. The procedure to stain the gel after the electrophoresis was as follows, first the gel was fixed with 10% acetic acid solution during 30 min at 40-50 rpm, and the gel was washed three times with deionized water for 5 min at the same agitation. Afterwards, the gel was submerged in AgNO$_3$ solution (1 g/L) during 30 min, without light incidence. The bands in the gel were developed with anhydrous sodium carbonate (23.3 g/L) solution during 15 min, afterwards the gel was submerged in the fixation solution (10% acetic acid) during 5 min and finally the gel was rinsed with deionized water. The gel bands were observed at visible light, and a photo with a digital camera was taken.

DGGE gels were analyzed with the Cross Checker v 2.91 software (Wageningen University, The Netherlands) to create a binary matrix, the Unweighted Pair Group Method Arithmetic (UPGMA) averages were calculated and corresponding dendograms showing the relationships between the DGGE profiles were constructed with the Phylogeny Inference Package v 3.69 (University of Washington, USA). The dominant bands were excised from the gel, eluted in 20µL of sterile deionized water overnight at room temperature. The eluted DNA was reamplified by PCR using 341F (without GC-clamp) and 907R primers. The PCR products from reamplification were sent to purification and sequencing to “Laboratorio Nacional de Biotecnología Agrícola, Médica y Ambiental” (LANBAMA, Mexico). Sequence data were analyzed with DNA Baser v.3.2.5 software (Heracle BioSoft, Arges, Romania) and submitted to the non-redundant nucleotide database at GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/) and Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp) for bacterial identification.
2.3 Results and discussion

2.3.1 Reactors performance

Four laboratory scale UASB reactors were operated in order to investigate the effect of different start-up strategies and different inoculum structure on both the VHPR and the biomass activity. Figure 2.2 shows the time course of the reactors performance. The gas production started at different times in each reactor, in the reactors with disaggregated granular sludge, R1 and R2, the gas production started after 40 to 70 hours of continuous operation (Figs. 2.2a and b), meanwhile in R3 and R4, inoculated with granular sludge, the gas production started during the first 12 hours after inoculation (Figs. 2.2c and d). In the reactors started-up with strategy A (R1 and R3) the gas composition in the first 9 days of continuous operation was mainly H₂, 98 and 76%, respectively, and the rest was CO₂, the VHPR was less than 0.3 L H₂/L-d (Figs. 2.2a and c). Despite heat treatment was applied to the seed sludge methane was produced in all reactors. From day 10 onwards, methane was produced in R1 and R3 at a higher volumetric rate (0.5 L CH₄/L-d) than hydrogen production (Figs 2.2a and c). In the reactors started-up with strategy B (R2 and R4), the H₂ content in the gas was 49 and 31%, respectively, and the rest was CO₂; methane production began on days 64 and 25 for R2 and R4, respectively (Figs. 2.2b and d). The highest VHPR achieved in R2 and R4 was 1.41 and 3.25 (point not shown) L H₂/L-d, respectively, which can be explained by the higher initial OLR applied.

During the start-up period (days 1-50) a stable hydrogen production was not expected, due to the variable operational conditions. In R1 the highest hydrogen producing period was between days 68 and 72, reaching a VHPR of 1.12 ± 0.19 L H₂/L-d, then the production decreased to an average value of 0.33 ± 0.2 up to day 84 (Fig. 2.2a). In R2 during days 59 to 64 the VHPR was 0.74 ± 0.14 L H₂/L-d, following a similar tendency like R1 decreasing to 0.38 ± 0.1 from day 80 to 84 (Fig. 2.2b). In R3 the VHPR was in average 0.11 ± 0.03 L H₂/L-d during all the stabilization period from days 51 to 84 (Fig. 2.2c), meanwhile in R4 the hydrogen production rate was 0.36 ± 0.1 L H₂/L-d in the same period, being a more stable hydrogen production compared to R1 and R2 (Fig. 2.2d).

The hydrogen production rates obtained in R1, R2, and R4, at the end of reactors operation, are higher than the VHPR obtained by Castello et al. (2009) (122 mL H₂/L-d), in a UASB
reactor at an OLR of 20 g COD/L-day with cheese whey as substrate and methane occurrence as in the present study; but are lower than the hydrogen production rate reported by Chang and Lin (2004), about 0.5 L H₂/L-day, under the same OLR using sucrose as substrate with only H₂ and CO₂ in the biogas.

In order to diminish the methane production in all reactors, from day 50 to 84 the buffer capacity in the influent was decreased and the pH was reduced from 5.9 to an average value of 5.4. However this strategy was not efficient to control the methanogenesis, similar results have been reported, where the pH was kept at an average value of 5 but the methane production did not ceased (Castello et al., 2009). A general tendency in all reactors when methane production began, was that methane content in the biogas increased gradually reaching higher percentages than H₂, which can be explained by an acclimation of the methanogens over the time even at low pH values, contrasting with the neutral pH considered for the optimum methanogenic systems operation (Taconi et al., 2008).

**Figure 2.2.** Profiles of the volumetric hydrogen production rate, VHPR (●); volumetric methane production rate, VMPR (◊) and operational conditions applied: hydraulic retention time, HRT (---) and organic loading rate, OLR (-----), in reactors R1 (a), R2 (b), R3 (c) and R4 (d).
According to Wang et al. (2007) reducing the pH up to 4.5 effectively suppressed methane production in UASB reactor inoculated with heat treated anaerobic sludge, possibly in the present study a higher decrease on the pH (one unit or more) would be a sustainable strategy to reduce the methanogenic activity.

The results confirmed that one or repeated heat treatments did not eliminate the methanogens from the inoculum, due to the protection structure provided by the granules as was established by Hu and Chen (2007), who found that a heat treatment was effective in a sewage sludge but it did not eliminate completely the methanogenic activity in granular sludge. In other study in which the seed sludge was heat treated, methane production was also observed (Wang et al., 2007). Potential strategies to efficiently inhibit methanogens are: heat treatment after disintegration of the granules; longer dry heat treatments at 105°C (1.5 to 2 hours) (Khanal et al., 2006; Wang and Wan, 2008); heat treatment followed by an acid treatment (2 hours of heat treatment followed by acid incubation at pH 2 or 3 during 24 hours) (Mohan et al., 2008; Zhang et al., 2008) and boiling the sludge during 5 h (Argun and Kargi, 2009).

Another important factor to consider is the solids retention time (SRT), for instance in a CSTR the HRT is equal to the SRT, which in principle allows the washout of cells not able to growth at the dilution rate applied. In contrast, within UASB reactors the SRT is longer than the HRT, this may explain why the methanogens were preserved even at the short HRT (6 h) applied to the reactors with strategy A (R1 and R3), unlike the results from previous experiments using the same seed sludge and similar heat treatment, but different reactor system such as a CSTR (Davila-Vazquez et al., 2009).

In the case of reactors started-up with strategy B, R2 and R4, the high initial concentration of VFA produced (up to 10 g COD/L) due to the higher initial substrate concentration applied during the start-up period, inhibited the methanogenic community (Wong et al., 2008) retarding the methane presence comparing with R1 and R3.

It can be considered that reactor R4 reached steady state after 60 days of operation (Fig. 2.2d). This time is shorter than the time reported by Mu and Yu (2006) (140 days) and Zhao et al. (Zhao et al., 2008) (8 months), but is similar to the time reported by Chang and Lin (2004) (50 days) to reach equivalent and stable hydrogen production rates (about 0.5 L
H₂/L-d), it that experiment the OLR was constant as in strategy B of the present study (20 g COD/L-d).

2.3.2 Metabolic byproducts

Figures 2.3 and 2.4 show the metabolites, hydrogen and methane production rates for R1, R3 and R2, R4, respectively. In all reactors the lactose consumption was higher than 99%. In reactors operated with strategy A (Fig. 2.3), the main metabolic product throughout the operation time was acetate, indicating a hydrogen production pathway (Hawkes et al., 2007). In R1 until day 62, the second more produced metabolite was propionate (Fig. 2.3a), which has been reported as a non-hydrogen production pathway (Hawkes et al., 2007; Lee and Rittmann, 2009). The propionate concentration decrease coincided with the hydrogen production increase. In reactor R3, the second more produced metabolite was butyrate, indicating that a mixed fermentation acetate-butyrate occurred, this production pathway has been reported as adequate for H₂ generation (Davila-Vazquez et al., 2008a; Hawkes et al., 2007), however the hydrogen production achieved in R3 was the lowest from the four experiments, being in average 3.09 mmol/d (Fig. 2.3b).

In reactors, R2 and R4, started-up with strategy B (Figure 2.4), unlike reactors R1 and R3, other reduced products such as lactate and formate were also produced but in low rates (less than 4 mmol/d). In R2 before the methane production started, the second more produced metabolite was ethanol after acetate (Fig. 2.4a), which has been reported as a hydrogen producer mixed metabolic pathway (Guo et al., 2008). Similarly to R2, in R4 acetate was the main metabolic by-product produced at a stable rate of 97.5 ± 11.7 mmol/d, followed by butyrate (Fig. 2.4b).

According to Dinamarca and Bakke (2009), the low H₂ production rate could be caused either by electrons derived from the competition between electron carriers, which are redirected to the formation of reduced products such as biomass and/or exocellular electron transfer; or molecular H₂ consumption by homoacetogenic bacteria or methanogens. Due to the low production of propionate or lactate during days 51 to 84 in all the reactors (in average 15.45 mmol/d), the reduced products option acting as electrons sink was discarded. The most feasible explanations, for a low production H₂ rate, are the hydrogen consumption
by homoacetogenic bacteria, which could also explain the high acetate production, or the hydrogen consumption by methanogens. To elucidate this, batch assays to evaluate the methanogenic and the homoacetogenic activities were carried out, the results are presented in section 2.3.4.

**Figure 2.3.** Profiles of the metabolites and gas production rates obtained from UASB reactors operated with strategy A: R1(a) and R3 (b); Acetate (●), Ethanol (▼), Propionate (□), Butyrate (Δ), H₂ (●) and CH₄ (○).

### 2.3.3 Electron balance

Figure 2.5 shows the electron balance of the reactors through the stabilization period (days 50-84). Reactors with disaggregated inoculum (R1 and R2) were less stable in electron distribution than reactors with granular inoculum (R3 and R4). The main identified electron sinks were acetate and butyrate, with average values of 18 and 16%, 19 and 13%, 20 and 25%, 15 and 11% of the available electrons for R1, R2, R3 and R4, respectively. In reactors with disaggregated inoculum ethanol was also a substantial electron sink, corresponding to 7 and 14% of the available electrons for R1 and R2, respectively. In these reactors the
electrons derived to hydrogen and methane were in average 1.75% and 2.5 %, respectively (Figs. 2.5a and b).

**Figure 2.4.** Profiles of the metabolites and gas production rates obtained from UASB reactors operated with strategy B: R2(a) and R4 (b); Acetate (●), Ethanol (▼), Propionate (□), Butyrate (△), Lactate (❖), Formate (◄), H₂ (●) and CH₄ (◊).

In R3 and R4 the electrons invested in hydrogen were less than 0.5% whereas in methane reached values up to 5.5% (Figs. 2.5c and d). The highest electron percentage in all reactors were the non identified products, in reactors R1 to R3 the average value was 43 % ± 1.7, in R4 this value was even higher reaching on average 61% of the available electrons. The non-identified products could correspond to biomass growth and exopolymers that were not quantified, and to the production of other metabolites not determined such as valerate, caproate, butanol and propanol (Hu and Chen, 2007; Kotsopoulos et al., 2006). A similar balance, but in batch conditions, showed that the electron percentage towards biomass growth can be around 12% (Lee and Rittmann, 2009). In the present experiment, it is assumed that most of the non determined electrons were directed to biomass growth;
despite the biomass withdrawn, the VSS concentration in the reactors increased to around 27 g VSS/L at the end of the experiments, which represents 35% increment from the inoculated VSS.

![Figure 2.5](image)

**Figure 2.5.** Electron balances in UASB reactors during the stabilization period in reactors R1 (a), R2 (b), R3 (c) and R4 (d); N.D. not determined.

### 2.3.4 Specific activities

The granular inoculum had higher SHA (36% more) than the disaggregated inoculum, according to the results of the specific activity batch assays presented in Table 2.2. The specific hydrogenogenic activities (SHA) were also determined with the biomass withdrawn from the reactors at days 33 and 82, meanwhile specific hydrogenotrophic methanogenic activity was determined at the same days only from R3 and R4, expecting the same tendency in reactor started-up with strategy A (R1) and those started-up with strategy B (R2) (Table 2.2). The biomass developed at day 33 in reactors with
disaggregated inoculum (R1 and R2) had lower SHA (57 to 65% decrement) compared to the inoculum. However, those values increased at day 82, in particular in R2 the SHA reached similar hydrogenogenic activity (1469.7 mL H$_2$/g VS-d) to the inoculum. In contrast, the SHA of R3 and R4 at day 82 diminished 90 and 60%, respectively compared to the SHA of the granular inoculum, hydrogen yields were also lower than the obtained in the granular inoculum, most probably as a consequence of the considerable hydrogenotrophic methanogenic activity detected in R3 and R4.

The SHMA increased over time in R3 and R4 from 26 mL CH$_4$/g VS-d in the granular inoculum, to 69.2 and 113 mL CH$_4$/g VS-d in R3 and R4, respectively. This increment in the hydrogenotrophic methanogenic activity is clearly reflected in R4 performance (Fig. 2.2d), where even at a constant OLR the volumetric methane production rate (VMPR) increased gradually. During the SHMA batch assays, acetate was not produced indicating the absence of homoacetogenic activity; when bromoethanesulfonate, a specific methanogenic inhibitor (Ryan et al., 2008), was added, neither methane nor acetate production was detected. Besides the SHMA assay, an acetoclastic methanogenic activity batch assay was carried out during 30 days of incubation (data not shown), and no methane production was detected neither with the granular inoculum nor with the biomass withdrawn from R3 and R4 at day 82. These results indicate that hydrogenotrophic methanogenic activity was not inhibited and was the main reason for the low hydrogen production in R3 and R4 and possibly in R1 and R2 also, due to the same inoculum source and the heat treatment applied. Similarly Kim et al. (2004), using a stoichiometric analysis, proposed that methane produced in a semi-continuous hydrogenogenic reactor was due to the hydrogen consumption at pH of 4.5; in our study the hydrogenogenic activity was evaluated through activity batch tests from the reactors biomass that were operated at pH 5.9 and 5.5 during days 33 and 82, respectively; the tolerance of hydrogen consuming methanogens at acidic pH has been observed previously in natural environments (Horn et al., 2003).

The lower SHA results and the different metabolites concentration from the reactor biomass at day 33 compared to their respective inocula (Table 2.2), indicated that a biomass wash-out occurred during the start-up period. In R1 and R2, at day 82 the main metabolic products in both reactors were acetate, propionate and butyrate at similar concentrations,
therefore is assumed that a hydrogenogenic biomass was selected in both reactors, which is reflected in the similar VHPR and metabolic products in continuous production after day 80. On the other hand, batch assays for R3 and R4 at day 82 indicated that acetate was the main metabolic pathway. Nonetheless, even the higher lactate production (a non hydrogen production pathway) and SHMA in R4, the hydrogenogenic activity was higher than in R3 and this was reflected in the reactor performance.

**Table 2.2.** Specific hydrogenogenic activity (SHA) and final metabolites concentration obtained in the batch assays conducted with the inoculum and biomass withdrawn from the UASB reactors at days 33 and 82. The specific hydrogenotrophic methanogenic activity (SHMA) is also shown.

<table>
<thead>
<tr>
<th>Biomass</th>
<th>Yield (mol H₂/mol hexoseeq)</th>
<th>SHA¹ (mL H₂/g VS-d)</th>
<th>Ace²</th>
<th>Prop³</th>
<th>But²</th>
<th>Lac³</th>
<th>For²</th>
<th>EtOH²</th>
<th>SHMA³ (mL CH₄/g VS-d)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Disaggregated Granules</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inoculum</td>
<td>1.11</td>
<td>1420.8 (19.1)</td>
<td>42.6</td>
<td>8.4</td>
<td>48.0</td>
<td>0.0</td>
<td>0.0</td>
<td>46.0</td>
<td></td>
</tr>
<tr>
<td>R1, day 33</td>
<td>0.44</td>
<td>489.6 (15.1)</td>
<td>73.2</td>
<td>28.1</td>
<td>24.2</td>
<td>10.2</td>
<td>0.0</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>R2, day 33</td>
<td>0.36</td>
<td>613.6 (2.6)</td>
<td>70.0</td>
<td>18.2</td>
<td>7.8</td>
<td>19.3</td>
<td>17.9</td>
<td>55.0</td>
<td></td>
</tr>
<tr>
<td>R1, day 82</td>
<td>0.50</td>
<td>1283.4 (70.2)</td>
<td>43.6</td>
<td>55.6</td>
<td>32.6</td>
<td>0.0</td>
<td>0.0</td>
<td>17.5</td>
<td></td>
</tr>
<tr>
<td>R2, day 82</td>
<td>0.59</td>
<td>1469.7 (53.7)</td>
<td>39.0</td>
<td>49.6</td>
<td>35.1</td>
<td>0.0</td>
<td>0.0</td>
<td>25.0</td>
<td></td>
</tr>
<tr>
<td><strong>Granules</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inoculum</td>
<td>1.31</td>
<td>1932.4 (190.3)</td>
<td>37.4</td>
<td>1.7</td>
<td>57.7</td>
<td>0.0</td>
<td>0.0</td>
<td>13.1</td>
<td></td>
</tr>
<tr>
<td>R3, day 33</td>
<td>0.22</td>
<td>486.3 (2.3)</td>
<td>79.5</td>
<td>13.4</td>
<td>39.8</td>
<td>0.0</td>
<td>31.5</td>
<td>15.1</td>
<td></td>
</tr>
<tr>
<td>R4, day 33</td>
<td>0.36</td>
<td>510.7 (0.8)</td>
<td>57.6</td>
<td>52.6</td>
<td>5.0</td>
<td>0.0</td>
<td>0.0</td>
<td>43.5</td>
<td></td>
</tr>
<tr>
<td>R3, day 82</td>
<td>0.12</td>
<td>180.8 (19.7)</td>
<td>93.7</td>
<td>18.9</td>
<td>21.3</td>
<td>22.4</td>
<td>0.0</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>R4, day 82</td>
<td>0.30</td>
<td>773.4 (41.6)</td>
<td>83.0</td>
<td>15.2</td>
<td>21.3</td>
<td>46.4</td>
<td>0.0</td>
<td>2.3</td>
<td></td>
</tr>
</tbody>
</table>

Average value (standard deviation), n=2; ¹ Specific hydrogenogenic activity; ² Ace-Acetate, Prop-Propionate, But-Butyrate, Lac-Lactate, For-Formate, EtOH-Ethanol; ³ Specific hydrogenotrophic methanogenic activity; ⁴ not determined; Gas production reported at 0.81 atm, 25°C.

**2.3.5 Bacterial community analyses by PCR–DGGE**

DGGE analysis of the PCR-amplified bacterial *16S* rRNA gene fragments, was used to compare the respective community fingerprints of the biomass developed in the reactors during 82 days of continuous operation and different startup strategies. In reactors inoculated with disaggregated sludge (Figure 2.6a), the inoculum and the biomass of R2 at day 33 were clustered together and biomass from R1 at day 33 was clustered to this group; whereas the biomass from day 82 in R1 and R2 are clustered together which agree with
their similar SHA. Even though the SHA results from R1 and R2 (day 82) show a similar potential than the inoculum (Table 2.2), the bacterial DGGE fingerprint showed a different community selection over the operation time. Therefore, the reactor operation time was determinant for the bacterial community selection, more than start-up strategies A and B in these reactors (R1 and R2).

Figure 2.6. Bacterial DGGE community fingerprints and corresponding similarity dendrograms and indexes from reactor inoculated with disaggregated sludge (a) and granular sludge (b). I corresponds to inoculum samples; I-1, I-2 and I-3 corresponds to heat treated inoculum 1, 2 or 3 times; R1-R4 corresponds to the reactors and the operation day of each sample 33 or 82. A, B and C are the sequenced bands.

Figure 2.6b shows the DGGE fingerprint from the reactors inoculated with granular sludge, in this case the communities developed at day 33 with start-up strategy A or B (R3 and R4) were 96% similar, and were clustered to the DGGE fingerprint of R3 at day 82. Whereas the bacterial community from R4 at day 82 was clustered separately from all the samples, unlike to reactors with disaggregated inoculum. According to this result, in reactors with granular inoculum the start-up strategy and the reactor operation during 82 days were determinant in the bacterial community selection, which agree with the difference in the SHA assays at day 82 (Table 2.2, R3 and R4) and the reactor performance (Figure 2.2c and d). Although the methanogenic community was not monitored in the present work, the DGGE fingerprint cluster analysis can be associated to the SHA results.

The DNA retrieved from the bands A, B and C shown in Figure 2.6b were amplified and sequenced (Table 2.3). The sequences of bands B and C were similar to sequences of the genus Clostridium (93 and 97% identity), were found during all operation time of R3 and R4 as was expected due to metabolites produced, since butyrate and acetate were the main
products (Davila-Vazquez et al., 2009). The sequence of band A was closely related to the sequence of *Lactobacillus casei* BD-1 (98% identity), present in R3 and R4 at day 33, and only present in R4 at day 82. Band A was not found in R3 at day 82 which pointed out a probable wash-out of *Lactobacillus* from R3. Microorganisms of this genus have been related previously to hydrogen producing reactors fed with dairy effluents (Castello et al., 2009), producing lactate by fermentation of sugars (Kandler and Weiss, 1986) which was only detected in reactors started-up with the strategy B (R2 and R4).

Table 2.3. Affiliation of the DGGE fragments showing the highest percentage of identity in the output result from the analysis in the non-redundant nucleotide database from NCBI using the BLAST program.

<table>
<thead>
<tr>
<th>Band</th>
<th>Closest relative</th>
<th>Gen bank accession number</th>
<th>Percentage of identity against GenBank database</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><em>Lactobacillus casei</em> BD-I</td>
<td>CP002618.1</td>
<td>98</td>
</tr>
<tr>
<td>B</td>
<td><em>Clostridium sp.</em></td>
<td>FJ805840.2</td>
<td>93</td>
</tr>
<tr>
<td>C</td>
<td><em>Clostridium ramosum</em></td>
<td>HM245949.1</td>
<td>97</td>
</tr>
</tbody>
</table>

2.4 Conclusions

The start-up strategy had no effect in the bacterial community in reactors inoculated with disaggregated sludge according to the cluster analysis, unlike to those inoculated with granular sludge.

Based on the similar performance of the UASB reactors R1, R2 and R4 in the last 14 days of the experiments, it can be concluded that the start-up strategies applied selected a hydrogen producing biomass. Nonetheless, judging by the SHA batch assays results, the strategy B produced a more active biomass and it can be considered that keeping a constant OLR and decreasing gradually the HRT is a suitable strategy for the development of a hydrogen producing microbial community in reactors fed with cheese whey.

Considering the inoculum structure, the disaggregated inoculum produced a more active hydrogenogenic biomass, but not granule formation was observed during the operational period. In order to conserve the granular structure and due to the stable reactor performance, the strategy used in R4 can be the most promising if the methanogenic
activity can be controlled. The pH decrease from 5.9 to 5.5 did not cause a reduction in the methanogenic activity. Although hydrogen was not the main product in the gas, the experiments showed a high hydrogenogenic potential because the methane detected in the reactors was produced from hydrogen, at a ratio of 1 mol of methane per 4 mol of hydrogen consumed. The challenge now, is to identify the operational parameters to block the hydrogenotrophic methanogenic activity, or to use more aggressive inoculum treatments.

2.5 References


Chapter 3

Decreasing methane production in hydrogenogenic UASB reactors

Summary

One of the problems in fermentative hydrogen producing reactors, inoculated with pre-treated anaerobic granular sludge, is the eventual methane production by hydrogen consuming methanogens. In this study, strategies such as reduction of pH and HRT, organic shock loads and repeated biomass heat treatment were applied to hydrogenogenic UASB reactors that showed methane production after certain time of continuous operation (between 10 and 60 days). The reduction of pH to 4.5 not only decreased methane production but also hydrogen production. Organic shock load (from 20 to 30 g COD/L d) was the more effective strategy to decrease the methane production rate (75%) and to increase the hydrogen production rate (172%), without stopping reactor operation. Repeated heat treatment of the granular sludge was the only strategy that inhibited completely methane production, leading to high volumetric hydrogen production rates (1.67 L H₂/L-d). However this strategy required stopping reactor operation; in addition homoacetogenesis, another hydrogen consuming pathway, was not completely inhibited. This work demonstrated that it was possible to control the methane production in hydrogen producing reactors using operational strategies.
3.1 Introduction

The environmental impacts derived from the use of fossil fuels, such as climate change and the finite reserves of these fuels, drive a search for renewable and carbon neutral energy sources. The production of hydrogen by dark fermentation from biomass is a sustainable alternative because organic residues and wastewater can be used, turning a pollution problem into an energy resource; moreover, H₂ has the highest energy content compared with any fuel and it can be used directly in fuel cells to produce electricity (Das and Veziroglu, 2008; Davila-Vazquez et al., 2008b; Rittmann, 2008).

Wastes with high content of biodegradable carbohydrates are potential feedstocks for fermentative hydrogen production, such as dairy industry effluents (Kapdan and Kargi, 2006). Annually, the cheese industry produces over 160 million tons of cheese whey (Guimaraes et al., 2010), in its dried form this by-product contains around 70% of lactose and has been used successfully for hydrogen production (Castello et al., 2009; Davila-Vazquez et al., 2009).

Continuous hydrogen producing reactors are usually inoculated by a complex consortium from an anaerobic reactor, the inoculum receives a treatment to select hydrogen producing bacteria and eliminate hydrogen consuming microorganisms (Castello et al., 2009). In such continuous reactors the biohydrogen yield and production rates achieved, depend on different operational factors such as pH, hydraulic retention time (HRT), solids retention time, and organic loading rate (OLR) among others (Buitrón and Carvajal, 2010; Chen et al., 2009; Davila-Vazquez et al., 2009). Frequently, in high cell density reactors, such as the upflow anaerobic sludge bed (UASB), the inoculum treatment is not enough to eliminate undesired microorganisms and eventually methane is produced. Part of the available electrons are directed to methane production lowering significantly the hydrogen productivity (Carrillo-Reyes et al., 2012; Castello et al., 2009). Methane production in UASB reactors may start several days or weeks after the reactor start-up (Carrillo-Reyes et al., 2012; Castello et al., 2009; Spagni et al., 2010). Different causes contribute to methane occurrence, such as operating the reactor at a pH near to neutrality (6-7) (Wang et al., 2007), a long solids retention time (Castello et al., 2009) and deficient inoculum
pretreatments that do not eliminate completely the methanogenic community (Argun and Kargi, 2009).

According to (Wang et al., 2007) the pH is a parameter that can determine the methane production in hydrogenogenic UASB reactors. At pH value of 5 and lower they did not find any methane production, having the highest hydrogen production rate at pH 4 (127 ± 9 mL H₂/L-h). Whereas, Castello et al. (2009) operated a UASB reactor at an average pH value of 5 during 200 days, methane being the main component in the biogas (6-20 % methane and less than 1 % hydrogen) even though they started-up the reactor at pH 3.3.

Previous works in methanogenic reactors have shown that organic shock loads create an imbalance accumulating volatile fatty acids (VFA), increasing the hydrogen partial pressure and diminishing or even eliminating the methane production (CordRuwisch et al., 1997). This strategy has been proved in hydrogenogenic UASB reactors to reduce the methane production, increasing the OLR from 7.6 to 12.7, 25.5 and finally to 38.2 g COD/L-d (Spagni et al., 2010). Although the two lowest OLR values were kept only during 5 days, methane started to be produced and it did not cease completely at the highest OLR values tested. Therefore, it is important to evaluate the organic shock load effect in hydrogenogenic reactors with a more stable microbial community where methane has been produced for longer periods of time.

The inoculum pretreatment selects hydrogen producing bacteria using its sporulation capability when facing unfavorable growth conditions, eliminating microorganisms that do not form endospores like methanogens (Duangmanee et al., 2007). Different inoculum pretreatments have been evaluated, being heat treatment the most commonly used (Akutsu et al., 2009; Hu and Chen, 2007). The efficiency of this pretreatment is influenced by the biomass structure. Hu and Chen (Hu and Chen, 2007) demonstrated that heat treatment was more effective to inhibit methanogenesis in a flocculent biomass than in a granular biomass. Recently, Duangmanee et al. (Duangmanee et al., 2007) applied repeated heat treatments to the recirculated biomass in a completely mixed reactor, favoring the stability of hydrogen production.

The above results show different approaches and some contrasting results in order to avoid methane production in hydrogenogenic reactors. Therefore, the aim of this work was to
evaluate systematically different strategies to inhibit the methanogenic activity in hydrogenogenic UASB reactors that had been in operation for extended periods of time. The strategies included operational conditions (pH, OLR and hydraulic retention time) and repeated heat treatment to the biomass. The reactors performance was evaluated through volumetric hydrogen and methane production rates, metabolites produced as well as hydrogenogenic and methanogenic specific activities.

3.2. Materials and Methods

3.2.1 Substrate and seed sludge

Cheese whey powder (CWP) solution was used as a synthetic dairy wastewater. The CWP was purchased from Grande Custom Ingredients Group (Wisconsin, USA). The soluble lactose content of CWP was 75.5% with 11.5% protein (w/w). An initial stock solution of 100 g CWP/L was centrifuged 10 minutes at 13000 rpm to remove insoluble material. After that, soluble chemical oxygen demand (COD) was determined. The medium contained a known amount of CWP-COD and was supplemented with (mg/L): NH₄H₂PO₄, 4500; K₂HPO₄, 125; MgCl₂·6H₂O, 100; ZnCl₂, 75; FeSO₄·6H₂O, 25; MnSO₄·7H₂O, 15; Na₂MoO₄·2H₂O, 12.5; CuSO₄·5H₂O, 5; CoCl₂·8H₂O, 3. Additional Na₂HPO₄ was added to increase the buffer capacity of the medium and controlling the pH at the desired value.

The original seed sludge was collected from a full-scale methanogenic UASB reactor treating wastewater from a confectionery factory in San Luis Potosí, México. The sludge received a heat treatment (boiled during 1 hour) and was inoculated in different lab scale hydrogenogenic UASB reactors (working volumes between 0.47 and 1.3 L) to reach volatile suspended solids (VSS) concentration from 13 to 20 g VVS/L. The reactors were fed with CWP as substrate and operated during extended periods of time where, eventually, methane started to be produced concomitantly with hydrogen, for periods not less than 20 days before the present experiments were performed.
3.2.2 Experimental set-up

Five different experiments (A to E) were carried out to evaluate the effect of different operational strategies in order to decrease the methane production in the aforementioned UASB reactors. Figure 3.1 shows the operational parameters before the present experiments were carried out and the parameters modified in each experiment (from day 0 onwards), as well as the length time of each one. The details of each experiment are described as follows:

Figure 3.1. Operational conditions before the present experiments were carried out and the parameters evaluated in each experiment (from day 0 onwards). a) Experiment A, b) Experiment B and C, c) Experiment D and E. — Hydraulic retention time, HRT; ○ Substrate concentration, S; … pH; --- Organic loading rate, OLR.
Experiment A: the effect of pH reduction was tested by decreasing the reactor pH from 5.63 to 5.0 and 4.5, and reestablishing the pH again to 5.0. OLR and HRT were kept at 20 g COD/L-d and 6 h respectively (Figure 3.1a).

Experiment B: the effect of the organic shock load was tested by increasing the OLR from 20 to 30 and finally to 40 g COD/L-d. The OLR was increased by incrementing the COD influent concentration while the HRT was kept constant at 6 h (Figure 3.1b).

Experiment C: the effect of the organic shock load was further tested by operating at the previous OLR (40 g COD/L-d), but by means of decreasing both the HRT and the COD influent concentration (Figure 3.1b).

Experiment D: the effect of the organic shock load was also tested in this experiment by increasing the OLR from 26 to 37 by means of raising the COD concentration in the influent at constant HRT (13 h). Afterwards the OLR was increased up to 48 g COD/L-d by decreasing the HRT to 10. Then the HRT was further decreased to 8 h, while reducing the COD concentration in the influent in order to maintain an OLR of 48 g COD/L-d (Figure 3.1c).

Experiment E: the granules of the experiment D were harvested, heat treated (boiled 1 hour) and re-inoculated to evaluate the effect of a second heat treatment, keeping the same operational parameters applied in the last stage of experiment D (Figure 3.1c).

The pH in the reactors during experiments B to E was 5.3 - 5.5.

### 3.2.3 Batch activity assays

The specific hydrogenogenic and methanogenic activities were carried out in batch tests using sludge withdrawn from the UASB reactors during experiments A to E. Table 3.1 shows the specific operational conditions and days where the sludge was sampled. All batch activity assays were carried out at 35 °C under agitation at 120 rpm.

Specific hydrogenogenic activity (SHA). SHA measurements were performed in duplicate in 120 mL glass serum bottles sealed with rubber septa according to the method described by Davila-Vazquez et al. (Davila-Vazquez et al., 2008a), at an initial pH value of 7.5. Each
serum bottle was inoculated with 4.5 g VSS/L and CWP was used as substrate at a concentration of 25 g COD/L. The SHA was calculated dividing the maximum hydrogen production rate by the VSS concentration.

Specific hydrogenotrophic methanogenic activity (SHMA). SHMA was measured in duplicate using 160 mL glass serum bottles, with 80 mL of working volume and 80 mL of headspace. Each serum bottle was inoculated with 4.5 g VSS/L and the headspace was displaced with a mixture of H$_2$/CO$_2$ (80/20 %). The pressure of the headspace was measured periodically during 5 days, calculating the stoichiometric methane production according to reaction 3.1:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad (3.1)$$

### 3.2.4 Analytical methods

Hydrogen, CH$_4$ and CO$_2$ were measured using a 1.0 mL Pressure-Lok® syringe (Valco Instruments, Houston, Texas, USA) by comparing a 500 μL sample from the reactors and serum bottles headspace with high purity standards (Alltech, Deerfield, Illinois, USA) in a gas chromatograph (GC, 6890N Network GC System, Agilent Technologies, Waldbronn, Germany) equipped with a thermal conductivity detector. The column used was a Hayesep D (Alltech, Deerfield, Illinois, USA) with the following dimensions: 10’ x 1/8”x 0.085”. Temperatures of the injection port, oven and the detector were 250, 60 and 250ºC, respectively. Nitrogen was used as carrier gas with a flow-rate of 12 mL/min. Pressure of the headspace from the hydrogenotrophic SHMA assays was measured with a manometer (Model 407910, Extech Instruments Corporation, Massachusetts, USA). Lactose, VFA, and ethanol were analyzed as previously described (Davila-Vazquez et al., 2008a). COD and VSS were analyzed according to Standard Methods (APHA/AWWA/WFE, 2005). All gas measurements are shown at standard conditions (273.15 K and 1 atm).
3.3 Results and Discussion

In this study, five different strategies to diminish the methane production in hydrogenogenic UASB reactors were evaluated. Figures 3.2 to 3.4 show the volumetric hydrogen and methane production rates and the metabolites concentration produced.

To evaluate the biomass activity, SHA and SHMA batch test were carried out at different operational conditions using biomass withdrawn for the reactors and the results are presented in Table 3.1. The acetoclastic methanogenic activity was also evaluated in the biomass from the experiment C and, after more than 30 days of incubation, no methane production was measured (results not shown); therefore it was assumed that only hydrogenotrophic methanogenic activity was present in all experiments.

3.3.1 Evaluation of the pH reduction effect

The first condition evaluated in experiment A was the pH reduction from 5.63 to 5. The total gas production (H₂ + CH₄ + CO₂) at pH 5 was rather similar to the production at pH 5.63, slightly increasing from 1.58 ± 0.51 to 1.69 ± 0.41 L/L-d. Even though the volumetric methane production rate (VMPR) varied from 0.26 ± 0.10 to 0.30 ± 0.06 L/L-d, the volumetric hydrogen production rate (VHPR) decreased from 0.51 to 0.31 L/L-d (Figure 3.2a).

When the pH was further decreased to 4.5 the total gas production diminished abruptly, with average values of 0.49 ± 0.29 L/L-d increasing the methane concentration. Due to the variation in the total gas and methane production, the pH was adjusted again to 5 (Figure 3.2a). This last pH change resulted in similar VHPR and VMPR compared to the previous condition at pH 5.

In this work the strategy of reducing the pH to 5.0 to avoid methane production was not efficient, unlike the findings reported by Wang et al. (2007), where pH 5 was enough to control the methane production. This was due probably to methanogens acclimatization to such low pH (Taconi et al., 2008). Additionally, contrary to the work reported by Yu and Mu (2006), reducing the pH to 4.5 did not favor hydrogen production and even caused a sharp drop in the total gas production. Similar results were found by Taconi et al. (2008),
operating a methanogenic reactor where low production rates were reported at pH between 4.7 and 5.0.

Figure 3.2. Volumetric production rates (a) and metabolites produced (b) at each condition of Experiment A (effect of pH reduction).

However a noticeable effect of the pH decrease was the change in the metabolic pathways (Figure 3.2b). Before pH was diminished to 5, the acetate concentration was higher than 15 mM in the reactor and during all the conditions of experiment A its concentration was kept between 7 and 15 mM. When pH was lowered from 5.6 to 5.0 the butyrate production increased reaching similar concentrations than acetate during the experiment. Propionate increased during the operation at pH 4.5, which corresponds to the low hydrogen production, as has been reported before (Lee and Rittmann, 2009).

The acetate-butyrate pathway has been reported as a hydrogen producer one (Davila-Vazquez et al., 2008a), although hydrogen production did not correspond to the metabolites concentration, which is explained by the hydrogen consumption by methanogens, as was previously proposed by Kim et al. (2004) for hydrogenogenic reactors using a stoichiometric balance. In this sense, Horn et al. (2003) showed that in natural acidic systems like peats, methane is only produced by hydrogenotrophic methanogens at pH 4.4,
suggesting that these microorganisms have a higher resistance to acidic conditions than acetoclastic methanogens. In the present study the methanogenic activity of hydrogen utilizing microorganisms was proved through batch tests (Table 3.1). The SHA and the SHMA in experiment A were similar at the beginning and at the end of the experiment, following the same trend than the reactor performance.

It has to be highlighted that the batch assays were not carried out under the same conditions to which the biomass was exposed in the reactor (i.e. substrate concentration, pH, H₂ and CO₂ partial pressure); therefore activity measurements only show the tendency followed by the specific activity over the different operational conditions evaluated.

Table 3.1. Results of the specific hydrogenogenic (SHA) and hydrogenotrophic methanogenic activities (SHMA) batch tests with the biomass withdrawn from the reactors at different days.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>pH</th>
<th>HRTa</th>
<th>OLRb</th>
<th>Operation Day</th>
<th>SHAc</th>
<th>SHMAd</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5.6</td>
<td>6</td>
<td>20</td>
<td>0</td>
<td>1561.4</td>
<td>(229.3)</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
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<td>C</td>
<td>5.3</td>
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<td>124</td>
<td>536.3</td>
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<td>421.0</td>
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<td>D</td>
<td>5.3</td>
<td>8</td>
<td>48</td>
<td>75</td>
<td>2513.7</td>
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<tr>
<td>E</td>
<td>5.5</td>
<td>8</td>
<td>48</td>
<td>133</td>
<td>1193.0</td>
<td>(84.3)</td>
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<td>48</td>
<td>146</td>
<td>279.3</td>
<td>(17.0)</td>
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Average value (standard deviation), n=2; a hydraulic retention time (h); b organic loading rate (g COD/L-d); c specific hydrogenogenic activity (mL H₂/g VS-d) and d specific hydrogenotrophic methanogenic activity (mL CH₄/g VS-d).

3.3.2 Evaluation of the organic shock load: effect of increasing influent substrate concentration

The increase in the OLR was evaluated in experiment B and in the first stage of experiment D (Figures 3.3 and 3.4). In experiment B the OLR was increased stepwise from 20 to 40 g COD/L-d. The total gas produced in the different OLRs had the following values: 2.49 ± 0.54, 2.32 ± 0.46 and 1.73 ± 0.47 L/L-d, for 20, 30 and 40 g COD/L-d, respectively. The first increment to 30 g COD/L-d had a positive effect in the hydrogen content in the gas,
increasing the VHPR in 172 %, and decreasing the VMPR in 75%. The increment from 30 to 40 g COD/L-d diminished the hydrogen concentration causing a lower VHPR ($0.27 \pm 0.14$ L/L-d), and a similar VMPR (Figure 3.3a). The SHA and SMA results from experiment B followed the same trend than the reactor performance (Table 3.1).

In the first stage of experiment D the OLR was increased from 26 to 37 g COD/L-d, causing an increment in both, the VHPR and the VMPR by 91 and 75%, respectively (Figure 3.4a), however the VMPR was higher than the VHPR. A recent study showed that an increment in the OLR favored the hydrogen production and decreased the methane production (Spagni et al., 2010), nevertheless in the present work this trend just worked for the first OLR increment in experiment B. An important difference between both studies is the period of time previous to the OLR increase, meanwhile Spagni et al. (2010) kept their reactor in a lower OLR of 7.6 g COD/L-d for only five days until the methane occurrence, in our experiments the reactors came from previous studies that lasted few months (Carrillo-Reyes et al., 2012), most probably developing a stable methanogenic community.

![Figure 3.3. Volumetric production rates of Experiment B and C (a) and metabolites concentration at each condition (b). Organic loading rate, OLR; Hydraulic retention time, HRT.](image_url)
To reach higher OLR, the substrate COD concentration in the influent was increased, which was reflected in the metabolites concentration produced (Figures 3.3b and 3.4b). As it is evident from these results, it seems that the metabolic pathway was not shifted at the conditions tested and, as a consequence, the observed reduction of VMPR (experiment B) may have been due to an inhibitory effect on the methanogenic activity caused by the volatile fatty acids accumulation, around 60 mM as was reached by Duangmanee et al. (2007) at a pH of 5.5, even though this activity was not completely eliminated.

3.3.3 Evaluation of the organic shock load: effect of decreasing HRT

For the sake of comparison with the aforementioned results (section 3.2), the effect of a higher OLR but with the same substrate concentration was evaluated in the second stage of experiment D; the HRT was reduced from 13 to 10 h with the consequent increase in OLR from 37 to 48 g COD/L-d (Figure 3.4a). Noteworthy, this change increased slightly the total gas production from $3.45 \pm 0.47$ to $4.3 \pm 0.92$ L/L-d, nonetheless the change did not produced significant changes neither in the VHPR and VMPR nor in metabolites concentration. These results may indicate that only the organic shock load due to the increase in the influent substrate concentration has a control effect in the methanogenic activity.

During the experiment C and in the third stage of experiment D (Figures 3.3 and 3.4) a HRT decrease was evaluated, from 6 to 3 and 10 to 8 h, respectively, keeping the same OLR (40 g COD/d) than in the previous stages. Results of experiment C shown that both VHPR and VMPR increased (figure 3.3a) as a result of an increment in the hydrogen and methane concentration in the biogas (15 to 16 % and 12 to 27 %, respectively), and as was expected, the methane production was favored due to the lower metabolites concentration (figure 3.3b). In experiment D there was no effect neither in the gas production (around 4 L/L-d), nor in both VHPR and VMPR.

In both cases there were changes in the metabolic pathway (Figures 3.3b and 3.4b), a decrease in the butyrate concentration was observed with the consequent decrease of the molar butyrate/acetate (B/A) ratio. In experiment C the B/A ratio decreased from 0.72 to 0.27, and in experiment D from 1.09 to 0.55. Zhao et al. (2008) reported that lower B/A
ratios in the range from 0.57 to 3.65 produce low hydrogen rates. In this study the metabolic change, measured as the change in the B/A ratio, produced an increase in the VHPR in the experiment C whereas in experiment D there was no effect. Besides the metabolic pathway, the metabolite concentrations have also to be considered. The decrease of the substrate concentration from 10 to 5 g COD/L (experiment C) had an expected decrease on the methane inhibition, in contrast with the decrease from 20 to 16 g COD/L in experiment D, where the methane production remained the same (Figure 3.4a). It is clear that more research is needed in order to elucidate the VFA inhibitory concentration for the methanogens in this kind of systems.

![Figure 3.4](image.png)

**Figure 3.4.** Volumetric production rates of Experiment D and E (a) and metabolites concentration at each condition (b). Organic loading rate, OLR; Hydraulic retention time, HRT; Heat treatment, H.T.

### 3.3.4 Heat treatment

During the experiment E (Figure 3.4) a heat treatment was applied to the biomass withdrawn from the last stage of experiment D and it was reinoculated into the reactor, operating again at the same HRT (8 h) and OLR (48 g COD/L d) of the last stage. This
strategy was efficient to prevent the methane production during the experiment, and the VHPR increased 79% in average. The VHPR reached values of 1.67 ± 0.06 L/L-d, however the gas production decreased gradually and during the last 10 days of operation the VHPR increased again up to 0.88 ± 0.17 L/L-d. The specific activities (Table 3.1) show a decrement in the SHA activity over the time; comparing the SHA immediately after the heat treatment and after 30 days of reactor operation there was a 76% decrement.

It has to be pointed out that during the SMA measurements, the pressure dropped gradually, indicating a consumption of a component of the gas phase in the serum bottles, even that methane was not detected in the head space at the end of the experiments, indicating a H₂ consumption by a different pathway. Besides the methanogenesis, the other possible hydrogenotrophic pathway is homoacetogenesis, which can be present in hydrogenogenic systems as was proposed by Dinamarca and Bakke (2009). These authors carried out different experiments in CSTRs with 8, 12 and 17 hours of HRT, reporting that longer sludge retention time produced faster hydrogen consumption, suggesting a link between the homoacetogenic activity, high biomass concentration and high biomass retention time. Recently Parameswaran et al. (2010) found similar coexistence of methanogens and homoacetogens in a microbial electrolysis cell inoculated with thickened anaerobic digested sludge and return activated sludge. They demonstrated with molecular techniques that once the methanogens were inhibited with bromoethanesulfonate (BES), the homoacetogenic bacteria were significant in the microbial community, in contrast when no BES was added in the feeding.

According to reaction 3.2, the homoacetogenic activities measured at day 133 and 146 from experiment E were 14.66 ± 0.09 and 18.29 ± 2.69 mL H₂ consumed/g VS-d, respectively, implying an increase in the homoacetogenic activity over time.

\[ 2\text{CO}_2 + 4\text{H}_2 \rightarrow \text{CH}_3\text{COOH} + 2\text{H}_2\text{O} \]  \hspace{1cm} (3.2)

The metabolites distribution (Figure 3.4b) showed a higher propionate production in the reactor, equivalent in average to the acetate production, although with a high variation during the experimental period. The high acetate concentration produced in the last 20 days of experiment E (47.5 ± 3.9 mM) did not correspond to the expected hydrogen production, this fact can be explained taking into account the homoacetogenic activity. Recently an
equation to calculate the acetate produced by homoacetogenic pathway was proposed (Arooj et al., 2008; Luo et al., 2011):

\[ A_{\text{homoacet}} = \frac{(2[\text{Ac}]+2[\text{But}]-[\text{Prop}]-[\text{H}_2])}{6} \]  

(3.3)

Figure 3.5 shows the proportion of acetate produced and hydrogen consumed by homoacetogenic pathway according to equation 3.3 and reaction 3.2, respectively. It has to be noticed that the homoacetogenic activity increased in the second half of experiment E, remaining stable and causing a considerable decrement in the hydrogen production potential of the reactor, from around 250 mmol/d to a real hydrogen production of 40 mmol/d.

![Figure 3.5. Theoretical acetate produced and hydrogen consumed by homoacetogens expressed as molar rates during Experiment E.](image)

### 3.4 Conclusions

The organic shock load was the more effective strategy to decrease the methane production, mainly due to the higher influent organic substrate concentration. Nonetheless, it is necessary to evaluate different ranges of organic substrate concentration to identify the limits of this strategy, considering that high VFA concentration could also be inhibitory for the hydrogen production due to the increase of undissociated acids. Once the methanogens are completely inhibited, the homoacetogenic activity diminishes considerably the
hydrogen production potential. Studies like this one are relevant considering that potential substrates for hydrogen production could take hydrogen-consuming microorganisms into the fermentation system.

3.5 References


Chapter 4

Community dynamics during methane decreasing strategies in UASB hydrogenogenic reactors

Summary

Methane occurrence is a common concern in hydrogen producing reactors. In the present study the microbial community structure was analyzed during the application of different operational strategies to decrease methane production in UASB hydrogen producing reactors. The pH reduction selected homoacetogenic organisms related to Blautia hydrogenotrophica and Oscillibacter valericigenes, and the hydrogen producer Enterobacter aerogenes. The organic loading rate increment from 20 to 30 g COD/L-d, selected the hydrogen producers Clostridium tyrobutyricum, Citrobacter freundii and E. aerogenes; further increments caused an inhibition in hydrogen production due to the high undissociated acids concentration. Repeated heat treatment to the biomass completely inhibited methane production, selecting hydrogen producers capable to sporulate, even though homoacetogens were also selected. The archaeal community was represented by hydrogenotrophs from genera Methanobrevibacter and Methanobacterium in all experiments. This study shows that operational strategies can select hydrogen producing bacteria, however methanogen activity is not completely inhibited, unless a second heat treatment is applied.
4.1 Introduction

Hydrogen is considered a clean fuel, which can be used directly in energy fuel cells to produce electricity and has the greatest energy content among conventional fuels. Moreover, hydrogen can be produced by dark fermentation of organic wastes, making this a promising technology for a renewable energy source (Kapdan and Kargi, 2006). For the continuous hydrogen production by dark fermentation, the use of mixed cultures for the continuous hydrogen production by dark fermentation is more feasible than pure cultures. Mixed cultures have increased adaptation capacity therefore complex substrates can be used, and do not require sterile conditions (Abreu et al., 2011; Luo et al., 2011). Nevertheless, the use of mixed cultures such as anaerobic sludge can promote the proliferation of microorganisms that consume the hydrogen produced (e.g., methanogens and homoacetogens), decreasing the system efficiency. This is a common problem in biomass fixed reactors such as UASB, even though the inoculum used has been subjected to a pretreatment to select the hydrogen producing microorganisms (Carrillo-Reyes et al., 2012; Castello et al., 2009; Spagni et al., 2010).

Some strategies to reduce methane production in hydrogenogenic reactors are: i) acidic pH (Wang et al., 2007), diminishing the pH below the methanogenic optimal (6-7); ii) applying a shock load, creating an imbalance from the methanogen step due to the accumulation of the volatile fatty acids (CordRuwisch et al., 1997; Spagni et al., 2010) and iii) repeated heat treatments (Duangmanee et al., 2007), enriching the biomass with spore forming microorganisms eliminating the methanogens.

Recently, Luo et al. (2011) analyzed the effect of different pretreatments of the inoculum, acid treatment, heat treatment and shock load in repeated batch tests, concluding that the inhibition of methanogenesis and homoacetogenesis was dependent on fermentation conditions, and not on inoculum pretreatment. However, in that work the methanogen community structure was not evaluated, only the bacterial one. Abreu et al. (2011) carried out a similar study, evaluating the effect of different pretreatments (heat treatment, BES and BES + chloroform) in EGSB reactors performance and in the bacterial community. Such treatments completely inhibited the methanogens and prompted the homoacetogenic activity.

In a previous work (Carrillo-Reyes et al., 2011) we evaluated the effect of acid pH, shock
loads and repeated heat treatment in UASB reactors to diminish the methane production. We concluded that the shock load was more efficient than the pH and HRT decrement. Nonetheless, once the methane production was completely inhibited with the heat treatment, the homoacetogenic activity became relevant diminishing the hydrogen producing potential by approximately fourfold.

There is a lack of studies that evaluate the archeal and bacterial community response during different strategies to reduce the methane production. It is necessary to establish if there is a link between the diversity and reactor performance and if any specie is determinant in the methane occurrence after the aforementioned strategies were applied. The use of 16S rDNA-based methods employing denaturing gradient gel electrophoresis (DGGE) (Davila-Vazquez et al., 2009), molecular cloning and sequencing (Abreu et al., 2010; Offre et al., 2009) can provide an accurate estimation of the bacterial and archeal community composition, distribution and diversity.

Therefore the aim of this work was to evaluate the community dynamics using molecular techniques during the application of different strategies to inhibit the methanogenic activity in hydrogen producing UASB reactors that had been in operation for extended periods of time using cheese whey powder (CWP) solution as a synthetic dairy wastewater.

4.2 Materials and methods

4.2.1 Substrate

The CWP was purchased from Grande Custom Ingredients Group (Wisconsin, USA). The soluble lactose content of CWP was 75.5% with 11.5% protein (w/w). An initial stock solution of 100 g CWP/L was centrifuged 10 minutes at 13000 rpm to remove insoluble material. After that, soluble chemical oxygen demand (COD) of the solution was determined. The medium contained a known amount of CWP-COD and was supplemented with a nutrient solution previously described (Carrillo-Reyes et al., 2012). Additional Na₂HPO₄ was added to increase the buffer capacity of the medium and controlling the pH at a desired value.
4.2.2 Reactors and inoculum

Three different UASB reactors were used to carry out the present experiments. The working volumes of the reactors was between 0.47 and 1.3 L, and were inoculated with heat treated anaerobic sludge from a confectionary factory at San Luis Potosí, México, to reach a solids concentration from 13 to 20 g VVS/L. The main objective of the reactors used was the hydrogen production from cheese whey, although eventually during their operation methane started to be produced concomitant to the hydrogen, for periods not less than 20 days before the present experiments were performed.

4.2.3 Experimental set-up

Five different experiments (A to E) were carried out to evaluate the effect of different methane decreasing operational strategies in the microbial community of the aforementioned UASB reactors. The details of each experiment are described as follows:

Experiment A: the effect of pH reduction was tested by decreasing the reactor pH from 5.63 to 5.0 and 4.5, and reestablishing the pH again to 5.0. OLR and HRT were kept at 20 g COD/L-d and 6 h respectively.

Experiment B: the effect of the organic shock load was tested by increasing the OLR from 20 to 30 and finally to 40 g COD/L-d. The OLR was increased by incrementing the COD influent concentration while the HRT was kept constant at 6 h.

Experiment C: the effect of the organic shock load was further tested by operating at the previous OLR (40 g COD/L-d), but by means of decreasing both the HRT and the COD influent concentration.

Experiment D: the effect of the organic shock load was also tested in this experiment by increasing the OLR from 26 to 37 by means of raising the COD concentration in the influent at constant HRT (13 h). Afterwards the OLR was increased up to 48 g COD/L-d by decreasing the HRT to 10. Then the HRT was further decreased to 8 h, while reducing the COD concentration in the influent in order to maintain an OLR of 48 g COD/L-d.
Experiment E: the granules of the experiment D were harvested, heat treated (boiled 1 hour) and re-inoculated to evaluate the effect of a second heat treatment, keeping the same operational parameters applied in the last stage of experiment D.

The pH in the reactors during experiments B to E was 5.3 - 5.5.

4.2.4 Analytical methods

Hydrogen, CH₄ and CO₂ were measured with a 1.0 mL Pressure-Lok® syringe (Valco Instruments, Houston, Texas, USA) by comparing a 500 μL sample from the reactors and serum bottles headspace with high purity standards (Alltech, Deerfield, Illinois, USA) using a gas chromatograph (GC, 6890N Network GC System, Agilent Technologies, Waldbronn, Germany) equipped with a thermal conductivity detector. The column used was a Hayesep D (Alltech, Deerfield, Illinois, USA) with the following dimensions: 10´ x 1/8’’ x 0.085”.

Temperatures of the injection port, oven and the detector were 250, 60 and 250 ºC, respectively. Nitrogen was used as carrier gas with a flow-rate of 12 mL/min. Lactose and VFA were analyzed by capillary electrophoresis and ethanol by gas chromatography as previously described (Davila-Vazquez et al., 2008a). COD and VSS were analyzed according to Standard Methods (APHA/AWWA/WFE, 2005). The biogas measurements are presented in standard conditions (273.15 K and 1 atm).

4.2.5 Microbial community analyses

DNA extraction. Ten milliliters of sludge withdrawn from each operational condition evaluated were kept at -20°C. Genomic DNA was extracted the DNA as described elsewhere (Wisotzkey et al., 1990).

PCR amplification. Amplification of the 16S rRNA gene from the purified nucleic DNA preparations was carried out by PCR using Taq DNA polymerase (DONGSHENG, China). Almost complete bacterial 16S rDNA was selectively amplified for cloning and sequencing using 27-F (5’-AGAGTTTGATCCTGGCCAG-3’) and 1492-R (5’-GGTTACCTTGGTTCAGCTT-3’) primers with the following thermocycling program: 94°C for 3 min; 35 cycles at 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min; and 72°C
For 10 min (Martin-Laurent et al., 2001). For DGGE a specific region of the 16S rDNA was amplified using the primer 357F-GC (5’-CGCCCGCCGCACGGGCCGCGGGGCACGGGGGCCTACGGGAGGC AGCAG-3’) and the reverse primer 907R (5’-CCGTCAATTCMTTTTGAGTTT-3’), reaction conditions were as follows: 96 °C for 4 min, followed by 10 cycles at 94°C for 30 s, 61°C for 1 min (with a reduction of 0.5 °C in each cycle), and 72°C for 1 min; in addition, 20 cycles at 94°C for 30 s, 56°C for 1 min and 72°C for 1 min; with a final extension at 72°C for 7 min were performed (Piña-Salazar, 2011).

For archaea, primers Arch109(K) (5’-ACKGCTCAGTAACAC GT-3’) and Uni1492-R (5’-CGGCTACCTTGTTACGAC-3’) were used for cloning with the following thermocycling program: 95ºC for 5 min; 25 cycles at 95ºC for 30 s, 52ºC for 40s, and 72ºC for 90 s; and 72ºC for 5 min (Sousa et al., 2007). Primers A109(T)-F (5’-ACTGCTCAGTAACACGT-3’) and 515-GC-R 5’CGCCCGGGCGCGCCCCCGGGCGGGGCGGGGCGGGGACGGGGGGATCGTATTACC GCGGCTGCTGGCAC-3’) were used for the archaeal DGGE analysis, with the following thermocycling program: 94 °C for 5 min; followed by 30 cycles at 94 °C for 30 s, 52 °C for 1 min, 68 °C for 1 min; followed by 7 min at 68 °C (Roest, 2007).

**DGGE analysis.** DGGE was performed with Dcode Universal Mutation Detection System (Biorad, Hercules, California, USA). 20 μL of the PCR products were loaded onto 8% polyacrylamide gels in 0.5 X TAE buffer (20 mM Tris–acetate, 10 mM sodium acetate, 0.5 mM EDTA, pH 8.0) with a denaturing gradient (urea–formamide) that ranged from 30 to 60% and 30 to 50%, for bacterial and archaeal community fingerprint, respectively. Electrophoresis was carried out at 60°C at a constant voltage of 70 V for 16 hours. The gel was fixed with acetic acid solution at 10% during 30 min at 40-50 rpm. After it was washed out three times with deionized water at the same agitation, then the gel was submerged in AgNO₃ solution (1 g/L) during 30 min, without light incidence. Later the gel was developed with sodium carbonate solution (23.3 g/L) during 15 min. Afterwards it was submerged in the fixation solution during 5 min, finally the gel was rinsed with deionized water. The gel bands were observed at visible light, and a photo with a digital camera was taken. DGGE gels were analyzed with the Cross Checker v 2.91 software (Wageningen University, The Netherlands) to create a binary matrix, corresponding dendrograms...
showing the relationships between the DGGE profiles were constructed with Phylogeny Inference Package v 3.69 (University of Washington, USA), using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Similarity between groups was calculated using the Dice coefficient.

Cloning. PCR products obtained with the primers pair 27-F and 1492-R, and Arch109(K)-F and Uni1492-R, were ligated into pGEM-T vector using the pGEM Easy Vector Systems kit (Promega), and introduced into competent Top10 E. coli. 12 positive transformants were selected (by blue/white screening) and grown in LB media supplemented with ampicillin. After cell lysis, inserts were amplified using the primer set M13-F (5’-CGCCAGGGTTTCCCCAGTCACGAC-3’) and M13-R (5’-AGCGGATAACATTTCCACACAGG-3’) and the obtained PCR products were analyzed in agarose gel (1%) in order to select clones with the right insert fragments. Amplicons of the correct size were screened by amplified ribosomal DNA restriction analysis (ARDRA), using the restriction enzymes MspI and HinfI, incubated during 2 hours at 37°C. The restriction fragments were analyzed by electrophoresis in 2.5% (w/v) agarose gel and visualized with ethidium bromide. Amplification with M13 primers of selected transformants, with different ARDRA patterns and corresponding to predominant bands in the DGGE community fingerprint, were subjected to DNA sequence analysis. The PCR products were sent to purification and sequencing to “Laboratorio Nacional de Biotecnología Agrícola, Médica y Ambiental” (LANBAMA, IPICYT, Mexico). The presence of some clones was inferred by the position of their DGGE fragment in the fingerprint, in relation with fragments in the same position in other samples.

Phylogenetic Analysis. 16S rRNA gene partial sequences were depurated with the BioEdit V7.1.3 software package (Hall, 1999). Consensus sequences were checked for potential chimera artifacts by the Pintail software V1.0 (Cardiff University, UK). Similarity searches for the partial 16S rRNA gene sequences were performed using the NCBI BLAST search program within the GenBank database. Phylogenic assignment of the sequences to higher-order taxa was performed using the RDP Naïve Bayesian Classifier. In order to dereplicate the sequences and to group the 'similar' sequences together in Operational Taxonomic Units (OTU), the FastGroupII tool was used (Yu et al., 2006). 16S rRNA sequences were further aligned by using the Clustal X V2.0 software (Larkin et al., 2007). The resulting alignments
were used for the construction of a 16S rRNA gene-based phylogenetic tree. Due to the low number of archaeal sequences recovered, only the bacterial phylogenetic tree is included.

4.2.6 Statistical analysis

In order to explore the effects of the operational parameters (pH, HRT, OLR) and the repeated heat treatment in the gas and metabolites production, data were analyzed using a cross-correlation matrix of the Spearman’s rank correlation coefficients (Ramette, 2007). Then, the significant correlations ($P < 0.05$) were tested with the non parametric multivariate analysis of variance (MANOVA) (Anderson, 2001), in order to elucidate the intensity of such relations. All statistical analyses were performed with the R environment (R Development Core Team, 2009). Spearman correlations and non parametric MANOVA were run using the R “Pspearman” and “Vegan” packages, respectively (Oksanen et al., 2012; Savicky, 2009).

4.3 Results and Discussion

In this study the microbial community dynamics was evaluated during five different strategies to diminish the methane production in UASB hydrogen producing reactors. Samples analyzed were taken at each evaluated condition. The average hydrogen and methane production rate are shown in Figure 4.1. The metabolites concentration for each experiment is shown in section 3.3 (Figures 3.3 to 3.4).

4.3.1 pH reduction

Figure 4.1a shows that the volumetric hydrogen production rate diminished from 0.51 (with high variability) to 0.25 L/L-d, from a pH value of 5.63 to 5; whereas the methane production rate remained the same varying from 0.26 to 0.32 L/L-d. The Spearman’s correlation coefficients of experiment A showed a positive significant association between pH and the H$_2$ production rate, acetate, and ethanol production. In other words, the positive association indicates that pH is directly proportional to metabolites concentration. In
contrast, butyrate production was associated negatively with the pH. Similar relations between pH and the hydrogen and metabolites production were found by Fang and Liu (2002) in a CSTR at pH values from 4.0 to 7.0. In this sense, according to the MANOVA results, the pH control explained 36.63% of the variance of metabolites and H₂ production in experiment A.

The similarity index of the bacterial fingerprint showed 50% of similitude between the first and the last condition evaluated in experiment A, explained by a bacterial community selection. Among all experiments, 23 bacterial OTUs (BacOTU) were identified. The Figure 4.2 shows the phylogenetic tree indicating the closest relative organisms.

**Figure 4.1** Volumetric production rates of Experiment A (a), Experiments B and C (b), and Experiments D and E (c). H.T. (Heat treatment).
The relation of Bacterial OTUs sequenced and the DGGE profile, showed that BacOTUs 1 and 2 that prevailed in experiment A, were related to Blautia hydrogenotrophica and Oscillibacter valericigenes, reported as homoacetogenic microorganisms (Fonty et al., 2007). The presence of homoacetogenic activity at pH values below 5.5 have been proved before, even though this is not the most favorable pH value (Calli et al., 2008).

A higher abundance of OTUs related to Propionibacterium acidipropionici and Lactobacillus casei, BacOTUs 5 and 6, respectively, were identified at the end of the experiment A (pH 5). The former a propionic acid producer (Zhang and Yang, 2009) and the latter a lactic acid producer, both contributed to the low hydrogen production due to the use of the available electrons to produce reduced compounds instead of hydrogen. Even though the low hydrogen production can be explained by the putative presence of hydrogen consuming and non producing hydrogen bacteria, in the last condition evaluated in experiment A (pH 5), it was inferred the presence of Enterobacter aerogenes, a hydrogen producing bacteria (Zhang et al., 2009), related with BacOTU 15.

Table 4.1 shows the archeal OTUs (ArcOTU) identified for experiments A to E. In the case of experiment A, all the ArcOTU were present in the DNA extracted, except by the ArcOTU 6. The species related belong to the Methanobacteriaceae family, from the genera Methanobrevibacter and Methanobacterium, both presenting hydrogenotrophic activity (Huber, 2001). As Figure 4.1a depicts, the volumetric methane production rate was only affected at pH 4.5, probably by the low hydrogen production, in the other pH values evaluated methane was produced at similar rates. Other studies for hydrogen production have only found methane activity at pH above 5, in completely mixed and fixed biomass reactors (Fang and Liu, 2002; Wang et al., 2007). Nevertheless, in an anaerobic digester methane activity was present at pH values between 4.0 to 5.3, which can be explained by an acclimated consortium and the presence of micro environments (Taconi et al., 2008).

4.3.2 OLR increments

During experiment B the OLR was increased from 20 to 30 and 40 g COD/L-d. According to the Spearman’s correlations, the increase in the OLR had a significant effect in the hydrogen, acetate, and propionate production.
Figure 4.2 Phylogenetic relationships of partial 16S rRNA gene sequences recovered from three clone libraries. The tree was inferred using the neighbor-joining algorithm, with the Tamura Nei model. *Escherichia coli* was used as the outgroup taxon. The scale bar represents 5% sequence divergence; values at the nodes are the percentages of 1,000 bootstrap replicates supporting the branching order; bootstrap values below 50% are not shown. A similarity threshold of over 97% was used for the same OTU assignment.
The MANOVA results showed a significant low variation value of 14.75 %, for the latter correlation. Figure 4.1b shows that only the first increment, from 20 to 30 g COD/L-d, had a positive effect in the hydrogen production rate.

The dendrogram (Figure 4.3a) shows that the bacterial communities at 20 and 40 g COD/L-d in experiment B were clustered together. In these conditions the hydrogen production rate was similar around 0.28 L/L-d (Figure 4.1b); the DGGE profile shows the same dominant bacterial species, whereas the methane production rate decreased from 0.71 to 0.22 L/L-d.

**Figure 4.3.** Bacterial DGGE community fingerprints and corresponding similarity dendrograms and indexes. a) Experiment B and C, b) Experiment D and E. The numbers correspond to the different Bacterial OTUs associated with the DGGE fingerprint.

The DGGE bacterial fingerprint from experiment B at 30 g COD/L-d was clustered separately from the community of the other conditions evaluated. At this condition was observed the highest and the lowest hydrogen and methane production rate (0.90 and 0.18 L/L-d) respectively, from the experiments B and C.

During the first stage of experiment B (20 g COD/L-d) BactOTUs 5 and 6 related to *Propionibacterium acidipropionici* and *Lactobacillus casei* were identified, which could reduce the hydrogen production efficiency, as was discussed above. Moreover, it was identified the BactOTU 4 related to anaerobic fermentative bacteria (*Butyricimonas synergistic* and *Parabacteroides johnsonii*) with non reported hydrogen production capacity.
(Sakamoto et al., 2007; Sakamoto et al., 2009). When the OLR was increased to 30 g COD/L-d the dominant BacOTUs 9, 14 and 8 were related to *Clostridium tyrobutyricum*, *Citrobacter freundii* and *Enterobacter aerogenes*, all hydrogen producers (Jo et al., 2008; Kumar and Vatsala, 1989; Zhang et al., 2009), corresponding to the highest hydrogen production rate in experiment B.

In the last condition of experiment B (40 g COD/L-d), even though BacOTUs 9, 8 and 11 were related to hydrogen producers such as *Clostridium tyrobutyricum*, *Enterobacter aerogenes* and *Clostridium ramosum* (Lin et al., 2006), it was also was present the BacOTU 22, related to *Clostridium ljungdahlii*, an homoacetogenic organism (Köpke et al., 2010), reducing the hydrogen production. Another factor that contributed to the low hydrogen production was the undissociated VFA concentration of 13.4 mM. According to Castro-Villalobos et al. (2012), a 10 mM concentration of undissociated fermentation by-products caused a biomass growth inhibition in a hydrogen producing system.

During experiment B all the ArcOTUs included in Table 4.1 were identified, proving that methane was produced by hydrogenotrophic activity. Methane production was decreased from 20 to 30 g COD/L-d, as was expected by the increment in the substrate concentration (Spagni et al., 2010). The Spearman’s correlations showed a negative significant relation between acetate and butyrate concentration and the volumetric methane production, confirming statistically this association. The last increment to 40 g COD/L-d did not affect the methane production in comparison with 30 g COD/L-d, in spite of the low hydrogen productivity (Figure 4.1b).

### 4.3.3 HRT decrements

In order to evaluate the effect of the HRT in the experiments C and D (Figures 4.1b and c) the HRT was diminished from 6 to 3 and from 10 to 8 h, respectively. Bacterial DGGE fingerprint corresponding to the HRT decrement in the experiment C had 60 % of similitude with the previous HRT evaluated (Figure 4.3a). This HRT decrement had a positive effect in the hydrogen and methane production (Figure 4.1b), favored by the low substrate concentration. According to the MANOVA, the HRT explained 19.7 % of the experiment variance in a significant way. Important changes in the community due to the
HTR reduction were the occurrence of the BacOTU 6 (*Lactobacillus casei*) and the disappearance of the BacOTU 11 (*Clostridium ramosum*), the latter a hydrogen producer.

Table 4.1. Affiliation of the Archaeal OTUs identified showing the highest percentage of identity in the output result from the analysis in the non-redundant nucleotide database from NCBI using the BLAST program.

<table>
<thead>
<tr>
<th>ArcOTU</th>
<th>pB sequence</th>
<th>Closest relative</th>
<th>GenBank accession number</th>
<th>Percentage of identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>983</td>
<td><em>Methanobreivibacter arborophilus</em></td>
<td>NR_042783</td>
<td>99</td>
</tr>
<tr>
<td>2</td>
<td>972</td>
<td><em>Methanobacterium congelense</em></td>
<td>NR_028175</td>
<td>98</td>
</tr>
<tr>
<td>3</td>
<td>900</td>
<td><em>Methanobacterium bryantii</em></td>
<td>NR_042781</td>
<td>96</td>
</tr>
<tr>
<td>4</td>
<td>767</td>
<td><em>Methanobacterium oryzae</em></td>
<td>NR_028171</td>
<td>95</td>
</tr>
<tr>
<td>5</td>
<td>959</td>
<td><em>Methanobacterium palustre</em></td>
<td>NR_041713</td>
<td>96</td>
</tr>
<tr>
<td>6</td>
<td>970</td>
<td><em>Methanobacterium alcaliphilum</em></td>
<td>NR_028228</td>
<td>97</td>
</tr>
<tr>
<td>7</td>
<td>839</td>
<td><em>Methanobacterium congelense</em></td>
<td>NR_028175</td>
<td>97</td>
</tr>
</tbody>
</table>

The bacterial community during the HRT decrement in experiment D had 60 % of similitude with the community at the previous condition, 48 g CDO/L-d at 10 h of HRT (Figure 4.3b). However, hydrogen methane production rates remained in similar values (Figure 4.1c). The BacOTUs 20 and 14 identified in this experiment were related to *Lactococcus lactis*, a lactic acid producer microorganism, and *Clostridium tyrobutyricum* a hydrogen producer, respectively (Jo et al., 2008). The ArcOTUs identified in both experiments (C and D) for the HRT decrements were all those listed in Table 1. Consequently, the HRT did not affect the methane community diversity, only affecting their activity in experiment C.

In experiment C, the HRT decrement implied a decreased of substrate concentration from 10 to 5 g COD/L, resulting in a decrement of the undissociated acids concentration below the inhibition threshold for hydrogen production (Castro-Villalobos et al., 2012); explaining the slight increment in the hydrogen production rate (Figure 4.1b). Otherwise, in experiment D the reduction of the substrate concentration was from 20 to 16 g COD/L, keeping the undissociated acids concentration above the inhibition threshold, showing similar hydrogen and methane productions rates (Figure 4.1c).
4.3.4 Heat treatment

The repeated heat treatment of experiment E was the only strategy that inhibited completely the methane production, increasing the hydrogen production rate from 0.49 to 1.23 L/L-d. Three samples of biomass were withdrawn from the reactor during this strategy at days 0, 15 and 30. The bacterial DGGE fingerprint shows that the samples taken at day 15 and 30 were clustered together with 82% of similitude (Figure 4.3b). The BacOTUs identified at day 0, corresponded to the hydrogen producing bacteria *Clostridium butyricum* (BacOTU 21) and to the homoacetogenic *Clostridium ljungdahlii* (BacOTU 22). The DGGE profile (Figure 4.3b) shows that the abundance of BacOTU 22 diminished at day 15, and the presence of the *Enterobacter aerogenes* can be inferred, corresponding to BacOTU 15, a hydrogen producing microorganism. At the end of the experiment, the bands corresponding to the BacOTUs 15 and 22 (*Enterobacter aerogenes* and *Clostridium ljungdahlii*) disappeared; *E. aerogenes* is a non spore forming bacteria, which explains its reduced abundance after the heat treatment. The BacOTUs 21 and 23, related to *Clostridium butyricum* and *Lactobacillus rhamnosus*, an acid lactic producer, prevailed during the whole experiment E; the former is a spore forming and the latter is commonly found in dairy effluents such as the substrate used in the present work (Castello et al., 2009). The presence of *Lactobacillus* genus has been reported widely in hydrogen producing systems, but their capacity to produce hydrogen has to be investigated due to contradictory results (Castello et al., 2011). As was mentioned in our previous work (Carrillo-Reyes et al., 2011), in experiment E the homoacetogenic activity was identified in batch test and increased over the time. Due to the spore forming capacity of homoacetogenic bacteria, these could survive to the heat treatment (Luo et al., 2011). Despite the lack of methanogenic activity, was amplified and cloned archeal 16S rDNA, explained by the low DNA degradation in the reactor.

4.4 Conclusions

The strategies applied to decrease the methane production had an effect in the microbial community. Among the operational strategies (pH reduction, shock load and HRT reduction), the shock load enhanced the hydrogen production rate due to the selection of
hydrogen producing microorganisms. However, extended operation times selected organisms that diverted the available electrons to the production of more reduced compounds such as propionate or lactate. The organic shock load strategy had a limitation, related to the high concentration of undissociated acids resulting in the inhibition of hydrogen production.

Only a second heat treatment, applied to the biomass, completely inhibited the methanogenic activity. However the sporulation capacity selected both hydrogen producing and homoacetogenic bacteria, the latter reducing the hydrogen producing potential of the reactor. The presence of microorganisms of the genus *Lactobacillus* (non-spore forming) after the heat treatment, highlighted the importance of the indigenous microorganisms load present in the substrate, indicating that the relevance of this microorganisms in the reactors performance has to be investigated.

This study shows that operational strategies to decrease methane production can select hydrogen producing bacteria. However, when methanogen activity is diminished or completely inhibited, homoacetogenesis bacteria evolve to the main hydrogen consumer activity in detriment of the hydrogen production potential.

### 4.5 References


Chapter 5

Implications of the hydrogen consuming microorganisms in two upflow anaerobic packed-bed reactors treating cheese whey

Summary

Low hydrogen yields in fermentative systems are attributed to the hydrogen consuming activity of homoacetogens, mainly in fixed biomass reactors. In order to determine the implication of this activity, two packed-bed reactors were fed with cheese whey at organic loading rates of 24 and 48 g COD/L-d. Batch assays were carried out to evaluate the specific hydrogen consuming activity using biomass withdrawn from the reactors. During the start-up, maximal hydrogen yields of 2.92 and 2.55 mol H₂/mol lactose consumed were achieved. However, a sudden decrease was observed, following a common trend in this kind of reactors. Batch assays showed homoacetogenesis in the inoculum (obtained from substrate natural fermentation) and its increment over the operation time, up to certain moment where it was replaced by hydrogenotrophic methanogenic activity. Once the methanogenic community was established, its specific activity remained with similar values throughout the reactors operation. DGGE fingerprints showed increasing bacterial and archaeal diversity over the time. The present work demonstrated that the hydrogen consuming activities, homoacetogenic or methanogenic, affected equally the systems evaluated.
5.1 Introduction

Hydrogen production by the fermentative pathway is considered a sustainable alternative for fossil fuels, due to its highest energetic content; moreover, it can be produced through the use of wastes or byproducts (Saxena et al., 2009). Cheese whey, a dairy byproduct, is a potential substrate for hydrogen production, because of its high content of readily biodegradable carbohydrates (Davila-Vazquez et al., 2009). The hydrogen production research has been focused on increasing the yield and productivity, evaluating different factors such as the substrate, reactor configuration, operational parameters and different sources of inoculum, among others (Valdez-Vazquez and Poggi-Varaldo, 2009).

Respect to the reactor configuration, fixed biomass reactors have been used under the principle that higher biomass concentration and higher organic loading rates can be applied, consequently increments in the hydrogen production are expected. In particular, the anaerobic packed-bed reactors have a simple construction, there is no need of mechanical agitation, and a low hydraulic retention time can be applied (Peixoto et al., 2011).

One of the highest hydrogen yields achieved in continuous hydrogen systems was 3.25 mol$_{\text{hydrogen}}$/mol$_{\text{glucose}}$, using a mixed culture as inoculum and a constant nitrogen flush to diminish the hydrogen partial pressure (Bastidas-Oyanedel et al., 2012). In practice, the theoretical yield of 4 moles of hydrogen per mol of hexose, is far to be reached in systems with mixed cultures and fed with real wastewaters.

The low hydrogen production yields can be caused by mixed pathways such as the acetate and butyrate, or by the production of reduced compounds such as propionate or lactate, acting as electron sinks (Dinamarca and Bakke, 2009). When the metabolites profile shows a predominant acetate fermentation and low hydrogen yield, some authors have proposed the occurrence of homoacetogenic activity, which is the autotrophic acetate production using hydrogen and carbon dioxide, therefore, diminishing the hydrogen production (Arooj et al., 2008; Carrillo-Reyes et al., 2011).

There are contradictory results about the relevance of the homoacetogenic activity, whereas Kraemer and Bagley (2008) established that the homoacetogenic activity was insignificant, several authors have attributed the low hydrogen yields to this pathway (Dinamarca and Bakke, 2009; Fontes Lima and Zaiat, 2012).
The homoacetogenic activity is promoted by a high hydrogen partial pressure and high cellular retention time. The hydrogen partial pressure threshold for the homoacetogenic activity (520-950 ppm) is easily reached in hydrogen producing systems. Moreover the homoacetogens are a very cosmopolitan trophic group present in anaerobic mixed cultures and natural environments (Cord-Ruwisch et al., 1988; Dinamarca et al., 2011). Our previous work confirmed the occurrence of homoacetogenic activity in batch assays using biomass withdrawn from a hydrogen producing UASB reactor (Carrillo-Reyes et al., 2011). Arooj et al. (2008) proposed a stoichiometric balance to calculate the hydrogen consumed by homoacetogenesis based on the theoretical yield and the acetate and butyrate concentration in the medium.

Homoacetogens are a versatile bacterial functional group, capable of developing an autotrophic or heterotrophic growth. It is suggested that homoacetogens will switch from heterotrophic to autotrophic metabolism when beneficial (Dinamarca et al., 2011; Oh et al., 2003).

The inoculum is an important factor to control in hydrogen producing reactors, commonly an anaerobic treated sludge is used, although, hydrogenotrophic methanogenic activity can be found. An alternative to avoid the presence of methanogens is the use of naturally fermented substrate as inoculum, which may favor the growth of the microorganisms present in the substrate and in the environment (Fontes Lima and Zaiat, 2012; Peixoto et al., 2011). According to our knowledge there are not systematic analyses of the relevance of homoacetogenic activity over the time in hydrogen producing reactors. The emergence of homoacetogenic activity could be a response of the hydrogen concentration in the reactor, therefore the specific hydrogen consumption activity could have values according to the hydrogen production of different reactors.

The aim of the present work was to evaluate the hydrogen consuming activity over the operation time in two hydrogen producing anaerobic packed bed reactors fed with cheese whey. The specific hydrogen consuming activity was measured in batch assays using biomass withdrawn from the reactor, the bacterial diversity was analyzed using PCR-DGGE techniques.
5.2 Materials and Methods

5.2.1 Substrate and mineral medium

Cheese whey powder (CWP) was purchased (Elegé®, Brasil Foods S.A.) and used as the only carbon and energy source. A CWP solution was prepared to the desired chemical oxygen demand (COD). A phosphate buffer and nutrients concentration were supplied as follows (mg/L): K$_2$HPO$_4$, 125; NH$_4$H$_2$PO$_4$, 1500; MgCl$_2$ 6H$_2$O, 100; FeSO$_4$ 7H$_2$O, 25; ZnCl$_2$, 75; MnSO$_4$ H$_2$O, 15; CuSO$_4$ 5H$_2$O, 5; CoCl$_2$ 6H$_2$O, 3; Na$_2$B$_4$O$_7$ 10H$_2$O, 0.15; Na$_2$MoO$_4$ 2H$_2$O, 12.5; NiCl$_2$ 6H$_2$O, 0.08; Na$_2$SeO$_3$, 0.01. The Na$_2$HPO$_4$ were adjusted between 1 and 2 g/L to maintain the reactor pH at around 4.9.

5.2.2 Reactor design

Two up-flow anaerobic packed-bed reactors were used (Figure 5.1). The reactors dimensions were: inner diameter of 80 mm, outer diameter of 88mm, length of 820 mm giving 2.69 L, 3.88 L and 4 L of liquid, bed, and total volume, respectively. The reactors were divided into three compartments (the entrance of the influent, the effluent exit and the fixed-bed), which were separated by a stainless steel mesh. Along the fixed-bed section there were 5 sampling ports homogeneously distributed, the influent section had an additional sampling port (Fig. 5.1). The substrate and a effluent recirculation were fed using two positive displacement pumps (Prominent® Concept Plus). The packing material used to attach the biomass was recycled low-density polyethylene (10x10x20 mm), a non-porous material with a surface area of approximately 7.94 m$^2$/g.

5.2.3 Inoculum

The inoculum for each reactor was obtained by the natural fermentation of a CWP solution (40 g COD/L), which was prepared with tap water and stored in an opened container for three days. This process favored the fermentation of the substrate by the microorganisms present in the environment and in the CWP. Between reactor A and B operation was a gap of 40 days, for each reactor an inoculum was prepared at room conditions.
In an independent assay, in order to elucidate the indigenous bacterial contribution of the substrate, the natural fermentation of the CWP was prompted in sterile conditions, with and without the nutrients solution cited in section 5.2.1. In an aseptic work area, CWP was added into a sterilized serologic bottle with sterile water or medium, and then it was sealed. These bottles were incubated at 25°C, after seven days the headspace composition was analyzed and the biomass was recovered for the molecular community analysis.

5.2.4 Reactors operation

The natural fermented inocula were recirculated in the reactors during three days to favor the microorganism attachment to the support. Then the continuous reactors operation began with a hydraulic retention time (HRT) of 2 hours, based on the liquid volume. A recirculation ratio of 1 was applied, and the temperature was controlled at 25 °C. Two organic loading rates (OLR) were evaluated: in reactor A, 24 g COD/L d was applied, whereas in reactor B, 48 g COD/L d was applied, with a substrate concentration of 2 and 4 g COD/L, respectively. Each reactor was operated during 40 days.

![Figure 5.1. Anaerobic-packed bed reactor design.](image-url)
5.2.5 Hydrogen consuming activity batch assays

At days 20, 35 and 40 of reactor A operation; and days 20, 29 and 40 of reactor B operation, biomass samples were withdrawn. To recover the biomass, 50 mL from each sampling port (6 in total) were withdrawn, mixed, and centrifuged. From each of the composed biomass samples and from the inoculum of each reactor, the hydrogen consuming activity batch assays were carried out as follows: in 315 mL bottles, 4.5 g VS/L of biomass was inoculated in 80 mL of working volume and 225 mL of headspace. Mineral medium (section 5.2.1) was used and the pH was adjusted with HCl 1 N to maintain similar conditions as in the reactor. The headspace was flushed with hydrogen (99.9 %) and the inner pressure was equalized to atmospheric pressure. Then 80 mL of headspace were removed with a syringe and replaced with CO₂ (99.9%), as the only carbon source, to keep a H₂/CO₂ ratio of 2/1.

Batch assay were performed in duplicate with a negative control without any biomass. The H₂/CO₂ consumption was followed measuring the headspace pressure using a manometer, and analyzing the headspace composition by gas chromatography. For each bottles the H₂/CO₂ was injected two times and its consumption monitored in order to report the maximum activity. The specific activity was calculated according to the maximum hydrogen consumption rate and the biomass concentration, and expressed as mL H₂ consumed/g SV-d. In order to compare the H₂/CO₂ consumption and the expected acetate consistent with the stoichiometry of reaction 5.1, the initial and final acetate concentrations were also measured.

\[4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \quad (5.1)\]

5.2.6 Analytical methods

The biogas produced in the reactors was recorded using a MilliGascounter (Ritter®) gas meter. The composition of the biogas (hydrogen, carbon dioxide and methane) was determined by gas chromatography (GC 2010 Shimadzu), using a thermal conductivity detector and argon as the carrier gas. The temperatures of the injector, detector and column were maintained at 30 °C, 200 °C and 230 °C, respectively.
Organic acids and solvents were analyzed with a Shimadzu® high pressure liquid chromatography system that was composed of an LC-10ADvp pump, an FCV-10ALvp solenoid valve, a CTO-10Avp oven (working temperature of 45-50 °C), an SCL-10Avp controller, an SPDM10Avp UV detector with a diode array of 205 nm and an Aminex HPX-87H ionic exchange column (0.3 m x 7.8 mm). The moving phase was 0.005 M H$_2$SO$_4$ and had a flow rate of 0.6 mL/min.

Carbohydrate concentrations were determined by the colorimetric method developed by Dubois et al., (1956). COD and volatile solids (VS) were determined in accordance with the Standard Methods for Examination of Water and Wastewater (APHA/AWWA/WFE, 2005).

### 5.2.7 DGGE

The total DNA was extracted from the biomass used in the batch assays using glass beads, phenol and chloroform method (Griffiths et al., 2000). For the bacterial analysis community the DNA templates were amplified via a polymerase chain reaction (PCR) using specific primers for the partial amplification of 16S rRNA genes for bacteria (968f and 1392r) and archaea (1100f and 1400r) domains (Kudo et al., 1997; Nübel et al., 1996). In both cases, a GC-clamp was added to the forward primer. The amplified DNA fragments were separated by denaturing gradient gel electrophoresis (DGGE), with denaturing concentrations of 45%-65%, and 40%-70% (urea and formamide), for bacteria and archaea, respectively.

Shannon–Wiener diversity indices (H) were calculated using the intensities of the bands on the DGGE fingerprints, expressed as the peak height in the densitometric curves, according to the equation: $H=-\sum(P_i \ln(P_i))$, where: $H$ is the diversity index and $P_i$ is the importance probability of the bands in a lane ($P_i = n_i/N$, where $n_i$ is the height of an individual peak and $N$ is the sum of all peak heights in the densitometric curves). The bacterial and archaean community changes were evaluated using the similarity and diversity indices results from the DGGE gel analysis.
5.3. Results and Discussion

Two fermentative packed-bed reactors fed with CWP were operated during 40 days, the specific hydrogen consuming activity was evaluated over the time in batch assays.

5.3.1 Reactor performance

Figures 5.2 and 5.3 depict the gas production rates and metabolites concentration, expressed as equivalent COD (to show the substrate’s electrons directed towards each compound), from reactor A and B, respectively. During the start up, both reactors showed a similar performance, reaching the highest hydrogen production at day 5, corresponding to a yield of 2.92 and 2.55 mol H\(_2\)/mol lactose consumed, for reactor A and B respectively. Before day 5, the main metabolites in both reactors were lactic acid and ethanol.

In packed bed reactors, a higher hydrogen production after the start-up and a following drop has been a common tendency (Figures 5.2a and 5.3a). For instance, Fontes Lima and Zaiat (2012), achieved a maximal volumetric hydrogen production rate (VHPR) of 6.95 L H\(_2\)/L-d, approximately 20 days after the start-up, applying 24 g COD/L-d. Nevertheless during 80 days of operation the average value was 2.9 ± 1.7 L H\(_2\)/L-d.

In Reactor A, between days 18 and 26, there was a stable stage with an average VHPR of 1.2 ± 0.4 L H\(_2\)/L-d, producing mainly butyric acid as metabolite. The highest VHPR reached in Reactor B was 6.83 L H\(_2\)/L-d, similar to the production rate (7.69 L H\(_2\)/L-d) obtained by Soares-Fernades (2008) using the same reactor configuration at 48 g COD/L-d, but fed with sucrose at 0.5 h of HRT. Even though the double OLR applied in reactor B compared to Reactor A, in Reactor B a decreasing trend in the VHPR between days 7 and 15 was observed, with an average production rate of 1.5 ± 0.52 L H\(_2\)/L-d. In this period in reactor B, the main metabolites produced were butyric acid and ethanol. A similar trend was also observed in the latter cited work (Soares-Fernades, 2008), where the VHPR dropped 50% after 30 days of operation, and the hydrogen production stopped definitively after 40 days.
Figure 5.2. Reactor A performance. Profile of volumetric gas production, percentage of lactose consumed and OLR (a), Metabolites concentration in COD equivalents (b and c).

Despite that the inoculum was expected to be free of methanogens, methane production started in both reactors at days 27 and 16 for reactors A and B, respectively. Due to the low HRT (2 h) applied in the reactors, the high volume of substrate solution (around 35 liters per day) made impractical its storage at 4 °C, favoring the fermentation of the substrate before it was fed into the reactor, suggesting the development of methanogens in the medium.
In reactor A, the VHPR remained at 0.87 ± 0.21 L H₂/L-d, between days 27 and 34, meanwhile the volumetric methane production rate (VMPR) was 0.1 ± 0.07 L CH₄/L-d (Figure 5.2a). During that period the main metabolites produced were ethanol and butyric acid. In reactor B, the VHPR dropped to 0.24 ± 0.12 L H₂/L-d during days 18 to 28. During the same period, the VMPR was 0.20 ± 0.08 L CH₄/L-d. Despite the low hydrogen production, the main metabolites produced were butyrate and acetate, in similar equivalent COD concentrations (Figs. 5.2b and c).

In order to diminish the methane production in both reactors by the effect of a shock load (Carrillo-Reyes et al., 2011), the OLR was increased 100 % at days 35 and 28 in reactor A and B, respectively (Figures 5.2a and 5.3a). In reactor A the VHPR increased to 1.85 L H₂/L-d at day 36; however, the shock load strategy had a negative effect in the VHPR in both reactors, decreasing to 0.22 and 0.16 L H₂/L-d in reactor A and B, respectively, during the last operation days. Moreover, the VMPR increased in reactor A to 0.44 L CH₄/L-d. The lactose consumption was higher than 97 % during the shock load, and the metabolites concentration showed an acetate-butyrate pathway for both reactors.

### 5.3.2 Hydrogen consumption batch assays

In order to evaluate the specific hydrogen consumption rate, batch assays were carried out using biomass withdrawn from the reactors. Due to the methane occurrence, the headspace analysis from the batch assays was used as indicator of homoacetogenic activity, when hydrogen consumption was detected and methane was not produced; and when methane production was detected this was an indicator of hydrogenotrophic methanogenic activity. Table 5.1 shows the specific hydrogen-consuming activity of each sample and the type of activity developed, either homoacetogenic or methanogenic.

The specific hydrogen consuming assays showed that the inoculum did not have methanogenic activity but had homoacetogenic activity. The first biomass sample taken from the reactors showed an increment of the homoacetogenic activity compared to the inoculum. Before the methane occurrence, the hydrogen partial pressure in reactors A and B was between 0.51-0.73 and 0.49-0.61 atm, respectively. Those partial pressures were
high enough to reach the H₂ threshold needed for homoacetogenesis (Cord-Ruwisch et al., 1988). Therefore, it was assumed that this activity was prompted at such conditions.

Figure 5.3. Reactor B performance. Profile of volumetric gas production, percentage of lactose consumed and OLR (a), Metabolites concentration in COD equivalents (b and c).

In the Reactor B, the total biomass withdrawn from the sample ports during days 6 to 15 (as purge sludge) was used to evaluate the hydrogen consuming activity, before the methane occurrence. On this sample, the first batch assay showed hydrogen consumption due to
homoacetogenic activity; in a second feed with H2/CO2 (8 days after the first feed), hydrogen consumption was due to methanogenic activity, increasing the hydrogen specific consumption rate (table 5.1). A similar result was found by Dinamarca et al. (2011), where homoacetogenic activity was present at the batch experiment start-up, and unintended methane started to be produced after 50 hours. The authors attributed the methane to acetoclastic activity. In contrast to the present study, where the hydrogenotrophic methanogenic activity was proved by the hydrogen consumption, and the methane production according to the headspace composition analysis.

The methane hydrogenogenic activity subsequent to the homoacetogenic activity, as shown in the batch assays from samples of day 6 to 15 of reactor B, can be explained by the lower generation time of homoacetogens (1.75 to 29 h) compared to the generation time of hydrogen consuming methanogens (> 6 h) (Zhang and Noike, 1994).

Table 5.1. Specific hydrogen consuming activity for the biomass withdrawn from the reactors.

<table>
<thead>
<tr>
<th>Reactor</th>
<th>Day</th>
<th>mmol H2/g VS d</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Inoculum</td>
<td>4.92(0.07)</td>
<td>Homoacetogenic</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>8.34(1.6)</td>
<td>Homoacetogenic</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>9.24(0.94)</td>
<td>Methanogenic</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>9.07(1.47)</td>
<td>Methanogenic</td>
</tr>
<tr>
<td>B</td>
<td>Inoculum</td>
<td>3.76(0.53)</td>
<td>Homoacetogenic</td>
</tr>
<tr>
<td></td>
<td>6-15(First)</td>
<td>4.83(0.51)</td>
<td>Homoacetogenic</td>
</tr>
<tr>
<td></td>
<td>6-15(Second)</td>
<td>6.28(0.79)</td>
<td>Methanogenic</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>6.53(0.49)</td>
<td>Methanogenic</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>6.59(2.82)</td>
<td>Methanogenic</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>7.34(0.17)</td>
<td>Methanogenic</td>
</tr>
</tbody>
</table>

1 (standard deviation, n=2); 2 Results corresponding to the first and the second H2/CO2 feed.

In both reactors the hydrogen consuming activities increased in comparison with the inoculum, whereas all the results from samples with methanogenic activity in both reactors did not showed significant difference, according to a statistical test ANOVA (α=0.05), f_{obs}=0.1893, f_{crit}= 6.5914. Therefore, the increasing methanogenic activity in the reactors can be explained by the increment in the biomass concentration, even though a constant purge of biomass in both reactors was carried out. In packed bed reactors, common
problems to overcome are the clogging of the packed-bed and the growth of unwanted microorganisms attached to the support (Peixoto et al., 2011).

Due to eventual methanogenic activity in the reactors and the batch assays results, it can be assumed that the hydrogen consuming activity by homoacetogenesis was replaced by the methanogenesis over the operation time. In reactor A the increment of the specific hydrogen consuming activity from day 21 (homoacetogenic) to day 35 (methanogenic), was only 10%; whereas in reactor B the increment from the sample with biomass of days 6 to 15 (homoacetogenic) to day 20 (methanogenic) was 35%. A higher increased in the specific hydrogen consumption due to methanogenesis in reactor B compare to reactor A, can be explained by the different inoculum community structure for each reactor, as is shown in the next section (Figure 5.4).

5.3.3 DGGE fingerprints

The bacterial and archaeal communities were analyzed by PCR-DGGE of the partial amplification of the 16S rDNA gene. Figure 5.4 shows the bacterial DGGE fingerprint and the similarity Dice index for the samples of both reactors. The Shannon–Wiener diversity index of each sample was calculated to have a numeric comparison point to relate the number of bands in the gel, corresponding to the number of different 16S rDNA amplified, and their relative abundances, according to the intensity of each band; results are depicted in Figure 5.5.

The fermented CWP with and without nutrients were clustered together, close to the inoculum of reactor B. Even though the inoculum of both reactors were prepared following the same procedure but at different days (section 5.2.3), the inoculum of reactor A was cluster separately from the other communities, showing a bacterial selection depending of the environmental conditions. The cluster distribution shows that at the end of reactor A operation (day 40), there was a different community selection compare to previous samples from the same reactor. In contrast to reactor B, the two last fingerprints (day 29 and 40) had a similarity higher than 80%.
The diversity indices of the bacterial community showed an enrichment of the community in the samples of both reactors and the fermented CWP with nutrients, compared to the fermented CWP without nutrients (Figure 5.5). In reactor A the highest diversity indices were found in the inoculum and at day 35. The low diversity found at day 21 can be related with the more stable stage of the reactor in terms of the VHPR ($1.2 \pm 0.4$ L H$_2$/L-d, days 18 to 26), before methane production. In Reactor B, the diversity indices remained in similar values. Although the source of the inoculum (natural fermentation), the values of the diversity indices of the bacterial communities ranked between 2.62 and 3.15, being similar to those found in a sewage sludge with an average of 3.04 (Xu et al., 2010); showing the high bacterial enrichment reached by the natural fermentation.

The fermented CWP assays developed a hydrogen producing activity (data not shown); in this sense, analysis of crude cheese whey community have shown the presence of low hydrogen and lactic acid producing bacteria (Castello et al., 2011).

The archaeal DGGE fingerprint is shown in Figure 5.6, in general all the samples showed low diversity and presented a predominant band. The inoculum of reactor A did not present methanogenic activity, however methanogenic DNA was amplified by PCR. The sample of day 40 in reactor A was clustered with the inoculum with a similarity index higher than 80 %. In reactor B, the last communities analyzed (days 20, 29 and 40) had a high similarity index, showing a prevalent archaeal community in the reactor.
Figure 5.5. Shannon-Wiener diversity indices from the bacterial and archaeal communities in the Fermented CWP (W N, with nutrients; W/O N, without nutrients); Reactor A and Reactor B

The diversity indices for the archaeal communities (Figure 5.5), showed the same tendency than values for the bacterial communities. The lower index was found at day 21 in Reactor A, when methanogenic activity wasn’t detected, neither in the reactor nor in the batch assays.

Figure 5.6. Archeal DGGE community fingerprints and corresponding similarity dendrograms and indexes from reactors A (a)

5.4 Conclusions
The hydrogen consuming homoacetogenic activity was present in the community developed by the natural fermented inoculum. The reactor conditions favored the
homoacetogenic community, increasing their specific activity. Possibly the substrate stock at room conditions prompted the growth of methanogenic microorganisms, establishing a community in the reactors as show by the DGGE fingerprints. Even though the low growth rate of methanogens and the high hydrogen threshold for homoacetogens, once the methanogenesis was present, it became the responsible of the hydrogen consuming activity. The change of homoacetogenesis to methanogenesis, did not represent an important increment in the specific hydrogen consuming activity. Unless methanogenesis can be avoided, this experience questions the viability of using raw cheese whey as substrate for hydrogen production at industrial scale.

5.5 References


Chapter 6

Perspectives, conclusions and final remarks
6.1 General discussion

In the present thesis, fixed biomass reactors using cheese whey powder as substrate were evaluated for hydrogen production. The start-up time is a limitation in granular systems, like the UASB reactors, related to the long time required to obtain a stable community, usually several months (Chang and Lin, 2004; Mu and Yu, 2006). In Chapter 2 different start-up strategies and inoculum structures were analyzed in UASB reactors. A conventional strategy based on the gradual decrease of the HRT from 24 to 6 hours in a period of 50 days, maintaining a constant OLR at 20 g COD/L-d was the more suitable strategy, according to the specific hydrogenogenic activity tests (1469.7 and 773.4 mL H₂/g VS-d for R2 and R4, respectively); The VHPR reached under these conditions was between 1 to 1.7 L/L-d in the first 30 days (R4, Figure 2.2d), using a treated granular sludge as inoculum.

In the start-up of the packed-bed reactor inoculated with natural fermented cheese whey (Figure 5.2) the VHPR reached values of 3 L/L-d (packed-bed reactor A). However, between days 18 to 26, there was a stable stage producing 1.2 ± 0.4 L H₂/L-d. The VHPR obtained in the granular system (UASB reactor) and in the biofilm based system (packed-bed reactor) at similar OLR (20 and 24 g COD/L-d, respectively), had equivalent values, in spite of the different HRT and inoculum. Other UASB reactors fed with cheese whey at 20 g COD/L-d, had lower VHPRs reporting less than 45 and 500 mL H₂/L-d (Castello et al., 2009; Castello et al., 2011). The latter comparison shows that according to the conditions evaluated in Chapter 3 and Chapter 5, the UASB and the packed bed reactors have the equivalent hydrogen producing potential. Recently Chojnacka et al. (2011) found that a granular based system produced hydrogen significantly more effectively than another containing only biofilm. Nevertheless, the efficiency of a biofilm system is determined by the characteristic of the support that allows the biomass adhesion.

Over the operation time of the reactors, microbial activities that diminished the hydrogen yield were identified, such as methanogenesis and homoacetogenesis. Both processes are a common concern of fixed biomass reactors (Castello et al., 2009; Moreno-Davila et al., 2011; Wang et al., 2007). The importance of the hydrogen consuming microorganisms was evaluated either measuring the hydrogen consuming activities in batch assays using the
biomass withdrawn from the reactors, or calculating the H\textsubscript{2} consumption according to stoichiometric relations (Reaction 3.3, figure 3.5). The community analysis showed that methane production was due to the presence of hydrogenotrophs from the genera *Methanobacterium* and *Methanobrevibacter*; whereas homoacetogens were related to *Blautia hydrogenotrophica, Oscillibacter valericigenes* and *Clostridium ljungdahlii*. Even though the unfavorable pH (4.5 – 5.5) for methanogens and homoacetogens in the evaluated reactors evaluated (Calli et al., 2008; Wang et al., 2007), these activities were present probably because the pH gradients formed in the granules and biofilms.

Although other works have proposed that methane is result of hydrogen consumption (Castello et al., 2009; Kim et al., 2004), the identification of hydrogenotrophic methanogens had not been done. The close relation between methanogen genera identified in the present work (*Methanobacterium* and *Methanobrevibacter*) suggests that a low methanogenic diversity that can support the unfavorable conditions for these species, such as pH in the hydrogen producing reactors.

Different operational strategies were applied to decrease the methane production in the UASB reactors with the concomitant hydrogen and methane presence. Increasing the OLR by higher substrate concentration was the more suitable operational strategy. A limitation of this strategy is due to the hydrogen production inhibition because of the high concentration of undissociated acids, according to thresholds concentrations reported previously (Castro-Villalobos et al., 2012; VanGinkel and Logan, 2005). The OLR increase strategy has been used previously in UASB hydrogen producing reactors (Spagni et al., 2010); moreover, similar failure is common in methanogenic digesters fed with cheese whey, favored by the high carbohydrate content and the low buffer capacity of the whey (Hassan and Nelson, 2012).

The only strategy that completely inhibited the methanogenesis, without a detrimental effect on hydrogen production, was a second biomass heat treatment. However this strategy may require stopping the reactor operation. As it was expected, the second biomass heat treatment selected spore forming bacteria as hydrogen producers but also homoacetogens, reducing significantly the hydrogen production potential according to the theoretical
hydrogen consumption. Therefore a more suitable strategy to avoid methanogens was evaluated, measuring the relevance of the homoacetogenic activity in batch assays.

The use of an inoculum obtained from naturally fermented cheese whey, that in principle did not have methanogens, was evaluated in packed-bed reactors. Nevertheless methane occurrence was detected, replacing the homoacetogenic activity over the operation time of the reactors; demonstrating that hydrogen consumption by homoacetogens can be as relevant as the methanogenic hydrogen consumption. In this sense, this is the first work where the homoacetogenic specific activities of continuous hydrogen producing reactors are evaluated.

Related to the unintended methane occurrence in packed bed reactors inoculated with naturally fermented cheese whey, it is possible that the high biodegradability of the cheese whey and its content of nutrients and vitamins enriched the indigenous microorganisms and those found in the environment (Prazeres et al., 2012). Similar results were found in a UASB reactor inoculated with kitchen waste compost fed with cheese whey, where no methane was expected, however, it was detected in a minor concentration after 56 days of operation (Castello et al., 2011). In this sense, the activity and relevance of the indigenous microorganisms in the cheese whey had to be elucidated. In the present study the indigenous bacteria of the CWP presented hydrogen production, proving that the microorganisms survived the drying process of the raw cheese whey. Otherwise, the presence of bacteria related to genus Lactobacillus (Figure 4.2) in the present work and others can reduce the viability of fermentable sugars (Castello et al., 2011). For instance, Stamatelatou et al., (2011) found lactic acid production in the cheese whey in spite it was stored at 4 °C.

This work contributes to establish the parameters that can control the methanogenic activity once it appears in hydrogenogenic systems. One of the most important results is that methanogenesis replaced to the homoacetogenesis, or vice versa, as happened in the packed-bed or UASB reactors, respectively. Also it was proved that the hydrogen production potential of fixed biomass reactors is restricted in similar magnitude by homoacetogenesis or methanogenesis. Therefore, in this work was elucidated the relevance
of the hydrogen consumption in hydrogenogenic fixed biomass reactors, giving a first approach to control this undesirable activity.

6.2 Conclusions and final remarks

The results of this work showed the importance of choosing adequate starting-up reactor operational conditions for hydrogen production. Due to the possibility of methanogens presence in different inocula, it is recommended using high substrate concentration (20 g COD/L) and an acidic pH around 5.0, as was applied in the strategy B presented in Chapter 2.

Once there was methane occurrence in the UASB hydrogenogenic reactor, the most suitable strategy to control de methane production was to increase the OLR by increasing the substrate concentration. In this sense, a recent work evaluated the possibility of shifting a methanogenic process for hydrogen production with grass silage as substrate, using the same strategies as in the present work (Pakarinen et al., 2011). These results demonstrate that such strategies have the potential to control methanogens in hydrogen producing reactors and according to the literature this strategy can be evaluated using other substrates.

About the inocula evaluated, during the start-up with heat treated anaerobic sludge and the naturally fermented cheese whey, similar hydrogen rates were produced in stable conditions. Even though, with the naturally fermented cheese whey yield peaks of 2.9 mol H₂/mol lactose, similar to the yields reported by Davila-Vazquez et al. (2009) were achieved, and correspond to the highest reported volumetric hydrogen production rates with cheese whey. This result shows the potential of using the natural fermentation strategy to inoculate hydrogen-producing reactors, moreover it is cheap and more suitable to use in real applications than heat-treating an anaerobic sludge.

Another limitation found in the present work is the homoacetogenic presence. As is established in the literature the hydrogen partial pressure threshold for this activity (520-950 ppm) is readily reached in hydrogen producing systems (Cord-Ruwisch et al., 1988). The homoacetogenic activity was assayed in different experiments in the present work, by batch assays and some organisms related to this activity were identified. Using different
reactor configurations, flushing the reactor with N₂, or even diminishing the pH have been proposed as strategies to control homoacetogenic activity; nevertheless, there is a lack of systematic studies focused on it, evaluating the real effect of the hydrogen partial pressure in the homoacetogenic activity in hydrogen producing systems. Another challenge to overcome in the study of homoacetogenic activity is the need of in situ evaluation. As was mentioned previously, the homoacetogenic bacteria is a versatile group capable to develop autotrophic or heterotrophic activity, therefore only an in situ activity test can give a conclusive result. In this sense using molecular biology techniques such as FISH (fluorescen in situ hybridisation), SIP (stable-isotopes probing), or RNA based target to the homoacetogenic functional gene can be useful (Radajewski et al., 2003).

An important parameter that was not evaluated in the present study is the cheese whey nutrient content. As has been mentioned, the cheese whey is rich in nutrients and vitamins (Prazeres et al., 2012). In the present study the medium was amended with nutrients and minerals; comparing with the works of Castello et al. (Castello et al., 2009; Castello et al., 2011) they did not enriched the cheese whey and their production was lower than the obtained in the present work at similar OLR. In this sense the optimum content of nutrients in hydrogen producing systems with this substrate has to be investigated.

Even though the limitations of using cheese whey to produce hydrogen were addressed in the present work, there is an increasing interest of using this process in the valorization of this waste, mainly for those medium and small size companies that cannot afford the cost of other technologies for valorization (such as spray dryers or ultrafiltration); having in mind also the potential reactor failure during the anaerobic digestion of cheese whey. Further research with the approaches outlined in this chapter are needed, in order to get closer to a real application of cheese whey to produce hydrogen.

6.3 References


