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

POSGRADO EN CIENCIAS APLICADAS

Biohydrogen production from lignocellulosic biomass hydrolysates: Evaluation on batch, semi-continuous and continuous systems

Tesis que presenta
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Para obtener el grado de
Doctor en Ciencias Aplicadas

En la opción de
Ciencias Ambientales

Director de la Tesis:
Dr. Felipe Alatriste Mondragón

San Luis Potosí, México. Enero de 2014

Constancia de aprobación de la tesis

La tesis "**Biohydrogen production from lignocellulosic biomass hydrolysates: Evaluation on batch, semi-continuous and continuous systems**" presentada para obtener el Grado de Doctor en Ciencias Aplicadas en la opción de Ciencias Ambientales fue elaborada por **Jorge Arreola Vargas** y aprobada el **31 de Enero de 2014** por los suscritos, designados por el Colegio de Profesores de la División de Ciencias Ambientales del Instituto Potosino de Investigación Científica y Tecnológica, A.C.

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Esta tesis fue elaborada principalmente en los Laboratorios de la División de Ciencias Ambientales del Instituto Potosino de Investigación Científica y Tecnológica, A.C., bajo la dirección del Dr. Felipe Alatriste Mondragón.

Durante la realización del trabajo el autor recibió una beca académica del Consejo Nacional de Ciencia y Tecnología (CONACyT 332540).

Este trabajo de investigación fue parcialmente financiado por el proyecto SEP-CONACYT CB 2009 01, 132483 "*Producción de biohidrógeno en reactores de alta densidad celular*".

Durante el periodo de la presente tesis el autor realizó dos estancias de investigación.

La primera estancia (Febrero-Agosto de 2011) se realizó en el Laboratorio de Investigación en Procesos Avanzados de Tratamiento de Aguas del Instituto de Ingeniería, Universidad Nacional Autónoma de México (Unidad Académica Juriquilla, Qro. México). Bajo supervisión del Dr. Germán Buitrón Méndez.

La segunda estancia (Junio-Noviembre de 2013) se realizó en el Dipartimento di Ingegneria Civile e Ambientale dell' Università di Firenze (Florencia, Italia). Bajo supervisión de los Drs. Riccardo Gori y Giulio Munz. Durante esta estancia, el autor recibió una beca Marie Curie por su participación en el proyecto CARbon BALAncing for nutrient control in wastewater treatment (CARBALA), Partnership Agreement PROJECT 295176, el cual se encuentra inscrito dentro del programa FP7 de la Unión Europea.

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sobre la Tesis intitulada:

Biohydrogen production from lignocellulosic biomass hydrolysates: Evaluation on batch, semi-continuous and continuous systems

que se desarrolló bajo la dirección de

Dr. Felipe Alatriste Mondragón

El Jurado, después de deliberar, determinó

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Dedicado a Aidee Medina Ulloa

Agradecimientos

Al Dr. Felipe Alatriste Mondragón por compartir conmigo su amplia experiencia, por sus invaluables consejos y por su apoyo en las situaciones difíciles.

A los Drs. Ma de Lourdes Berenice Celis García y Elías Razo Flores por su continuo apoyo y aportes a este trabajo.

A los Drs. Alberto López López y Hugo Oscar Méndez Acosta por sus valiosos comentarios que ayudaron a enriquecer este trabajo.

A los Drs. Germán Buitrón, Giulio Munz y Riccardo Gori por recibirme en sus grupos de trabajo y por su buena disposición durante mis estancias en la UNAM y en la Universidad de Florencia.

A la M. C. Dulce Isela de Fátima Partida Gutiérrez, al M. C. Guillermo Vidríales Escobar, al M. C. Juan Pablo Rodas Ortíz y a la I.Q. Ma del Carmen Rocha Medina por su apoyo técnico.

Al personal del Laboratorio Nacional de Biotecnología
Agrícola, Médica y Ambiental (LANBAMA).

A mis amigos, dentro y fuera de IPICyT, por formar parte de mi vida y por su continuo apoyo durante el transcurso de este doctorado.

A mi familia, por su invaluable apoyo y sus muestras de cariño.

Finalmente, agradezco a mi esposa “Aidee Medina Ulloa”, quien a pesar de las adversidades, continúa brindándome apoyo y amor.
Gracias por estar a mi lado en este efímero viaje llamado vida, te amo.

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Abstract

Biohydrogen production from lignocellulosic biomass hydrolysates: Evaluation on batch, semi-continuous and continuous systems

Keywords: Acid hydrolysis, enzymatic hydrolysis, fermentation, mixed culture, oat straw

Hydrogen is considered as the fuel of the future because of its high energy content (122 kJ/g) and because water is the only byproduct of its use. Moreover, the production of hydrogen via fermentation of organic wastes is carbon neutral. In this regard, lignocellulosic biomass has been recognized as a potentially attractive feedstock for the fermentative hydrogen production, since it is abundant and rich in carbohydrates. However, up to now, most of the reported studies on hydrogen production from lignocellulosic biomass have been carried out on batch mode; therefore, studies on semi-continuous and continuous systems are required in order to improve the understanding and further development of the process. This thesis studied the effect of lignocellulosic biomass hydrolysates over the hydrogen production on batch, semi-continuous and continuous systems. Oat straw was used as a lignocellulosic biomass model. Firstly, it was found that a sequential acid-enzymatic hydrolysis resulted effective to solubilize sugars from the hemicellulose and cellulose fractions of the oat straw. Then, the feasibility to produce hydrogen from acid and enzymatic oat straw hydrolysates was demonstrated in batch assays. Nonetheless, lower hydrogen molar yield (HMY) was obtained with the acid hydrolysate (1.1 mol H_2/mol reducing sugars) as compared to the enzymatic hydrolysate (2.4 mol H_2/mol reducing sugars). Lower performance of the acid hydrolysate was found partially due to a lower HMY from arabinose, whereas the better performance of the enzymatic hydrolysate was found partially due to fermentation of the commercial enzymatic preparation (Celluclast 1.5L), which contributed to the hydrogen production. Afterwards, the feasibility to produce hydrogen from both hydrolysates was also demonstrated in an anaerobic sequencing batch reactor (ASBR). However, it was observed that the initial feeding with model substrates (glucose/xylose) promoted high HMY (2 mol H_2/mol sugar consumed) and high hydrogen production rate (HPR, 278 mL H_2/L-h); whereas the gradual substitution of the model substrates by hydrolysates led to lower HMY and HPR, 0.8 mol H_2/mol sugar consumed and 29.6 mL H_2/L-h, respectively. Furthermore, PCR-DGGE analysis showed that *Clostridium pasteurianum* (99% of similarity) was the most abundant specie when model substrates were fed and that microbial population became more diverse when hydrolysates were fed. Due to the observed performance in the ASBR, the evaluation of the inoculum effect became relevant. Thus, the effect of five different inocula (anaerobic granular sludge, anaerobic flocculent sludge, maize silage, triticale silage and aerobic sludge) was evaluated over the hydrogen production in batch assays. Best performance was obtained with triticale silage, which was selected as inoculum for the hydrogen production from simple (glucose/xylose) and complex substrates

(acid and enzymatic oat straw hydrolysates) in a continuous system, a trickling bed reactor (TBR). Results showed that the enzymatic hydrolysate was a suitable substrate for hydrogen production, since its HMY was similar to the obtained with glucose, 1.6 mol H_2/ mol sugar consumed and 1.7 mol H_2/ mol sugar consumed, respectively. However, hydrogen was not produced when the acid hydrolysate was fed, which was putatively due to the presence of oligosaccharides, phenolic compounds and furfurals. Also, during this experiment a high HPR was obtained when glucose was fed (840 mL H_2/L-h). Finally, bacteria similar to *Clostridium* genus were identified as the putative responsible for the hydrogen production during the TBR operation.

This work demonstrates the feasibility to produce hydrogen from lignocellulosic biomass hydrolysates in batch, semi-continuous and continuous systems. However, the observed negative effect of the acid hydrolysate components over the hydrogen performance need to be further investigated. Furthermore, it is also necessary to optimize the hydrogen production from the enzymatic hydrolysates and study the feasibility to use the fermentation by-products in downstream processes.

Resumen

Producción de biohidrógeno a partir de hidrolizados de biomasa lignocelulósica: Evaluación en sistemas en lote, semi-continuo y continuo

Palabras clave: hidrólisis ácida, hidrólisis enzimática, cultivo mixto, fermentación, paja de avena

El hidrógeno es considerado el combustible del futuro debido a su alto contenido energético (122 kJ/g) y a que el único subproducto de su uso es el agua. Aunado a estas características, la producción de hidrógeno mediante fermentación de residuos orgánicos es carbono neutral. En este sentido, la biomasa lignocelulósica es reconocida como una materia prima potencialmente atractiva para la producción fermentativa de hidrógeno, ya que es abundante y rica en carbohidratos. No obstante, a la fecha, la mayoría de los estudios publicados sobre la producción de hidrógeno a partir de biomasa lignocelulósica han sido llevados a cabo en sistemas por lote, por lo cual, estudios en sistemas semi-continuos y continuos son necesarios para mejorar el entendimiento y futuro desarrollo del proceso. Por lo anterior, esta tesis se enfocó en estudiar el efecto de los hidrolizados lignocelulósicos sobre la producción de hidrógeno en sistemas en lote, semi-continuo y continuo. En el presente estudio, la paja de avena se utilizó como un modelo de biomasa lignocelulósica. Primeramente, se encontró que una hidrólisis secuencial ácida-enzimática es eficaz para solubilizar los azúcares de las fracciones de hemicelulosa y celulosa de la paja de avena. Además, se demostró la factibilidad de producir hidrógeno a partir de los hidrolizados ácidos y enzimáticos en ensayos por lote. Sin embargo, el hidrolizado ácido obtuvo menor rendimiento molar de hidrógeno (RMH) que el hidrolizado enzimático, 1,1 mol H_2/mol de azúcar y 2,4 mol de H_2/mol de azúcar, respectivamente. El menor RMH del hidrolizado ácido se debió parcialmente a un bajo RMH de la arabinosa, mientras que el mejor rendimiento del hidrolizado enzimático se debió parcialmente a la fermentación de la preparación comercial enzimática (Celluclast 1.5L), lo cual contribuyó a la producción de hidrógeno. Posteriormente, se demostró la viabilidad de producir hidrógeno a partir de ambos hidrolizados en un reactor anaerobio en lote secuencial (ASBR). No obstante, se observó que la alimentación inicial con sustratos modelo (glucosa/xilosa) facilitó la obtención de valores altos de RMH (2 mol H_2/mol azúcar) y de velocidad de producción de hidrógeno (VPH, 278 ml H_2/L-h); mientras que la sustitución gradual de estos sustratos modelo por hidrolizados, llevó a la obtención de valores de RMH y VPH menores, 0,8 mol H_2/mol azúcar y 29,6 ml H_2/L-h, respectivamente. Además, análisis mediante PCR-DGGE mostraron que *Clostridium pasteurianum* (99% de similitud) fue la especie más abundante durante la alimentación con sustratos modelo, mientras que durante la alimentación con hidrolizados la población microbiana fue más diversa. Debido al desempeño del ASBR, la evaluación del efecto del inóculo cobró relevancia. Por lo tanto, se evaluó el efecto de cinco diferentes inóculos (lodo anaerobio granular, lodo anaerobio flocúlento, ensilado de maíz,

ensilado de triticale y lodo aerobio) sobre la producción de hidrógeno en ensayos en lote. El mejor desempeño fue obtenido con el ensilado de triticale, el cual fue seleccionado como inóculo para la producción de hidrógeno a partir de sustratos simples (glucosa/xilosa) y complejos (hidrolizados ácidos y enzimáticos de paja de avena) en un sistema en continuo, un filtro percolador (TBR). Los resultados mostraron que el hidrolizado enzimático es un sustrato adecuado para la producción de hidrógeno, ya que su RMH fue similar al obtenido con glucosa, 1,6 mol H_2/mol azúcar y 1,7 mol H_2/mol azúcar, respectivamente. En contraste, el hidrolizado ácido suprimió la producción de hidrógeno, lo cual probablemente se debió al contenido de oligosacáridos, compuestos fenólicos y furfurales en este hidrolizado. Además, durante este experimento se obtuvo una alta VPH alimentando glucosa (840 ml H_2/L-h). Finalmente, especies similares al género *Clostridium* fueron identificadas como las probables responsables de la producción de hidrógeno durante la operación del TBR.

Este trabajo demuestra la viabilidad de producir hidrógeno a partir de hidrolizados de biomasa lignocelulósica en sistemas en lote, semi-continuo y continuo. Sin embargo, investigaciones futuras son necesarias para disminuir el efecto negativo de los hidrolizados ácidos sobre la producción de hidrógeno. Además, es necesario optimizar la producción de hidrógeno a partir de los hidrolizados enzimáticos y estudiar la factibilidad de utilizar los subproductos de fermentación en procesos subsecuentes.

Chapter 1

Fermentative hydrogen production from lignocellulosic feedstock

Summary

Hydrogen production via fermentation of organic wastes is attractive because of its environmental and energetic properties. In the last decade, several studies have reported advances on this topic, mainly evaluating different reactor conditions and type of substrates. Regarding the type of substrate, lignocellulosic biomass has been recognized as an attractive and potential feedstock for fermentative hydrogen production, since it is abundant and inexpensive. Nonetheless, lignocellulosic biomass pretreatment is required prior to fermentation. The present chapter reviews the main factors that influence the fermentative hydrogen production from lignocellulosic feedstock.

1.1 Introduction

High dependence on fossil fuels to supply world energy needs has triggered environmental problems and energy crisis. Environmental problems associated with the use of fossil fuels include air, water and soil pollution; but also the increase in CO_2 concentration in the atmosphere, which is considered the main cause of global warming and associated climate change [1, 2]. These negative effects have promoted the search of alternative energy sources such as solar, wind, hydraulic, biomass, and others. Among these alternatives, the use of lignocellulosic biomass to produce fuels is especially attractive, because it is abundant and is included in the global carbon cycle of the biosphere [2, 3].

Two possibilities to obtain energy from lignocellulosic biomass are the thermochemical and biochemical pathways [3]. The thermochemical pathway involves high temperature degradation of biomass in an oxidized or reduced atmosphere to release the inherent energy (combustion), or to produce fuel intermediates, such as synthesis gas (syngas) and pyrolysis

liquids. Meanwhile, the biochemical pathway is used to produce biofuels and involves depolymerization of biomass polysaccharides and fermentation of the resulting sugars by microorganisms, being less energy intensive [3].

Main biofuels obtained from biochemical pathway processes are ethanol, butanol, methane and hydrogen (H_2). Hydrogen is considered as the most promising biofuel due to its energetic and environmental benefits, such as: high energy content (122 kJ/g), production of water as the only byproduct of its use and potential use in fuel cells to produce electricity [4]. Studies on biochemical hydrogen production have been mainly focused on dark fermentation processes, since hydrogen can be produced at higher rates than in photosynthetic processes [5]. Furthermore, a higher range of organic substrates can be metabolized and main byproducts (volatile fatty acids, VFA) may be used in downstream processes in order to produce methane [6], or electricity [7].

During fermentative hydrogen production, anaerobic bacteria metabolize organic compounds in order to obtain adenosine triphosphate (ATP) for maintenance and growth. Due to the lack of an external electron acceptor during the fermentation process, the electron transport chain is not usable to obtain ATP, unlike respiration processes. Thus, ATP is only produced by substrate level phosphorylation in the Embden-Meyerhoff-Parnas pathway (i.e. glycolysis). During this process, produced electrons need to be disposed in order to keep the electric neutrality in the cell; thus, protons (H^+) may be used as electron acceptors [8, 9]. If this occurs, electrons are transferred to electron carriers, producing two moles of $NADH_2$ and two moles of reduced ferredoxin; and then, these compounds are oxidized by hydrogenases, reducing H^+ to H_2. Production of acetate from pyruvate also occurs during this pathway. It is important to point out that hydrogen production from $NADH_2$ is only possible if the hydrogen partial pressure is under 60 Pa ($6*10^{-4}$ atm); otherwise, $NADH_2$ is oxidized through other pathways, producing different reduced compounds such as butyrate (Fig. 1.1) [10].

Therefore, acetate pathway is the most favorable for hydrogen production. When this pathway takes place, the oxidation of 1 mol of hexose would yield 4 mol of H_2 and 2 mol of acetate. However, the occurrence of other metabolic pathways will lead to obtain lower hydrogen molar yields (HMYs), such as in most of the reported studies [8-10].

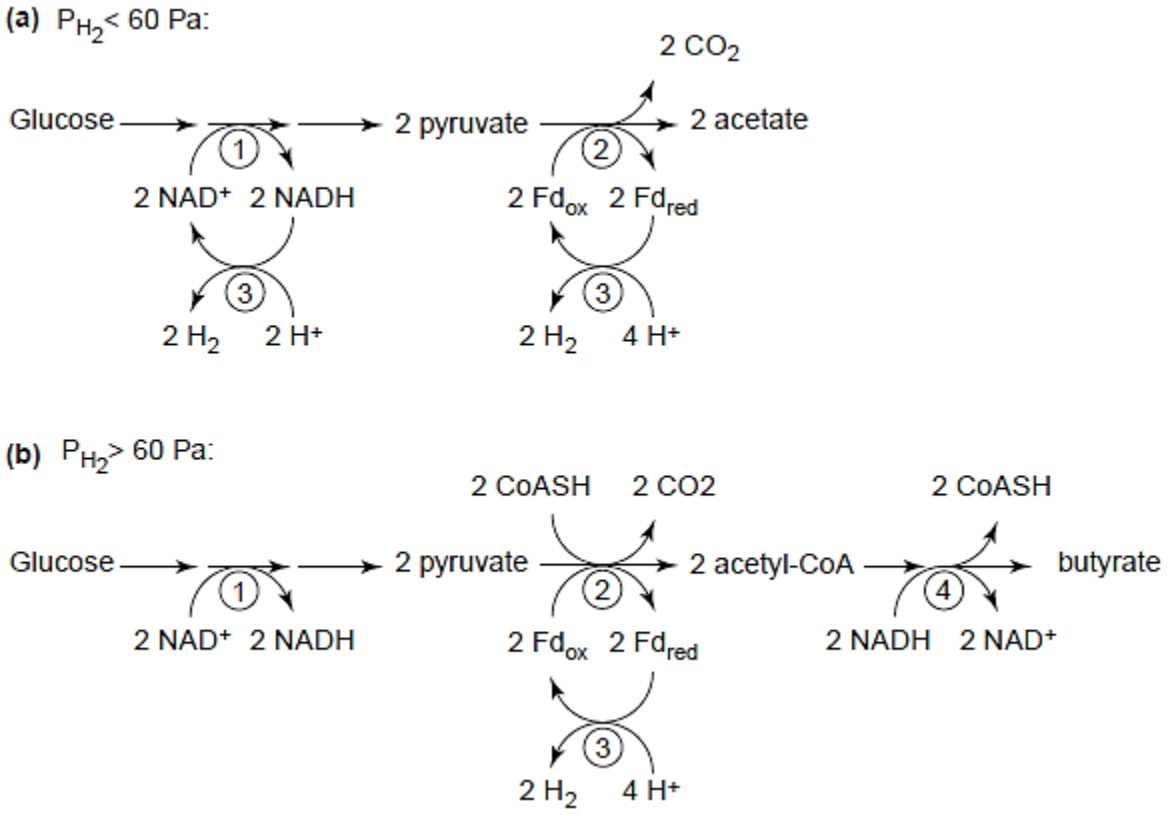

Fig. 1.1 Main metabolic pathways observed during the fermentative hydrogen production. (a) Acetate pathway, (b) butyrate pathway. 1: production of pyruvate and $NADH_2$ through glycolysis; 2: production of acetate and reduced ferredoxin through the oxidative decarboxylation of pyruvate by pyruvate:ferredoxin oxidoreductase; 3: formation of H_2 by hydrogenases; 4: formation of butyrate through $NADH_2$ oxidation, [10].

1.2 Factors influencing fermentative hydrogen production

The improvement of the HMY is an important research area of the fermentative hydrogen production; nonetheless, the increase of the volumetric hydrogen production rate (VHPR) is also relevant, since it does not have theoretical limitations and is the main parameter for potential application of hydrogen in fuel cells [2]. This section review important factors that affect both parameters (HMY and VHPR).

1.2.1 Inoculum

The inoculum is one of the most important factors that affect HMY and VHPR, since it mainly determines the initial microbial community in the fermentative system. Even though

some works have reported the use of pure cultures as inoculum, most of the studies have reported the use of mixed cultures [2, 9]. Main advantages of mixed cultures over pure cultures are the wide range of potential substrates and the possible use of these substrates under unsterile conditions. Up to now, anaerobic sludge is the most widely reported inoculum [9]. However, a disadvantage of the use of anaerobic sludge as inoculum is that it is necessary a pretreatment to eliminate hydrogen-consuming bacteria [2, 9]. Common reported pretreatment methods for this type of inoculum are the acid and heat-shock [2, 9].
Current molecular techniques (PCR-DGGE, cloning, T-RFLP, etc.) have advanced the knowledge of the microbial communities present during fermentative hydrogen production [11-14]. These analyses have revealed that *Clostridia* genus is the main responsible for the hydrogen production, followed by *Enterobacteria, Micrococci, Thermoanaerobacterium, Thermobacteroides, Ruminococcus, Anaerotruncus, Megasphaera* and *Pectinatus* [2]. These hydrogen-producing bacteria are widely spread in different environments, such as soil, wastewater treatment plant sludge (aerobic and anaerobic), compost, etc. [9, 15, 16].

1.2.2 Reactor configuration

Based on the biomass growth, the reactor configuration can be divided in two types: suspended biomass reactors and fixed biomass reactors. Regarding suspended biomass reactors, complete stirred tank reactor (CSTR) is the most reported configuration for hydrogen production [2, 9]. This configuration has the advantage of a good mass transfer of substrate towards the microbial population. However, CSTR has the disadvantage of biomass wash-out when operation is carried out at low hydraulic retention time (HRT). This problem may be overcome using an anaerobic sequencing batch reactor (ASBR), where the biomass is settled prior to liquid discharging [17]. Regarding fixed biomass reactors, these are proposed for hydrogen production because of their capability to operate at high organic loading rates (OLR), which should promote high VHPR. This type of reactors retain high amount of biomass in granular or biofilm systems. The most reported fixed biomass reactor is the up-flow anaerobic sludge blanket (UASB) [2]. Nonetheless, the use of other types of fixed biomass reactors such as the trickling bed reactor (TBR) would also provide other type of advantages such as the low partial pressure of hydrogen in the biofilm [18].

1.2.2.1 ASBR

An ASBR is a suspended biomass reactor that is operated semi-continuously by means of repeated cycles. Each single cycle consists of four stages: filling, reaction, settling and discharging. The advantages of ASBRs over continuous feeding mode reactors are the high degree of process flexibility, the better control of the microbial population and the decoupling of the solids retention time (SRT) from the HRT [17]. This type of reactor has been widely used in wastewater treatment processes, and recently some works have reported its use on fermentative hydrogen production [17, 19-22]. Nonetheless, even though these works have studied the effect of different operational parameters over the hydrogen production (pH, HRT, temperature, substrate concentration, cycle duration, etc.); up to now, no report has evaluated the use of an ASBR for the hydrogen production from lignocellulosic feedstock.

1.2.2.2 TBR

A TBR, also called biotrickling filter or biofilter, is a fixed biomass reactor that is operated continuously. During the operation of this type of reactor, bacteria grow and form a biofilm on a packing material, while a continuous substrate fluid layer is trickled over the biofilm which is surrounded by a gaseous phase. Thus, this configuration promotes high cell density and easy hydrogen release, which facilitates the evaluation of high OLR and avoids high partial pressure of hydrogen in the biofilm. Both characteristics could help to obtain high VHPR and HMY. However, in spite of the TBR advantages, only a few reports regarding hydrogen production in TBRs have been published [18, 23-26]. The main concern of the TBR relies on the excessive growth of biomass on the packing material, which may cause clogging of the reactor. Nonetheless, the selection of an adequate packing material could help to solve this issue.

1.2.3 Temperature

The temperature is an important factor that influences the fermentative hydrogen production, since microbial populations are different at mesophilic or thermophilic conditions [2]. It has been reported that in an appropriate range, increasing the temperature could increase the hydrogen production, which is attributed to thermodynamic

considerations. For example, comparing mesophilic and thermophilic conditions in two CSTRs (35 and 55°C), Gavala et al. [27] found that the thermophilic reactor had higher HMY and specific hydrogen production. However, the use of high temperatures could also contribute to proteins denaturalization and increases in the energy cost [28].

1.2.4 pH

The pH is another important factor that influences the fermentative hydrogen production, since it may affect the hydrogenases activity as well as the metabolism [9]. Furthermore, the pH may also contribute to inhibit the methanogens growth and enhance the stability of the hydrogen producing reactors [2]. According to literature, the optimal pH for hydrogen production is between 4.5 and 6.5 [2, 9, 28].

Batch studies generally report higher initial pH in order to avoid acidification of the medium [2]. According to Van Ginkel and Logan [29], the pH influences the state of dissociation of the produced VFA, since the undissociated forms are present in greater quantities at low pH (lower than 4.5). These undissociated forms are able to cross the cell membrane at this low pH and dissociate in the cell at the higher internal pH, releasing protons inside the cell. The uptake of protons in this form causes cell damage and it is known as an important factor that influences the change from hydrogen to solvent production [30]. During solventogenesis, microbial population converts the substrate into acetone, butanol and ethanol, instead of hydrogen and VFAs. Nonetheless, during this process, also the produced VFAs can be reutilized from the culture medium to produce the mentioned solvents [31]. This characteristic is important because the produced VFAs during the fermentative hydrogen production could be used in downstream processes to produce butanol, which is also an attractive alternative to fossil fuels [31].

1.3 Substrates for fermentative hydrogen production

Substrates for the fermentative hydrogen production are selected according to features such as the cost, availability, carbohydrates content and biodegradability. Different studies have used model substrates (glucose, sucrose, starch, etc.) in order to investigate the effect of different factors over hydrogen production [2, 9]. Nonetheless, complex substrates as wastewaters from the food and beverage industry, and lignocellulosic biomass from energy

crops or agricultural residues, have also been studied as potential substrates for hydrogen production processes [2, 9, 32-34]. Lignocellulosic biomass is a great source of carbohydrates; however, the use of energy crops for biofuel production is greatly discussed due to its ecological and food implications [35]. Thus, the use of agricultural residues stands as an excellent option.

1.3.1 Lignocellulosic biomass constituents

Major constituents of the lignocellulosic biomass are cellulose, hemicellulose and lignin (Fig. 1.2) [36, 37]. Their relative amounts depend of the plant species, age, stage of growth and other conditions. Chemical composition of these compounds is discussed below.

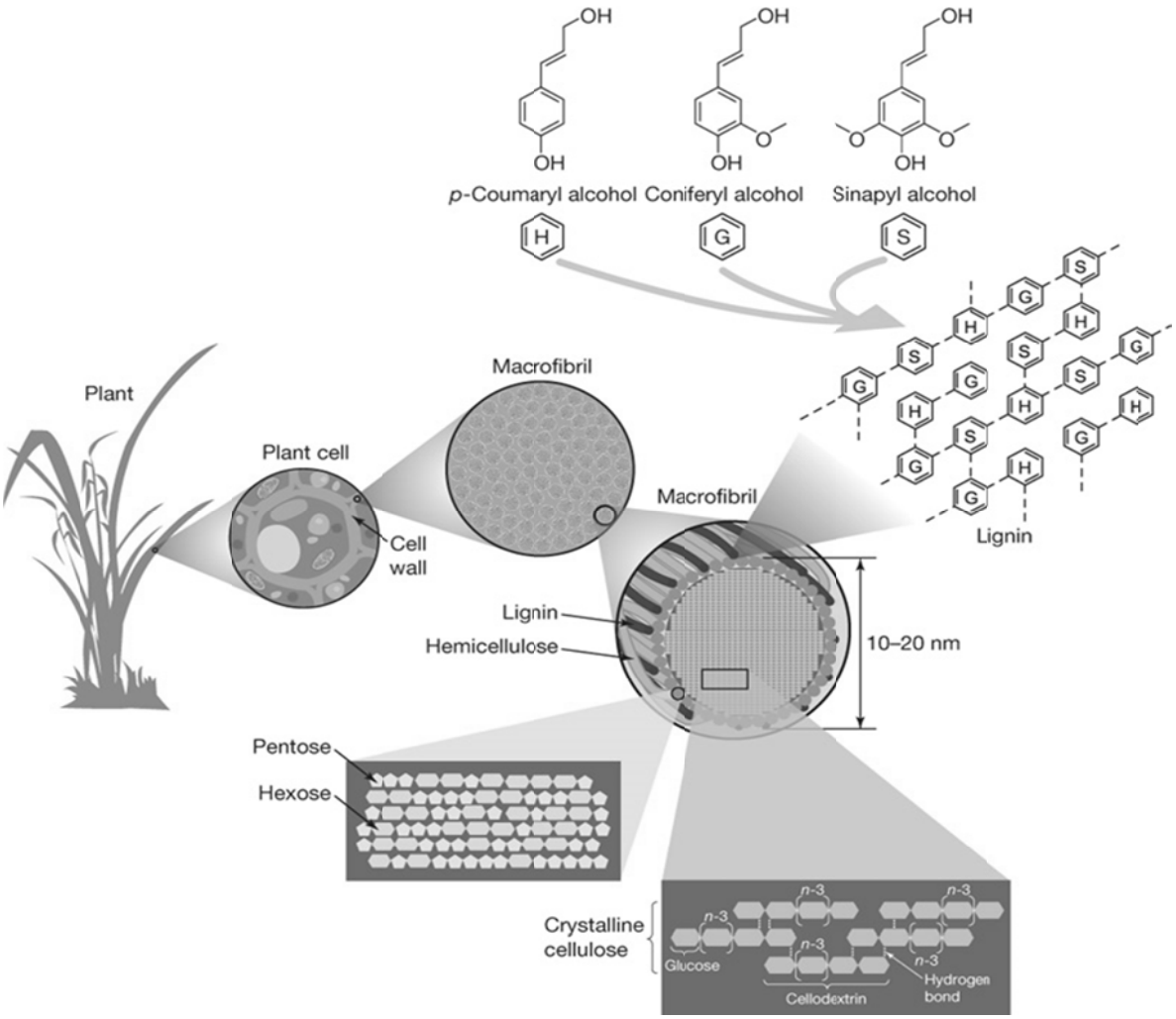

Fig. 1.2 Lignocellulosic biomass composition. Cellulose, hemicellulose and lignin are organized into macrofibrils, giving structural stability to the plant cell wall [37].

1.3.1.1 Cellulose

Cellulose is the main structural constituent in most of the plant cell walls. The structure of cellulose consists in a linear polysaccharide of glucose subunits, linked each other by β-1-4 glycosidic bonds. In turn, long-chain cellulose polymers are also linked together by hydrogen and van der Waals bonds, which cause the packing of cellulose into microfibrils [36]. Hemicelluloses and lignin cover these microfibrils, forming macrofibrils (Fig. 1.2). Cellulose may be present in both, crystalline and amorphous forms. Generally, crystalline cellulose comprises the major proportion of cellulose, whereas small percentage of unorganized cellulose chains forms the amorphous cellulose.

Glucose can be produced from cellulose through the rupture of the glycosidic bonds; however, the rupture of the bonds in crystalline cellulose is more difficult than in amorphous cellulose [36]. Released glucose could be used in fermentation for hydrogen production.

1.3.1.2 Hemicellulose

Hemicellulose is the second most common polysaccharide in nature (after cellulose) and represents about 20-35% of lignocellulosic biomass [38]. The main feature that differentiates hemicellulose from cellulose is that hemicellulose is a polysaccharide composed of different monosaccharides and includes ramifications with short lateral chains. These monosaccharides include pentoses (xylose, rhamnose, and arabinose), and hexoses (glucose, mannose, and galactose) (Fig. 1.2). In minor proportion, there are also uronic acids (4-omethylglucuronic, D-glucuronic, and D-galactouronic acids) [36-38]. It has been reported that hardwood hemicelluloses contain mostly xylans (xylose, other pentoses and uronic acids), whereas softwood hemicelluloses contain mainly glucomannans (hexoses) [38].

Unlike cellulose, hemicellulose polymers are easily hydrolysable, since polymers do not aggregate even though they co-crystallize with cellulose chains [36]. The fermentation of sugars obtained from hemicellulose is essential to increase the yield conversion of lignocellulosic materials into biofuels (such as hydrogen) and other value-added fermentation byproducts.

1.3.1.3 Lignin

Lignin is present in the cell wall, imparting structural support, impermeability, and resistance against microbial attack. It has been reported that herbaceous plants such as grasses have the lowest contents of lignin, whereas softwoods have the highest lignin contents [36]. Unlike cellulose and hemicellulose, lignin is not a polysaccharide, it is composed of a large molecular structure, containing cross-linked phenolic compounds (Fig. 1.2). Three phenyl propane molecules are the main constituents of lignin: coniferyl alcohol (guaiacyl propanol), coumaryl alcohol (p-hydroxyphenyl propanol) and sinapyl alcohol (syringyl propanol).

Lignin represents the main barrier to access the cellulose matrix; thus, its destabilization or degradation is required prior to cellulose solubilization.

1.3.2 Lignocellulosic biomass pretreatments

Even though hemicellulose and cellulose represent an important source of sugars, their potential as substrate for hydrogen production is hindered by the low biodegradability of the lignocellulosic matrix. Therefore, sugar solubilization processes such as dilute acid hydrolysis, alkaline hydrolysis, enzymatic hydrolysis, steam explosion, ammonia fiber explosion (AFEX), ozonolysis, organosolv, etc. are needed prior to fermentation. The first three treatments (dilute acid, alkaline and enzymatic hydrolysis) are especially interesting due to the mild conditions of the process [36].

In order to be effective, a pretreatment must meet the following requirements: (1) improve the formation of sugars or the ability to subsequently form sugars by further hydrolysis, (2) avoid the degradation or loss of carbohydrate, (3) avoid the formation of byproducts that are inhibitory to the fermentation processes (such as furfural, hidroxy methyl furfural (HMF), phenolic compounds, etc.) and (4) be cost-effective [36].

1.3.2.1 Dilute acid hydrolysis

Due to its reported effectiveness to solubilize hemicellulose and to enhance cellulases activity over remaining fiber, dilute acid hydrolysis (mainly with H_2SO_4 or HCl, 1-5 % v/v) has been widely used to pretreat different lignocellulosic biomasses, ranging from hardwoods to grasses and agricultural residues [36, 39]. Generally, the process is carried

out at temperatures ranging from 90 to 200 °C, at lower temperatures longer time of hydrolysis are required and vice versa (from minutes to hours). The mechanism of action of this type of hydrolysis is through protonation of the oxygen in the hemicellulose glycosidic bonds and further break up, which releases hemicellulose oligosaccharides and monosaccharides [40, 41].

Disadvantages of dilute-acid hydrolysis are: cost of the process, which is usually higher than other processes such as steam explosion or AFEX; required neutralization for the downstream enzymatic hydrolysis and/or fermentation processes; and generation of potential microbial inhibitors such as furfural or HMF [36].

1.3.2.2 Alkaline hydrolysis

Bases such as sodium, potassium, calcium, and ammonium hydroxides have been used for lignocellulosic biomass pretreatment. Alkaline hydrolysis plays a significant role in exposing the cellulose, since it cleaves lignin by means of a nucleophilic attack on the carbonyl group [42]. The lignin removal increases enzyme effectiveness by eliminating nonproductive adsorption sites and by increasing access to cellulose. Alkaline hydrolysis is typically carried out in combination with oxidant agents (hydrogen peroxide, chloride, etc.) at ambient conditions, but pretreatment times are longer (typically days) than dilute acid hydrolysis [36].

Even though recovering or regeneration of the salts is possible, a disadvantage of alkaline hydrolysis is the loss of released sugar, since these types of hydrolysates, very likely, are not suitable substrates for fermentation processes due to the presence of lignin by-products.

1.3.2.3 Enzymatic hydrolysis

Due to mild conditions of the process and high conversion yields, the enzymatic hydrolysis is one of the most reported methods used to solubilize cellulose [36, 45]. During enzymatic hydrolysis the conversion of cellulose to glucose is carried out by cellulases. The cellulases employed in cellulose depolymerization consist mainly of three main enzyme groups: endo-glucanases, exo-glucanases, and β-glucosidases. Endo-glucanases initiate the hydrolysis by randomly breaking β-1-4 bonds of the cellulose polymers to create free-chain ends. Then, exo-glucanases attack the free chain ends to produce cellobiose, a glucose disaccharide;

finally, cellobiose units are digested by β-glucosidases to produce glucose. Several species of bacteria such as *Clostridium, Cellumonas, Thermomonospora, Bacillus, Bacteriodes, Ruminococcus, Erwinia, Acetovibrio, Microbispora*, and *Streptomyces*, and fungi such as *Tricoderma, Penicillium, Fusarium, Phanerochaete, Humicola,* and *Schizophillum spp.*, are capable to produce cellulases. Among these, cellulases from *Trichoderma reesei* have been the most widely studied and employed [43].

The main disadvantage of the enzymatic hydrolysis is the requirement of a pretreated biomass for enhancing the action of cellulases. Nonetheless, the application of sequential hydrolysis such as acid-enzymatic or acid-alkaline-enzymatic has demonstrated to be effective for hemicellulose and cellulose solubilization in different agricultural residues [39, 44, 45].

1.4 Fermentative hydrogen production from lignocellulosic biomass hydrolysates

Even though several studies have been reported on the fermentative hydrogen production, only few studies have used lignocellulosic biomass hydrolysates as substrates [2, 5, 9, 33, 34]. Furthermore, most of these studies have been conducted in batch systems, using corn stover [46], cornstalks [47], sugar cane bagasse [48] or rice straw [49] hydrolysates as substrate. Up to now, there are no studies evaluating lignocellulosic biomass hydrolysates as substrate in semi-continuous systems and only a few in continuous systems.

Those studies carried out in continuous systems have achieved different VHPR, which may be due to differences on the type of reactor, conditions, type of lignocellulosic feedstock and type of pretreatment. As example Kongjan et al. [50], using wheat straw thermal hydrolysate in a CSTR achieved 7.7 mL H_2/L-h. Kongjan and Angelidaki [51], using also wheat straw thermal hydrolysate, but in an UASB, achieved 34.2 mL H_2/L-h. Finally, Arriaga et al. [23], using oat straw acid hydrolysate in a TBR achieved 81.4 mL H_2/L-h. In contrast, reported VHPR when model substrates are used are much higher, from 1500 to 15600 mL H_2/L-h [2]. This difference makes relevant the study of the fermentative hydrogen production from lignocellulosic biomass hydrolysates in order to have a better understanding of the process and improving the VHPR.

1.5 Justification

Even though hydrogen production via fermentation of organic wastes is recognized as an attractive alternative to fossil fuels, most of the current studies on this topic have used synthetic substrates based on sugars of easy degradation [2, 4]. Thus, in recent years special attention has been paid to studies dealing with substrates that could be used in full scale [33, 34]. In this regard, agricultural residues are recognized as a commercial feasible feedstock because of their chemical composition, abundance and low cost.

Currently, hydrolytic procedures are capable to solubilize most of the sugars from hemicellulose and cellulose fractions of the agricultural residues [39, 44, 45], producing hydrolysates with potential use as substrates for fermentative hydrogen production. However, even though some studies carried out on batch systems have reported advances on this topic, studies in semi-continuous and continuous systems are limited. Therefore, the present work focused on the evaluation of fermentative hydrogen production from oat straw hydrolysates in batch, semi-continuous and continuous systems (oat straw was used as an agriculture residue model).

1.6 Hypothesis

Because of the chemical composition of the oat straw hydrolysates, it will be possible to produce hydrogen from fermentation of these substrates in batch, semi-continuous and continuous systems. However, due to the fact that different biomass concentrations can be obtained in batch, semi-continuous and continuous systems, differences on hydrogen production performance are expected among these systems.

1.7 General objective

The main aim of this thesis was to evaluate the production of hydrogen from oat straw hydrolysates in batch, semi-continuous and continuous systems. Furthermore, in order to contribute to a better understanding of the processes, this thesis also aimed to study the effect of different type of inocula and to describe the microbial communities developed during the fermentative hydrogen production in semi-continuous and continuous systems.

1.8 Specific objectives

a) To evaluate the effect of different sequential hydrolysis procedures over sugar solubilization from oat straw.

b) To evaluate in batch assays the feasibility to produce hydrogen by fermentation of acid and enzymatic oat straw hydrolysates and to elucidate the role of the major components present in the hydrolysates over the hydrogen production.

c) To evaluate the feasibility to produce hydrogen from fermentation of acid and enzymatic hydrolysates in a semi-continuous reactor (an ASBR) and to correlate the reactor performance with changes on microbial population.

d) To determine the effectiveness of different types of inocula to produce hydrogen.

e) To compare the effect of model substrates and oat straw hydrolysates over the hydrogen production in a continuous reactor (a TBR) and to correlate the reactor performance with changes on microbial population.

1.9 Structure of the thesis

The present chapter (Chapter 1) gives an overview to the state of the art on the fermentative hydrogen production from lignocellulosic feedstock.

Chapter 2 describes the evaluation of different sequential hydrolysis procedures (acid-enzymatic *vs* acid-alkaline-enzymatic) for oat straw sugar solubilization. It also presents the assessment of hydrogen production in batch assays, using acid and enzymatic oat straw hydrolysates from the best sequential hydrolysis procedure, and describes the role of the major components of the hydrolysates (hexoses, pentoses, oligosaccharides, microbial inhibitors, buffer and commercial enzymatic preparation) over the hydrogen production.

In Chapter 3, a feasibility study of hydrogen production in an ASBR from acid and enzymatic oat straw hydrolysates is presented. Correlation between reactor performance and changes in the microbial community is also presented.

In Chapter 4, the effect of five different inocula (anaerobic granular sludge, anaerobic flocculent sludge, maize silage, triticale silage and aerobic sludge) over the hydrogen production, using glucose and acid and enzymatic oat straw hydrolysates as substrates is described.

In Chapter 5, a comparative study of the hydrogen production from complex substrates (acid and enzymatic oat straw hydrolysates), and model substrates (glucose/xylose) in a TBR is presented. Description of the microbial community changes occurred during the process is also included in this chapter.

In the final chapter (Chapter 6) a global discussion of the results obtained in this thesis is presented, accompanied by final conclusions and perspectives.

1.10 References

[1] Kapdan IK, Kargi F. Bio-hydrogen production from waste materials. Enzyme Microbial Technol 2006; 38:569-82.

[2] Davila-Vazquez G, Arriaga S, Alatriste-Mondragon F, De León-Rodríguez A, Rosales Colunga LM, Razo-Flores E. Fermentative biohydrogen production: trends and perspectives. Rev Environ Sci Biotechnol 2008; 7:27-45.

[3] Boateng AA, Weimer PJ, Jung HG, Lamb FS. Response of thermochemical and biochemical conversion processes to lignin concentration in alfalfa stems. Energ Fuel 2008; 22:2810-5.

[4] Hellenbeck PC. Fermentative hydrogen production: Principles, progress, and prognosis. Int J Hydrogen Energy 2009; 34:7379-89.

[5] Ren N, Wang A, Cao G, Xu J, Gao L. Bioconversion of lignocellulosic biomass to hydrogen: Potential and challenges. Biotechnol Adv 2009; 27:1051-60.

[6] Cota-Navarro CB, Carrillo-Reyes J, Davila-Vazquez G, Alatriste-Mondragón F, Razo-Flores E. Continuous hydrogen and methane production in a two-stage cheese whey fermentation system. Water Sci Technol 2011; 64:367-74.

[7] Liu H, Cheng S, Logan BE. Production of electricity from acetate or butyrate using a single-chamber microbial fuel cell. Environ Sci Technol 2005; 39:658-62.

[8] Lee HS, Rittmann B. Evaluation of metabolism using stoichiometry in fermentative biohydrogen. Biotechnol Bioeng 2009; 102:749-58.

[9] Wang J, Wan W. Factors influencing fermentative hydrogen production: A review. Int J Hydrogen Energy 2009; 34:799-811.

[10] Angenent LT, Karim K, Al-Dahhan MH, Wrenn BA, Domínguez-Espinosa R. Production of bioenergy and biochemicals from industrial and agricultural wastewater. Trends Biotechnol 2004; 22:477-85.

[11] Kim SH, Shin HS. Effects of base-pretreatment on continuous enriched culture for hydrogen production from food waste. Int J Hydrogen Energy 2008; 33:5266-74.

[12] Kim DH, Han SK, Kim SH, Shin HS. Effect of gas sparging on continuous fermentative hydrogen production. Int J Hydrogen Energy 2006; 31:2158-69.

[13] Shin HS, Youn JH, Kim SH. Hydrogen production from food waste in anaerobic mesophilic and thermophilic acidogenesis. Int J Hydrogen Energy 2004; 29:1355-63.

[14] Li RY, Zhang T, Fang HHP. Application of molecular techniques on heterotrophic hydrogen production research. Bioresour Technol 2011; 102:8445-56.

[15] Wang JL, Wan W. Comparison of different pretreatment methods for enriching hydrogen-producing cultures from digested sludge. Int J Hydrogen Energy 2008; 33: 2934-41.

[16] Cheong DY, Hansen CL. Bacterial stress enrichment enhances anaerobic hydrogen production in cattle manure sludge. Appl Microbiol Biotechnol 2006; 72:635-43.

[17] Arooj MF, Han SK, Kim SH, Kim DH, Shin HS. Effect of HRT on ASBR converting starch into biological hydrogen. Int J Hydrogen Energy 2008; 33:6509-14.

[18] Van Groenestijn JW, Geelhoed JS, Goorissen HP, Meesters KPM, Stams AJM, Claassen PAM. Performance and population analysis of a non-sterile trickle bed reactor inoculated with Caldicellulosiruptor saccharolyticus, a thermophilic hydrogen producer. Biotechnol Bioeng 2009; 102:1361-67.

[19] Buitron G, Carvajal C. Biohydrogen production from tequila vinasses in an anaerobic sequencing batch reactor: effect of initial substrate concentration, temperature and hydraulic retention time. Bioresour Technol 2010;101:9071-7.

[20] Sreethawong T, Chatsiriwatana S, Rangsunvigit P, Chavadej S. Hydrogen production from cassava wastewater using an anaerobic sequencing batch reactor: effects of operational parameters, COD:N ratio, and organic acid composition. Int J Hydrogen Energy 2010;35:4092-102.

[21] Chen WH, Sung S, Chen SY. Biological hydrogen production in an anaerobic sequencing batch reactor: pH and cyclic duration effects. Int J Hydrogen Energy 2009;34:227-34.

[22] Wu X, Yao W, Zhu J. Effect of pH on continuous biohydrogen production from liquid swine manure with glucose supplement using an anaerobic sequencing batch reactor. Int J Hydrogen Energy 2010;35:6592-9.

[23] Arriaga S, Rosas I, Alatriste-Mondragón F, Razo-Flores E. Continuous production of hydrogen from oat straw hydrolysate in a biotrickling filter. Int J Hydrogen Energy 2011; 36:3442-9.

[24] Jeon BS, Um YS, Lee SM, Lee SY, Kim HJ, Kim YH, Gu MB, Sang BI. Performance analysis of a proton exchange membrane fuel cell (PEMFC) integrated with a trickling bed bioreactor for biological high-rate hydrogen production. Energ Fuel 2008; 22:83-6.

[25] Oh YK, Kim SH, Kim MS, Park S. Thermophilic biohydrogen production from glucose with trickling biofilter. Biotechnol Bioeng 2004, 88:690-8.

[26] Zhang H, Bruns MA, Logan BE. Biological hydrogen production by Clostridium acetobutylicum in an unsaturated flow reactor. Water Res 2006; 40:728-34.

[27] Gavala HN, Skiadas LV, Ahring BK. Biological hydrogen production in suspended and attached growth anaerobic reactor systems. Int J Hydrogen Energy 2006; 31:1164-75.

[28] Carrillo-Reyes J. Producción de hidrógeno en reactores de biomasa fija, implicaciones de microorganismos hidrogenotróficos. 2013; San Luis Potosí, S.L.P., México: IPICyT, División de Ciencias Ambientales, PhD Thesis.

[29] Van Ginkel S, Logan BE. Inhibition of biohydrogen production by undissociated acetic and butyric acids. Environ Sci Technol 2005; 39:9351-6.

[30] Jones DT, Woods DR. Acetone-Butanol Fermentation Revisited. Microbiol Rev 1986; 50:484-524.

[31] Chen WH, Chen SY, Chao SJ, Jian ZC. Butanol production from the effluent of hydrogen fermentation. Water Sci Technol 2011; 63:1236-40.

[32] Vrije TC, Bakker RR, Budde MAW, Lai MH, Mars AE, Claassen PAM. Efficient hydrogen production from the lignocellulosic energy crop *Miscanthus* by the extreme thermophilic bacteria *Caldicellulosiruptor saccharolyticus* and *Thermotoga neapolitana.* Bioresour Technol 2009; 2:12.

[33] Guo XM, Trably E, Latrille E, Carrere H, Steyer JP. Hydrogen production from agricultural waste by dark fermentation: A review. Int J Hydrogen Energy. 2010; 35: 10660-73.

[34] Cheng CL, Lo YC, Lee KS, Lee DJ, Lin CY, Chang JS. Biohydrogen production from lignocellulosic feedstock. Bioresour Technol 2011; 102:8514-23.

[35] Zegada-Lizarazu W, Monti A. Energy crops in rotation. A review. Biomass Bioenerg 2011; 35:12-25.

[36] Kumar P, Barrett DM, Delwiche MJ, Stroeve P. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind Eng Chem Res 2009; 48:3713-29.

[37] Rubin, E. Genomics of cellulosic biofuels. Nature 2008;454: 841-5.

[38] Saha BC. Hemicellulose bioconversion. Ind Microbiol Biotechnol 2003; 30:279-91.

[39] Lloyd TA, Wyman CE. Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids. Bioresour Technol 2005; 96:1967-77.

[40] Carrasco F, Roy C. Kinetic study of dilute-acid prehydrolysis of xylan-containing biomass. Wood Sci Technol 1992; 26:189-208.
[41] Herrera A, Téllez-Luis SJ, Ramírez JA, Vázquez M. Production of xylose from sorghum straw using hydrochloric acid. J Cereal Sci 2003; 37:267-74.
[42] Hocking MB, Crow JP. On the mechanism of alkaline hydrogen-peroxide oxidation of the lignin model p-hydroxyacetophenone. Can J Chem 1994; 72:1137-42.
[43] Taherzadeh MJ, Karimi K. Enzyme-based hydrolysis processes for ethanol from lignocellulosic materials: a review. Bioresources 2007; 2:707-38.
[44] Gomez-Tovar F, Celis LB, Razo-Flores E, Alatriste-Mondragón F. Chemical and enzymatic sequential pretreatment of oat straw for methane production. Bioresour Technol 2012; 116:372-8.
[45] Curreli N, Agelli M, Pisu B, Rescigno A, Sanjust E, Rinaldi A. Complete and efficient enzymic hydrolysis of pretreated wheat Straw. Process Biochem 2002; 37:937-41.
[46] Datar R, Huang J, Maness PC, Mohagheghi A, Czernik S, Chornet E. Hydrogen production from the fermentation of corn stover biomass pretreated with a steam-explosion process. Int J Hydrogen Energy 2007; 32:932-9.
[47] Ma S, Wang H, Wang Y, Bu H, Bai J. Bio-hydrogen production from cornstalk wastes by orthogonal design method. Renew Energ 2011; 36:709-13.
[48] Pattra S, Sangyoka S, Boonmee M, Reungsang A. Biohydrogen production from the fermentation of sugarcane bagasse hydrolysate by Clostridium butyricum. Int J Hydrogen Energy 2008; 33:5256-65.
[49] Lo YC, Lu WC, Chen CY, Chang JS. Dark fermentative hydrogen production from enzymatic hydrolysate of xylan and pretreated rice straw by Clostridium butyricum CGS5. Bioresour Technol 2010; 101:5885-91.
[50] Kongjan P, O-Thong S, Kotay M, Min B, Angelidaki I. Biohydrogen production from wheat straw hydrolysate by dark fermentation using extreme thermophilic mixed culture. Biotechnol Bioeng 2010; 105:899-908.
[51] Kongjan P, Angelidaki I. Extreme thermophilic biohydrogen production from wheat straw hydrolysate using mixed culture fermentation: effect of reactor configuration. Bioresour Technol 2010; 101:7789-96.

Chapter 2

Oat straw sugar solubilization and hydrogen production from hydrolysates: role of hydrolysates constituents

Summary

Oat straw sugar solubilization and hydrogen production from hydrolysates and hydrolysates constituents were investigated. Sequential hydrolysis (acid-enzymatic or acid-alkaline-enzymatic) were assessed to solubilize sugars from hemicellulose and cellulose. Acid hydrolysis, using HCl, resulted effective to solubilize sugars from hemicellulose (81%) and also facilitated the activity of cellulases over remaining fiber. Alkaline hydrolysis, using KOH/$NaClO_2$/KOH or NaOH/H_2O_2, slightly increased the cellulose solubilization. Sugar recoveries ranged from 69 to 79% for the different sequential hydrolysis tested. Hence, hydrolysates from sequential acid-enzymatic hydrolysis were used as substrates for hydrogen production in batch assays. The enzymatic hydrolysate produced a higher hydrogen molar yield (2.39 mol H_2/mol reducing sugars) than the acid hydrolysate (1.1 mol H_2/mol reducing sugars). Hydrogen production from hydrolysates constituents was also evaluated. It was found that lower hydrogen production from the acid hydrolysate was partially due to a lower hydrogen yield from arabinose and not to the microbial inhibitors of the acid hydrolysate. Also, it was found that the commercial enzymatic preparation (Celluclast 1.5L) was easily fermented, and greatly contributed to the hydrogen production in the enzymatic hydrolysate test; this is the first study that provides experimental evidence of hydrogen production from fermentation of the enzymatic preparation.

Adapted from: Arreola-Vargas J, Razo-Flores E, Celis LB, Alatriste-Mondragón F. Oat straw sugar solubilization and hydrogen production from hydrolysates: role of hydrolysates constituents. Submitted to Renewable Energy.

2.1 Introduction

Hydrogen is considered as an excellent alternative to fossil fuels and as the future energy carrier. In addition, fermentative hydrogen production from organic wastes is recognized as an environmental friendly, cost effective, and sustainable process for energy production. Due to these reasons, several studies have reported advances on this topic [1, 2].

Lignocellulosic biomass, such as agricultural by-products, could be a commercially feasible feedstock for hydrogen production, because of its composition, abundance and low cost. The main components of lignocellulosic biomass are cellulose, hemicellulose and lignin; although, protein, pectin and fat are also present in minor proportion [3]. Even though cellulose and hemicellulose are an excellent source of sugars, the direct use of agricultural by-products as substrates for hydrogen production is hindered by the low biodegradability of the lignocellulosic matrix [4]. To overcome this limitation, sequential hydrolysis may be applied over biomass in order to solubilize sugars from hemicellulose and cellulose fractions [5, 6].

Dilute acid hydrolysis has proved to be one of the most effective methods for solubilizing the different sugars from hemicellulose (arabinose, galactose, glucose, mannose, xylose, etc.) [5, 6]. Moreover, when mild conditions are used for the acid hydrolysis, the resulting acid hydrolysates may contain lower concentrations of phenolic and furfural compounds than other types of hydrolysates, obtained under harsher conditions [7, 8]. This is important because phenolic and furfural compounds are considered as microbial inhibitors (MI) [9, 10].

On the other hand, delignification processes such as alkaline hydrolysis, are typically used to enhance the accessibility of cellulose to hydrolytic enzymes, which allows obtaining a higher glucose concentration in the enzymatic hydrolysate [11, 12]. Therefore, the application of sequential acid-alkaline-enzymatic hydrolysis could improve the sugar solubilization yields from hemicellulose and cellulose fractions of the lignocellulosic material.

In this regard, Curreli et al. [5] and Gomez-Tovar et al. [6] reported high overall sugar yields by applying sequential acid-alkaline-enzymatic hydrolysis over wheat and oat straw, respectively. However, Lloyd and Wyman [13] also reported high overall sugar yields by applying only sequential acid-enzymatic hydrolysis over corn stover, suggesting that

delignification by alkaline hydrolysis may not be necessary when hydrolytic sequential treatments are applied.

On the other hand, regarding the feasibility of using lignocellulosic acid and enzymatic hydrolysates as substrate for hydrogen production, just a few studies have been reported on this matter [14-16]. However, none of these studies have reported the use of sequentially obtained acid and enzymatic hydrolysates, which may improve the overall hydrogen yield from the raw material. Moreover, no report has evaluated the role of the main hydrolysates constituents over the hydrogen production. Due to the complexity of the acid and enzymatic hydrolysates, it is important to know the role of the major components present in each hydrolysate over the hydrogen production. Major components present in hydrolysates include individual sugars (arabinose, galactose, glucose, mannose, xylose), oligosaccharides, MI, commercial enzymatic preparation, and citrate buffer. This knowledge would contribute to the understanding and further improvement of the hydrogen production processes from lignocellulosic hydrolysates.

Therefore, the first objective of this study was to evaluate three different sequential hydrolysis procedures for sugar solubilization from oat straw (used as an agricultural by-product model). Sequential hydrolysis 1 and 2 included dilute acid hydrolysis, two different alkaline hydrolysis (in order to evaluate delignification capability) and enzymatic hydrolysis; sequential hydrolysis 3 included acid hydrolysis followed by enzymatic hydrolysis, i.e. no alkaline hydrolysis was applied. The second objective of this work was to assess the feasibility of using acid and enzymatic oat straw hydrolysates (from the most efficient sequential hydrolysis procedure) as substrate for hydrogen production; furthermore, the role of the major components present in each hydrolysate over the hydrogen production was also evaluated.

2.2 Materials and methods

2.2.1 Oat straw

Oat straw was commercially available (Forrajera Marquez Company, San Luis Potosí, México). A farm mill was used to reduce oat straw particle size, and the product was sifted to obtain an average length size of 2 cm. Before hydrolysis, the oat straw was washed and dried at 60 ºC overnight.

2.2.2 Sequential hydrolysis

All the sequential hydrolysis procedures included an initial dilute acid hydrolysis with HCl and a final enzymatic hydrolysis with a commercial enzymatic preparation, Celluclast 1.5L (Novozyme, SIGMA C2730); differences were due to the alkaline hydrolysis. As shown in Table 2.1, sequential hydrolysis 1 and 2 (SH1 and SH2, respectively) included dilute acid hydrolysis, followed by alkaline hydrolysis with KOH/$NaClO_2$/KOH or with NaOH/H_2O_2 respectively and enzymatic hydrolysis. Sequential hydrolysis 3 (SH3) included dilute acid hydrolysis and enzymatic hydrolysis (i.e. no alkaline hydrolysis was applied). All chemicals used were reagent grade.

Table 2.1 Sequential hydrolysis procedures applied to the oat straw

Sequential Hydrolysis (SH)	Acid hydrolysis[a]	Alkaline hydrolysis[b]	Enzymatic hydrolysis[c]
SH1	HCl	KOH/$NaClO_2$/KOH	Celluclast 1.5L
SH2	HCl	NaOH/H_2O_2	Celluclast 1.5L
SH3	HCl	None	Celluclast 1.5L

[a] Conditions described on section 2.2.1; [b] conditions described on section 2.2.2; [c] conditions described on section 2.2.3.

2.2.2.1 Dilute acid hydrolysis

Dilute acid hydrolysis was carried out as described by Gomez-Tovar et al. [6]. Briefly, dried oat straw was resuspended at 5% (w/v) in a 2% HCl solution and then heated 2 h at 90ºC. At the end of the treatment, the hydrolysate was filtered through cheesecloth. Fiber residue was rinsed with water until pH 7 was reached in the rising water and then dried at 60ºC overnight.

2.2.2.2 Alkaline hydrolysis

Depending on the applied sequential hydrolysis (Table 2.1), the fiber residue from dilute acid hydrolysis was further treated with two different alkaline hydrolysis procedures. In the SH1, alkaline hydrolysis consisted of a three step procedure adapted from Zuluaga et al. [11]. Thus, the first step consisted on dispersing the fiber residue at 4% (w/v) in a KOH solution at 5% (w/v) for 14 h at room temperature; then, the fiber residue was treated in a second step with 1% (w/v) $NaClO_2$ and heated at 70ºC and pH 5 for 1 h; finally, a third step treatment with KOH 5% solution at the same conditions of the first step was conducted. At

each step of the treatment, the fiber residue was separated from the liquid and then washed with water until neutral pH was reached in the rising water. In the SH2, the alkaline hydrolysis was adapted from Curreli et al. [5]. Fiber residue was dispersed at 4% (w/v) in a 1% (w/v) NaOH solution and incubated for 24 h at room temperature. Then, H_2O_2 was added (0.3% (v/v) final concentration) and hydrolysis continued for 24 h at room temperature.

At the end of both hydrolytic procedures, hydrolysates were filtered through cheesecloth and fiber residues were rinsed with water until neutral pH was reached in the rising water and dried at 60°C overnight.

2.2.2.3 Enzymatic hydrolysis

Fiber residues from both alkaline hydrolysis (SH1 and SH2) or from the dilute acid hydrolysis (SH3) were dispersed at 4% (w/v) in a 50 mM citrate buffer at pH 4.5. Then, Celluclast 1.5L was added at a concentration of 0.9 mg protein/mL medium, equivalent to 40 Filter Paper Units (FPU)/g of fiber. Hydrolysis was carried out with constant agitation for 10 h at 45 °C. At the end of the hydrolytic procedure, the hydrolysate was filtered through cheesecloth and the residual fiber was rinsed until neutral pH was reached in the rinsing water and dried at 60°C overnight.

2.2.2.4 Characterization of hydrolysates and fiber residues

The acid and enzymatic hydrolysates were characterized in terms of concentration of reducing sugars. Also, the type and concentration of individual sugars in the hydrolysates were determined by capillary electrophoresis. Furfural, hydroxymethylfurfural (HMF), vanillin and syringaldehyde were also determined. The oat straw and the fiber residues from acid, alkaline and enzymatic hydrolysis were characterized in terms of cellulose, hemicellulose and lignin composition. All determinations were carried out as indicated in Section 2.4 (Analytical methods).

2.2.3 Hydrogen production

2.2.3.1 Inoculum and mineral medium

Anaerobic granular sludge from a full-scale up-flow anaerobic sludge blanket (UASB) reactor was used as inoculum for hydrogen production in batch assays. The UASB reactor treated wastewater from a confectionery factory in San Luis Potosí, México. Prior to inoculation, and in order to inactivate hydrogen consuming microorganisms, the granular sludge was thermally treated, powdered and stored as reported by Buitrón and Carvajal [17]. Powder was used as inoculum at a concentration of 4.5 g VSS/L. Composition of the mineral medium used in the batch assays was as follows (g/L): $NH_4H_2PO_4$, 4.5; Na_2HPO_4, 11.9; K_2HPO_4, 0.125; $MgCl_2{\cdot}6H_2O$, 0.1; $MnSO_4{\cdot}6H_2O$, 0.015; $FeSO_4{\cdot}5H_2O$, 0.025; $CuSO_4{\cdot}5H_2O$, 0.005; $ZnCl_2$, 0.075. All chemicals used were reagent grade.

2.2.3.2 Batch assays

The effect of acid and enzymatic hydrolysates as substrates for hydrogen production was evaluated in batch assays. Furthermore, the effect of the major constituents of both hydrolysates over the hydrogen production was also evaluated. These assays included individual sugars (glucose, xylose, arabinose, mannose and galactose), disaccharides (lactose and cellobiose), commercial enzymatic preparation (Celluclast 1.5L), citrate buffer (CB) and MI.

A concentration of 4.7 g reducing sugars/L was used for the acid and enzymatic hydrolysates assays; thus, for the individual sugars and disaccharides a concentration of 4.7 g/L for each sugar or disaccharide was also used. For the cases of Celluclast 1.5L and citrate buffer assays, equivalent concentrations to those present in the enzymatic hydrolysate assay were evaluated. Finally, in order to evaluate the effect of MI over the hydrogen production, an assay containing glucose + MI was carried out. MI tested in this assay were furfural, HMF, vanillin and syringaldehyde at equivalent concentrations to those present in the acid hydrolysate assay.

All batch assays were carried out in 120 mL serum vials with a working volume of 80 mL; each vial contained inoculum, mineral medium and the substrates previously indicated. Initial pH for all the assays was adjusted to 7. After sealing the vials with rubber stoppers and aluminum crimps, the headspace was purged with nitrogen gas for 15 seconds. Vials

were placed in a horizontal shaker at 150 rpm and 35ºC. Gas production and composition of the headspace were measured periodically, as described in Section 2.4 (Analytical methods). All the assays were carried out by triplicate.

2.2.3.3 Kinetic analysis

The cumulative hydrogen production during batch experiments were fitted to a modified Gompertz model, using equation (1) and KaleidaGraph 4.0 (Synergy software). This equation has been widely used to model gas production data [16, 17].

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

Where H (t) (mL) is the total amount of hydrogen produced at culture time t (h), H_{max} (mL) is the maximum cumulative amount of hydrogen produced, R_{max} (mL/h) is the maximum hydrogen production rate, and λ (h) is the lag time before exponential hydrogen production. Hydrogen produced is reported at standard conditions (0 ºC and 1 atm).

2.2.4 Analytical methods

Type and concentration of hexoses, pentoses and volatile fatty acids were determined by capillary electrophoresis as described previously [15]. Furfural and phenolic compounds (furfural, HMF, vanillin, syringaldehyde) concentrations were measured by HPLC. A 4.6 x 150 mm 5-micron column (Zorbax Eclipse XDB-C18, Agilent Technologies, Santa Clara, CA, USA) was used. A mixture of water/acetonitrile (92/8%) was used as mobile phase at a flow rate of 0.8 mL/min and a temperature of 40°C. The pH of samples and standards was adjusted to 4.4 before injection. Compounds were detected at 280 nm with a diode array detector. Furfural and phenolic compounds were measured in four different hydrolysate samples. Average and standard deviation are reported.

Cellulose, hemicellulose and lignin were determined using a semiautomatic fiber analyzer (ANKOM Technology, Macedon, NY, USA) which is based on the methodology reported by Van Soest et al. [18]. Content of protein and activity of the Celluclast 1.5L was determined as described by Bradford [19] and by Ghose [20], respectively. Reducing sugars were determined by the dinitrosalicylic acid (DNS) method [21], using glucose as standard.

For hydrogen production, the volume of gas produced was measured by a liquid displacement device and gas composition was measured by a GC-TCD, as described previously [15]. COD and VSS concentrations were determined according to standard methods [22].

2.3 Results and discussion

2.3.1 Sequential hydrolysis

2.3.1.1 Effect of sequential hydrolysis on fiber composition

The weight percentage (on dry basis) of the lignocellulosic components of oat straw, before being subjected to any treatment, was 34.8 ± 1.3 cellulose, 26.7 ± 1.2 hemicellulose and 8.7 ± 0.6. Hence, 61.5% of the oat straw was made up by polysaccharides and the rest by lignin and other components. According to Fig. 2.1, dilute acid hydrolysis was very effective to solubilize hemicellulose (81%), which agrees with values reported in the literature [3, 5-6]. Fig. 2.1 also shows that alkaline hydrolysis in the SH2 (with $NaOH/H_2O_2$) was not as effective to remove lignin as alkaline hydrolysis in the SH1 (with $KOH/NaClO_2/KOH$), 14% against 48%, respectively. Possible reasons for the poor lignin removal in SH2 are the low concentration of the reactants and/or an inadequate hydroxide to peroxide ratio, required for producing hydroperoxide anion, which helps to cleave lignin by means of nucleophilic attack of the carbonyl group [23]. Higher lignin removal observed in SH1 could be due to both, the high concentration of reactants and/or a better performance of $NaClO_2$ as oxidant. However, a drawback of the alkaline hydrolysis in SH1 is that a higher percentage of cellulose was removed as compared to SH2 (23% *vs*. 6% respectively). In both cases, solubilized cellulose is lost in the alkaline hydrolysates which very likely are not suitable substrates for hydrogen production due to the presence of lignin by-products.

On the other hand, Fig. 2.1 shows that a narrow range of cellulose removal was obtained during enzymatic hydrolysis for the three sequential procedures (71, 61 and 56% for SH1, SH2 and SH3, respectively). This narrow range indicates that the assayed delignification processes (alkaline hydrolysis) only increased slightly the accessibility to cellulose as compared to the sole acid hydrolysis.

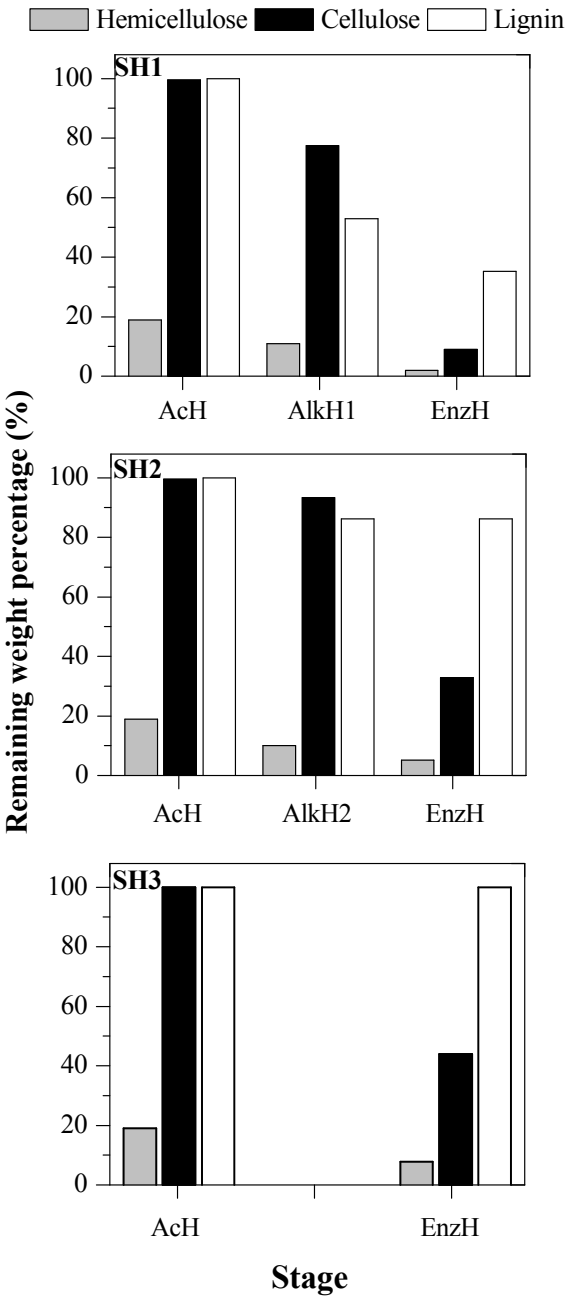

Fig. 2.1 Remaining weight percentage of the lignocellulosic components in fiber residues after each hydrolytic procedure. Remaining weight percentage values are expressed as percentage of the initial weight of each lignocellulosic component in the untreated oat straw. AcH: dilute acid hydrolysis; AlkH1 or AlkH2: alkaline hydrolysis with $KOH/NaClO_2/KOH$ or with $NaOH/H_2O_2$ respectively; EnzH: enzymatic hydrolysis. SH1: sequential hydrolysis 1; SH2: sequential hydrolysis 2; SH3: sequential hydrolysis 3

2.3.1.2 Sugar composition and sugar yield of the acid and enzymatic hydrolysates

Table 2.2 shows that xylose was the main sugar in the acid hydrolysate, followed by glucose, arabinose, mannose and galactose. These sugars have been reported as the main components of hemicellulose [3], indicating that hemicellulose removal observed during acid hydrolysis (Fig. 2.1) was due to solubilization of its main sugars. Sugar composition in the acid hydrolysate is consistent with previous studies [6, 15] and seemed to be a suitable substrate for hydrogen production. On the other hand, glucose was the main sugar in all the enzymatic hydrolysates, and only small amounts of xylose were detected, which probably came from residual hemicellulose (Table 2.2). The highest reducing sugar concentration was achieved by enzymatic hydrolysate from SH1 (20.7 g/L), which is consistent with the highest cellulose removal (Fig. 2.1). Presence of oligosaccharides in all the hydrolysates (including the acid hydrolysate) is hypothesized, since the sum of the individual sugars resulted lower than the reducing sugar concentration (Table 2.2).

Table 2.2 Sugar composition and sugar yield of acid and enzymatic oat straw hydrolysates

Hydrolysate	RS (g/L)	Yield (g RS/g oat straw)	Sugar composition (mg/L)				
			Mannose	Xylose	Glucose	Arabinose	Galactose
Acid	15.6 (± 2.1)	0.31	585 (± 28)	3686 (± 396)	1525 (± 206)	1300 (± 109)	459 (± 95)
Enzymatic [a]	20.7 (± 4.3)	0.18	Nd	1102 (± 95)	11700 (± 1854)	Nd	Nd
Enzymatic [b]	12.5 (± 1.3)	0.12	Nd	1351 (± 146)	4494 (± 357)	Nd	Nd
Enzymatic [c]	9.8 (± 3.7)	0.13	Nd	1275 (± 58)	3796 (± 279)	Nd	Nd

[a] obtained from SH1; [b] obtained from SH2; [c] obtained from SH3; RS: Reducing sugars; Nd: Not detected.

Considering the amount of reducing sugars (in grams) recovered in the acid and enzymatic hydrolysates from SH1, the yields were 0.31 g and 0.18 g per g of oat straw, respectively (Table 2.2). Therefore, the overall reducing sugar yield was 0.49 g/g oat straw. According to the lignocellulosic composition of the oat straw, 61.5% was made up by carbohydrates. Thus, the overall reducing sugar yield is equivalent to 79% recovery of the total

carbohydrate present in the raw material. SH2 and SH3 overall yields were 0.43 g and 0.44 g of reducing sugars per g of oat straw, respectively; which corresponds to 69% and 71% recovery of the total carbohydrate present in the raw material. It is intriguing that in spite of the highest sugar concentration obtained in the enzymatic hydrolysate from SH1 (20.7 g/L) as compared to that one from SH3 (9.8 g/L), only slight differences were found in the overall yield (79% *vs.* 71%, for SH1 and SH3 respectively). This slight difference may be due to the fact that during the alkaline hydrolysis procedures, lignocellulosic material is lost (Fig. 2.1).

Thus, our results agree with Lloyd and Wyman report [13], in which a sugar yield of 92.5% was achieved by applying an acid hydrolysis (140°C) followed by an enzymatic hydrolysis. Both studies indicate that the acid hydrolysis has the capacity to solubilize sugars from hemicellulose and to further facilitate the enzymatic cellulose degradation, pointing out that delignification by an alkaline hydrolysis is not necessary. Therefore, based on the small differences obtained in the overall sugar yields and on the less energy and chemicals required in SH3, this sequential hydrolysis procedure was selected as the most adequate process to solubilize sugars from oat straw for further experiments on hydrogen production.

2.3.1.3 Furfural and phenolic compounds in hydrolysates

The presence of some MI in acid and enzymatic hydrolysates from SH3 was determined. For acid hydrolysate, HMF and furfural were detected at concentrations of 133.2 ± 23.3 and 0.60 ± 0.45 (mg/L), respectively. These values are lower than the concentrations found by Kongjan et al. [24] or Cao et al. [9] in acid hydrolysates (250 and 600 mg/L for furfural and 140 and 250 mg/L for HMF, respectively). Phenolic compounds as vanillin and syringaldehyde were also measured. Vanillin concentration was 3.59 ± 0.89 mg/L, while syringaldehyde was not detected. The concentration of vanillin was also lower than the value of 60 mg/L reported by Cao et al. [9]. Lower values of MI in our acid hydrolysate were probably due to the lower temperature used for the acid hydrolysis (90ºC); in contrast to 121ºC used by Cao et al. [9] or 180 °C by Kongjan et al. [24]. As expected, none of these MI was found in the enzymatic hydrolysates.

2.3.2 Hydrogen production batch assays

For all the hydrogen production batch assays containing sugars, it was found that sugar removal was over 99%, implying that most of the sugars (monosaccharides and oligosaccharides) were metabolized by the microbial communities. Final pH was approximately 5.5 (± 0.3) and H_2 represented 55 to 70% of the gas in the head space, in balance with carbon dioxide.

2.3.2.1 Hydrogen production from acid and enzymatic hydrolysates and effect of the MI.

Fig. 2.2 shows that enzymatic hydrolysate produced the highest cumulative volume of hydrogen (110 mL), followed by glucose, glucose + MI and acid hydrolysate. Similar cumulative hydrogen values for glucose and glucose + MI assays indicates that MI, tested at equivalent concentrations than in the acid hydrolysate assay (HMF 40.11 mg/L; furfural 0.18 mg/L; vanillin 1.08 mg/L, which correspond to the concentrations after dilution with the mineral medium), did not have an inhibitory effect on hydrogen production. Higher concentrations of MI, produced during acid hydrolysis at harsher conditions, have been reported as inhibitory for hydrogen production [9, 10].

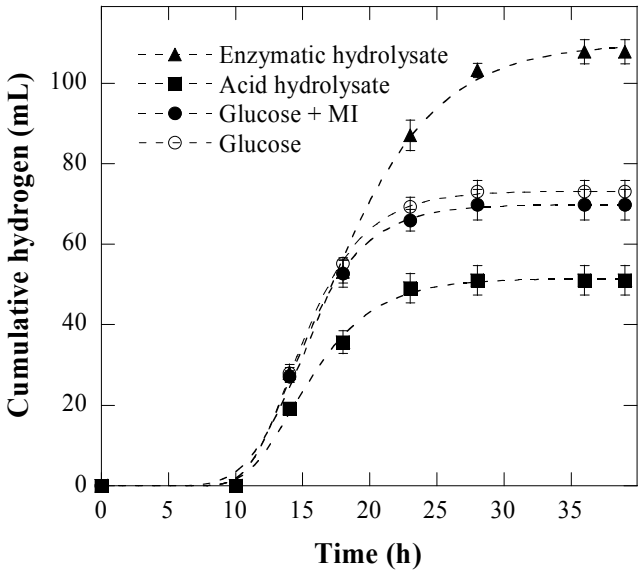

Fig. 2.2 Profiles for hydrogen production from hydrolysates, glucose and glucose + MI. Symbols are the average of three experiments; standard deviations are represented by error bars. Dotted curves are the fitting obtained with the modified Gompertz equation.

Data in Fig. 2.2 were adjusted to the Gompertz model; using Equation (1). Table 2.3 shows that all R^2 values were 0.99, indicating a good fitting to the model. The shortest lag phase (λ) was obtained when glucose was used as substrate and the maximum volumetric hydrogen production rate (VHPR) was similar for glucose, glucose + MI and enzymatic hydrolysate (approximately 110 mL H_2/L-h); the acid hydrolysate presented the lowest VHPR (70 mL H_2/L-h). Moreover, Table 2.3 shows that the enzymatic hydrolysate achieved the highest hydrogen molar yield (HMY), 2.39 mol H_2/mol reducing sugars; which was not an expected result since glucose is the easiest biodegradable substrate. HMY for the enzymatic hydrolysate is comparable with the highest values reported in the literature, up to 3 mol H_2/mol reducing sugar [4]. Nonetheless, due to the fact that experiments were carried out at equivalent reducing sugar concentration, it was probable that other components of the enzymatic hydrolysate (citrate buffer and/or the Celluclast 1.5L) were fermented and contributed to the hydrogen production; this issue is further discussed in section 2.3.2.2.

Table 2.3 Fitting parameters of the Gompertz equation for hydrogen production from hydrolysates, glucose and glucose + MI

Substrate	H_{max} (mL H_2)	R_{max} (mL H_2/h)	λ (h)	R^2	VHPR (mL H_2/L-h)	HMY (mol H_2/mol reducing sugars)
Glucose	73.16	8.84	8.96	0.99	110.50	1.59
Glucose + MI	69.85	8.80	10.88	0.99	110.01	1.53
Enzymatic hydrolysate	109.93	8.87	11.13	0.99	110.88	2.39
Acid hydrolysate	51.46	5.63	10.82	0.99	70.38	1.10

H_{max} is the maximum hydrogen production potential; R_{max} is the maximum hydrogen production rate; λ is the lag-phase time; VHPR is the volumetric hydrogen production rate; HMY is the hydrogen molar yield. Reported values are average of triplicates.

Main metabolic pathways involved in hydrogen production are the acetate and butyrate pathways, with theoretical yields of 4 and 2 moles of H_2 per mol of glucose, respectively [16, 25]. However, several studies have reported the formation of other compounds during fermentative hydrogen production [15, 17, 26]. Table 2.4 shows that the main metabolic by product produced during the present study was acetate; however, small amounts of lactate,

butyrate and ethanol were also present (acid hydrolysate also produced small amounts of propionate). All of the latter compounds represent hydrogen sinks that prevent achievement of the theoretical yield, which agrees with the HMY obtained in these batch assays (Table 2.3). Table 2.4 presents the amount of metabolites in mmol in order to facilitate the tracing of the possible stoichiometry during the different experiments.

Table 2.4 Metabolic products and COD balance obtained during fermentation of hydrolysates, glucose and glucose + MI

Metabolites	mmol				% COD distribution			
	Glucose	Glucose + MI	Enzymatic h.	Acid h.	Glucose	Glucose + MI	Enzymatic h.[a]	Acid h.[a]
Formiate	Nd	Nd	Nd	Nd	0	0	0	0
Acetate	1.98 (± 0.11)	1.92 (± 0.24)	4.09 (± 0.29)	1.69 (± 0.19)	31.25	30.27	64.40	26.62
Propionate	Nd	Nd	Nd	0.12 (± 0.04)	0	0	0	3.42
Lactate	0.61 (± 0.03)	0.60 (± 0.05)	0.37 (± 0.09)	0.34 (± 0.05)	14.57	14.22	8.70	8.16
Butyrate	0.04 (± 0.01)	0.08 (± 0.03)	0.43 (± 0.18)	0.06 (± 0.02)	1.41	3.34	16.95	2.30
Ethanol	0.21 (± 0.02)	0.20 (± 0.03)	0.20 (± 0.03)	0.23 (± 0.05)	5.13	4.70	4.79	5.51
Hydrogen	3.27 (± 0.07)	3.17 (± 0.08)	4.91 (± 0.09)	2.29 (± 0.06)	13.10	12.70	19.67	9.17
Substrate consumption	2.06 (± 0.01)	2.06 (± 0.01)	2.06 (± 0.04)	2.06 (± 0.03)	-100	-100	-100	-100
Balance					-34.53	-34.77	14.51	-44.83

Reported values are average of triplicates and standard deviations are reported in parentheses. Nd: Not detected. [a]: Balance input only accounted the reducing sugars of the hydrolysates

Table 2.4 also shows COD balances. Negative balances are due to biomass growth, exopolymers and not determined metabolites, such as valerate, caproate, butanol, propanol, etc. [26]. The positive balance for the enzymatic hydrolysate suggest that citrate present in the buffer and/or some of the components present in Celluclast 1.5L (sorbitol or protein

from the enzyme) were also fermented, since balance input only accounted for the reducing sugars present in the hydrolysates. This is further discussed in the section 2.3.2.2.

2.3.2.2 Contribution of acid and enzymatic hydrolysates constituents on hydrogen production

The acid hydrolysate produced less hydrogen than glucose, even though both assays contained the same amount of sugars and no inhibition by MI was found (Fig. 2.2). A possible explanation for this observation was that some sugars in the acid hydrolysate (i.e hexoses and pentoses) produced smaller hydrogen yields than glucose. Regarding this possibility, recent studies have reported the production of hydrogen from different monosaccharaides (mannose, galactose, glucose, xylose, and arabinose) [25, 27-29]. However, none of these reports have compared hydrogen production from these monosaccharides with the hydrogen production from lignocellulosic hydrolysates under the same experimental conditions.

Fig. 2.3 (A and B) shows the hydrogen production from some monosaccharaides and disaccharides. Hydrogen production from glucose was already discussed in section 2.3.2.1. Fig. 2.3A shows that hexoses, such as mannose and galactose, produced similar cumulative hydrogen productions; which resulted also similar to those obtained with glucose (Fig. 2.2). As already mentioned, differences among reducing sugar concentrations and sum of monosaccharaides concentrations (Table 2.2), indicates that disaccharides and other oligosaccharides were present in the hydrolysates. Microorganisms need to invest energy for enzyme production in order to hydrolyze oligosaccharides; because of that, it was relevant to evaluate hydrogen production from model oligosaccharides. Due to the fact that model long chain oligosaccharides were not available, the production of hydrogen from model disaccharides was evaluated. Fig. 2.3A shows that hydrogen productions form cellobiose and lactose were similar to hydrogen productions from monosaccharaides.

Acid hydrolysate also contained high concentration of pentoses (xylose and arabinose); thus, it was important to evaluate their effect over hydrogen production. Fig. 2.3B shows that xylose produced similar amounts of hydrogen than hexoses (approximately 70 mL). However, arabinose produced approximately half of the hydrogen produced by hexoses and xylose, which agrees with previous studies [28, 29]. Therefore, lower hydrogen

performance of the acid hydrolysate, as compared with glucose (Fig. 2), was partially due to the presence of arabinose. Also, the potential presence of oligosaccharides containing this sugar could contribute to the lower hydrogen production of the acid hydrolysate.

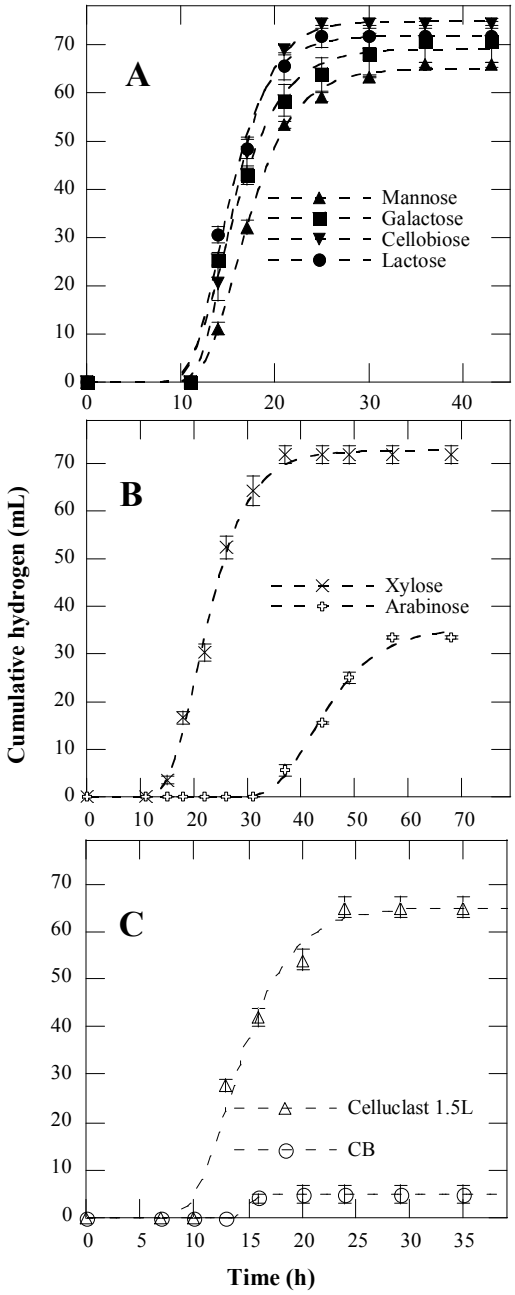

Fig. 2.3 Profiles for hydrogen production from main constituents of the acid and enzymatic hydrolysates. A) hexoses and di-hexoses, B) pentoses, C) Celluclast 1.5L and citrate buffer (CB). Values are the average of three experiments; standard deviations are represented by error bars. Dotted curves are the fitting obtained with the modified Gompertz equation.

As already mentioned, the fact that the enzymatic hydrolysate produced a higher amount of hydrogen as compared with glucose (Fig. 2.2), led to the hypothesis that citrate or some of the constituents of Celluclast 1.5 L were fermented and contributed to hydrogen production. Fig. 2.3C shows that the assay containing the sole Celluclast 1.5L as substrate, produced almost the same amount of hydrogen than hexoses, disaccharides and xylose (approximately 65 mL), whereas the assay with citrate buffer produced negligible amounts of hydrogen. Thus, the highest hydrogen production obtained with the enzymatic hydrolysate (Fig. 2.2) was due to the contribution of Celluclast 1.5L. This is an interesting finding because main components of Celluclast 1.5L are protein and sorbitol. It has been reported that sorbitol or protein can be fermented to hydrogen [30, 31]. However, this is the first report that provides experimental evidence of the actual contribution of a commercial enzymatic preparation to the hydrogen production. A further experiment with sorbitol as control, demonstrated that hydrogen production from the Celluclast 1.5L assay was due to the presence of this compound (data not shown). This experiment clarified the highest HMY obtained with the enzymatic hydrolysate (Fig. 2.2).

Table 2.5 Fitting parameters of the Gompertz equation for hydrogen production from main constituents of the acid and enzymatic hydrolysates

Substrate	H_{max} (mL H_2)	R_{max} (mL H_2/h)	λ (h)	R^2	VHPR (mL H_2/L-h)	HMY (mol H_2/mol reducing sugars)
Mannose	65.01	7.20	12.54	0.99	90	1.41
Galactose	69.12	7.45	11.01	0.99	93.13	1.50
Cellobiose	74.89	10.41	12.11	0.99	130.13	1.62
Lactose	71.84	9.37	11.14	0.99	117.13	1.56
Xylose	72.75	5.18	14.43	0.99	64.75	1.33
Arabinose	35.45	1.91	35.15	0.99	23.88	0.65
Celluclast	66.18	8.81	10.41	0.99	110.13	-
CB	5.10	2.35	13.90	0.99	29.38	-

H_{max} is the maximum hydrogen production potential; R_{max} is the maximum hydrogen production rate; λ is the lag-phase time; VHPR is the volumetric hydrogen production rate; HMY is the hydrogen molar yield; CB: citrate buffer. Reported values are average of triplicates.

Table 2.5 shows the fitting to the Gompertz model for the different constituents of the acid and enzymatic hydrolysates. All R^2 values were 0.99, indicating a good fitting to the model.

Highest VHPR and HMY were achieved by cellobiose, whereas arabinose achieved the lowest values. Arabinose also presented the longest lag phase.

Finally, Table 2.6 shows that acetate was the main metabolite produced during fermentation of the hydrolysates constituents. The presence of other metabolites such as lactate, butyrate and ethanol was also detected. Table 2.6 also shows that most of the COD balances were similar to those in Table 2.4. As already mentioned, negative balances are due to biomass growth, exopolymers and other metabolites not determined [26]. Table 2.6 also corroborated that positive balance for the enzymatic hydrolysate in Table 2.4 was due to fermentation of other hydrolysate constituents, besides sugars.

Table 2.6 Metabolic products and COD balance during fermentation of main constituents of the acid and enzymatic hydrolysates

	mmol								% COD distribution							
Metabolites	Mannose	Galactose	Cellobiose	Lactose	Xylose	Arabinose	C 1.5L	CB	Mannose	Galactose	Cellobiose	Lactose	Xylose	Arabinose	C 1.5L[a]	CB[a]
Formiate	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0	0	0	0	0	0	0	0
Acetate	1.98 (± 0.27)	1.30 (± 0.02)	1.54 (± 0.03)	1.56 (± 0.04)	1.62 (± 0.09)	1.65 (± 0.08)	1.10 (± 0.07)	3.20 (± 0.14)	31.13	20.46	24.25	24.61	25.56	26.08	11.55	60.46
Propionate	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0	0	0	0	0	0	0	0
Lactate	0.71 (± 0.14)	0.69 (± 0.04)	0.67 (± 0.03)	0.62 (± 0.05)	0.68 (± 0.10)	0.14 (± 0.01)	0.98 (± 0.10)	0.14 (± 0.05)	16.77	16.39	15.85	14.71	16.18	3.35	15.54	3.96
Butyrate	0.18 (± 0.01)	0.07 (± 0.01)	0.13 (± 0.03)	0.10 (± 0.02)	0.11 (± 0.02)	0.08 (± 0.01)	0.08 (± 0.01)	0.11 (± 0.03)	7.31	2.78	5.28	3.81	4.54	3.26	2.18	5.35
Ethanol	0.40 (± 0.02)	0.34 (± 0.03)	0.37 (± 0.07)	0.33 (± 0.05)	0.32 (± 0.04)	0.26 (± 0.04)	0.69 (± 0.03)	0.09 (± 0.03)	9.71	8.09	8.93	7.88	7.81	6.36	11.01	2.48
Hydrogen	2.90 (± 0.08)	3.09 (± 0.10)	3.34 (± 0.04)	3.21 (± 0.06)	3.25 (± 0.05)	1.58 (± 0.04)	2.95 (± 0.09)	0.23 (± 0.06)	11.61	12.38	13.38	12.86	13.02	6.33	7.87	1.11
Substrate consumption	2.06 (± 0.01)	2.06 (± 0.03)	2.06 (± 0.01)	2.06 (± 0.01)	2.44 (± 0.01)	2.44 (± 0.02)	-	-	-100	-100	-100	-100	-100	-100	-100	-100
Balance									-23.5	-39.9	-32.3	-36.1	-32.9	-54.6	-51.9	-26.6

Reported values are average of triplicates and standard deviations are reported in parentheses. CB: citrate buffer; C 1.5L: Celluclast 1.5L; Nd: Not detected; [a]: COD balances were made supposing that initial COD was completely consumed.

2.4 Conclusions

Dilute acid hydrolysis was very effective to solubilize hemicellulose from oat straw and to further facilitate the action of cellulases over remaining fiber, evidencing that delignification by alkaline hydrolysis was not necessary for oat straw. Due to this result, a sequential acid-enzymatic hydrolysis procedure was selected to solubilize sugars from oat straw for hydrogen production.

Feasibility to use oat straw acid or enzymatic hydrolysates as substrate for hydrogen production was demonstrated; nonetheless, differences on hydrogen yields were observed. It was found that the lowest hydrogen yield, obtained by the acid hydrolysate, was partially due to the presence of arabinose and not to the presence of MI. On the other hand, it was found that the highest hydrogen yield, obtained by the enzymatic hydrolysate, was partially due to hydrogen production from the fermentation of the commercial enzymatic preparation (Celluclast 1.5L).

2.5 References

[1] Davila-Vazquez G, Arriaga S, Alatriste-Mondragon F, De León-Rodríguez A, Rosales Colunga LM, Razo-Flores E. Fermentative biohydrogen production: trends and perspectives. Rev Environ Sci Biotechnol 2008; 7:27-45.

[2] Ren N, Wang A, Cao G, Xu J, Gao L. Bioconversion of lignocellulosic biomass to hydrogen: Potential and challenges. Biotechnol Adv 2009; 27:1051-60.

[3] Kumar P, Barrett DM, Delwiche MJ, Stroeve P. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind Eng Chem Res 2009; 48:3713-29.

[4] Cheng CL, Lo YC, Lee KS, Lee DJ, Lin CY, Chang JS. Biohydrogen production from lignocellulosic feedstock. Bioresour Technol 2011; 102:8514-23.

[5] Curreli N, Agelli M, Pisu B, Rescigno A, Sanjust E, Rinaldi A. Complete and efficient enzymic hydrolysis of pretreated wheat Straw. Process Biochem 2002; 37:937-41.

[6] Gomez-Tovar F, Celis LB, Razo-Flores E, Alatriste-Mondragón F. Chemical and enzymatic sequential pretreatment of oat straw for methane production. Bioresour Technol 2012; 116:372-8.

[7] Zhao Y, Lu WJ, Wang HT, Yang JL. Fermentable hexose production from corn stalks and wheat straw with combined supercritical and subcritical hydrothermal technology. Bioresour Technol 2009; 100:5884-9.

[8] Kongjan P, Angelidaki I. Extreme thermophilic biohydrogen production from wheat straw hydrolysate using mixed culture fermentation: effect of reactor configuration. Bioresour Technol 2010; 101:7789-96.

[9] Cao GL, Ren NQ, Wang AJ, Guo WQ, Xu JF, Liu BF. Effect of lignocellulose-derived inhibitors on growth and hydrogen production by *Thermoanaerobacterium thermosaccharolyticum* W16. Int J Hydrogen Energy 2010; 35:13475-80.

[10] Quéméneur M, Hamelin J, Barakat A, Steyer JP, Carrère H, Trably E. Inhibition of fermentative hydrogen production by lignocellulose-derived compounds in mixed cultures. Int J Hydrogen Energy 2012; 37:3150-9.

[11] Zuluaga R, Putaux JL, Cruz J, Vélez J, Mondragon I, Gañan P. Cellulose microfibrils from banana rachis: Effect of alkaline treatments on structural and morphological features. Carbohydr Polymer 2009; 76:51-9.

[12] Yamashita Y, Shono M, Sasaki C, Nakamura Y. Alkaline peroxide pretreatment for efficient enzymatic saccharification of bamboo. Carbohydr Polymer 2010; 79:914-20.

[13] Lloyd TA, Wyman CE. Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids. Bioresour Technol 2005; 96:1967-77.

[14] Lo YC, Lu WC, Chen CY, Chang JS. Dark fermentative hydrogen production from enzymatic hydrolysate of xylan and pretreated rice straw by *Clostridium butyricum CGS5*. Bioresour Technol 2010; 101:5885-91.

[15] Arriaga S, Rosas I, Alatriste-Mondragón F, Razo-Flores E. Continuous production of hydrogen from oat straw hydrolysate in a biotrickling filter. Int J Hydrogen Energy 2011; 36:3442-9.

[16] Fangkum A, Reungsang A. Biohydrogen production from sugarcane bagasse hydrolysate by elephant dung: Effects of initial pH and substrate concentration. Int J Hydrogen Energy 2011; 36:8687-96.

[17] Buitrón G, Carvajal C. Biohydrogen production from tequila vinasses in an anaerobic sequencing batch reactor: Effect of initial substrate concentration, temperature and hydraulic retention time. Bioresour Technol 2010; 101:9071-7.

[18] Van Soest PJ, Rovertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci 1991; 74:3583-97.

[19] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248-54.

[20] Ghose TK. Measurement of cellulase activities. Pure Appl Chem 1987; 59:257-68.

[21] Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal Chem 1959; 31:426-8.

[22] APHA, AWWA, WEF. Standard methods for the examination of water and wastewater. 20th ed. Washington DC, USA: American Public Health Association, American Water Works Association, Water Environment Federation; 1998.

[23] Hocking MB, Crow JP. On the mechanism of alkaline hydrogen-peroxide oxidation of the lignin model p-hydroxyacetophenone. Can J Chem 1994; 72:1137-42.

[24] Kongjan P, O-Thong S, Kotay M, Min B, Angelidaki I. Biohydrogen production from wheat straw hydrolysate by dark fermentation using extreme thermophilic mixed culture. Biotechnol Bioeng 2010; 105:899-908.

[25] Almarsdottir AR, Tarazewicz A, Gunnarsson I, Orlygsson J. Hydrogen production from sugars and complex biomass by *Clostridium species*, AK14, isolated from Icelandic hot spring. Icel Agric Sci 2010; 23:61-71.

[26] Carrillo-Reyes J, Celis LB, Alatriste-Mondragon F, Razo-Flores E. Different start-up strategies to enhance biohydrogen production from cheese whey in UASB reactors. Int J Hydrogen Energy 2012; 37:5591-601.

[27] Ren Y, Wang J, Liu Z, Ren Y, Li G. Hydrogen production from the monomeric sugars hydrolyzed from hemicellulose by *Enterobacter aerogenes*. Renew Energ 2009; 34:2774-9.

[28] Li J, Ren N, Li B, Qin Z, He J. Anaerobic biohydrogen production from monosaccharides by a mixed microbial community culture. Bioresour Technol 2008; 99:6528-37.

[29] Mangayil R, Santala V, Karp M. Fermentative hydrogen production from different sugars by *Citrobacter sp.* CMC-1 in batch culture. Int J Hydrogen Energy 2011; 36:15187-94.

[30] Shin JH, Yoon JH, Ahn EK, Kim MS, Sim SJ, Park TH. Fermentative hydrogen production by the newly isolated *Enterobacter asburiae* SNU-1. Int J Hydrogen Energy 2007; 32:192–9.

[31] Xiao B, Han Y, Liu J. Evaluation of biohydrogen production from glucose and protein at neutral initial pH. Int J Hydrogen Energy 2010; 35:6152-60.

Chapter 3

Hydrogen production from acid and enzymatic oat straw hydrolysates in an anaerobic sequencing batch reactor: performance and microbial population analysis

Summary

Feasibility of hydrogen production from acid and enzymatic oat straw hydrolysates was evaluated in an anaerobic sequencing batch reactor at 35 ºC and constant substrate concentration (5 g chemical oxygen demand/L). In a first experiment, hydrogen production was replaced by methane production. Selective pressures applied in a second experiment successfully prevented methane production. During this experiment, initial feeding with glucose/xylose, as model substrates, promoted biomass granulation. Also, the highest hydrogen molar yield (HMY, 2 mol H_2/mol sugar consumed) and hydrogen production rate (HPR, 278 mL H_2/L-h) were obtained with these model substrates. Gradual substitution of glucose/xylose by acid hydrolysate led to disaggregation of granules and lower HPR and HMY. When the model substrates were completely substituted by enzymatic hydrolysate, the HMY and HPR were 0.81 mol H_2/mol sugar consumed and 29.6 mL H_2/L-h, respectively. Molecular analysis revealed a low bacterial diversity in the stages with high hydrogen production. Furthermore, *Clostridium pasteurianum* (99 % of similarity) was identified as the most abundant species in stages with a high hydrogen production. Despite that feasibility of hydrogen production from hydrolysates was demonstrated, lower performance from hydrolysates than from model substrates was obtained.

Adapted from: Arreola-Vargas J, Celis LB, Buitrón G, Razo-Flores E, Alatriste-Mondragón F. Hydrogen production from acid and enzymatic oat straw hydrolysates in an anaerobic sequencing batch reactor: Performance and microbial population analysis. Int J Hydrogen Energy 2013; 38:13884-94.

3.1 Introduction

The type of substrate and type of reactor are factors that substantially affect fermentative hydrogen production parameters, i.e. the hydrogen production rate (HPR) and the hydrogen molar yield (HMY) [1, 2]. Thus, evaluation of different organic wastes as substrates for hydrogen production has become relevant [1-5]. Agricultural by-products may be a potential substrate for hydrogen production at commercial scale, given that they are abundant, easily available and inexpensive [3-5]. However, the direct conversion of this biomass to hydrogen is limited by the low biodegradability of the lignocellulosic matrix. Due to this reason, pretreatment of the agricultural by-products is needed in order to release the biodegradable sugars contained in the hemicellulose and cellulose fractions of this biomass [6, 7]. Common treatments applied before the production of biofuels from lignocellulosic biomass are acid, alkaline, enzymatic and hydrothermal hydrolysis. Sole or in combination, these types of hydrolysis have been used prior to the fermentative production of hydrogen from wheat straw [8, 9], sugarcane bagasse [10, 11], cornstalk and corn stover [12-15], rice straw [16] and oat straw [17].

Regarding the type of reactor, fermentative hydrogen production has been conducted in a variety of reactors operated under continuous feeding mode [1, 2]. However, it has been reported that hydrogen production in anaerobic sequencing batch reactors (ASBR) has some advantages over continuous feeding mode [18-22]. These advantages include high degree of process flexibility, better control of the microbial population due to the cyclic operation and the decoupling of the solids retention time (SRT) from the hydraulic retention time (HRT). Up to now, there is no report on the use of an ASBR for the production of hydrogen from lignocellulosic hydrolysates.

Therefore, the aim of this research was to study the feasibility of fermentative hydrogen production in an ASBR from oat straw hydrolysates. Oat straw was used as an agricultural by-product model. In order to solubilize the hemicellulose and cellulose fractions of the oat straw, it was sequentially hydrolyzed by means of a dilute acid hydrolysis followed by an enzymatic hydrolysis. The effect of both hydrolysates (acid and enzymatic) on the hydrogen production performance was evaluated. Performance of the processes was also correlated with changes in the microbial community.

3.2 Materials and methods

3.2.1 Experimental strategy

ASBR was initially fed with a mixture of glucose/xylose 1:1 on COD basis (5 g/L total COD). Then, the mixture was substituted in a step-wise mode with increasing amounts of acid and enzymatic oat straw hydrolysates.

In a first experiment (Experiment A), hydrogen production was initially observed, but complete suppression of hydrogen and an increase on methane production was observed. This result led to a second experiment (Experiment B) where several selective pressures against methanogens were applied. Table 3.1 summarizes the operational periods for both experiments; each condition was maintained for at least 20 cycles. Steady state was assumed after three similar values of hydrogen production and sugar removal were achieved; once steady state was reached a new condition was evaluated. Hydrogen produced throughout this study is reported at standard temperature and pressure conditions (0 ºC and 1 atm).

3.2.2 Inoculum and mineral medium

Experiment A: anaerobic granular sludge from a full-scale up-flow anaerobic sludge blanket (UASB) reactor was used as inoculum for hydrogen production. The UASB reactor treats wastewater from a confectionery factory in San Luis Potosí, México. Prior to inoculation, the granular sludge was thermally treated, powdered and stored as previously described [19]. The powder was used as inoculum in the bioreactor at a concentration of 5.5 g/L (4.5 g VSS/L). The mineral medium composition for this experiment was as follows (g/L): $NH_4H_2PO_4$, 4.5; Na_2HPO_4, 0.635; K_2HPO_4, 0.125; $MgCl_2 \cdot 6H_2O$, 0.1; $MnSO_4 \cdot 6H_2O$, 0.015; $FeSO_4 \cdot 5H_2O$, 0.025; $CuSO_4 \cdot 5H_2O$, 0.005; $CoCl_2 \cdot 5H_2O$, 0.003; $Na_2MoO_4 \cdot 2H_2O$, 0.0125; $ZnCl_2$, 0.075.

Experiment B: inoculum for this experiment was taken from the biomass obtained at the end of the experiment A. Prior to inoculation, the biomass was thermally treated again to eliminate hydrogen consumers and powdered at the same conditions than in experiment A. Mineral medium was similar to that in experiment A, with the difference that $CoCl_2 \cdot 5H_2O$ and $Na_2MoO_4 \cdot 2H_2O$ were eliminated from the medium.

Table 3.1 Operational stages of the ASBR during experiments A and B.

Stage	Purpose	Operation period (d)	Influent substrate concentration (g COD/L)	Equivalent HRT (h)	pH	Bioreactor operation mode
Experiment A						
I	Start-up	1-13	5[a]	24	5.5	ASBR
II	Acid hydrolysate effect	14-32	3.75[a] +1.25[b]	24	5.5	ASBR
Experiment B						
I	Start-up	1-7	5[a]	6	4.5	CSTR
II		7-12	5[a]	8	4.5	ASBR
III	Acid hydrolysate effect	12-14	4.5[a] + 0.5[b]	8	4.5	ASBR
IV		15-18	4[a] + 1[b]	8	4.5	ASBR
V		19-22	3.5[a] + 1.5[b]	8	4.5	ASBR
VI		23-27	3[a] + 2[b]	8	4.5	ASBR
VII		28-34	2.5[a] + 2.5[b]	8	4.5	ASBR
VIII	Effect of the acid and enzymatic hydrolysates mixture	34-37	2.5[b] + 2.5[c]	8	4.5	ASBR
IX	Enzymatic hydrolysate effect	38-42	5[c]	8	4.5	ASBR

[a]Model substrate: mixture of glucose-xylose (1:1).
[b]Acid hydrolysate.
[c]Enzymatic hydrolysate.

3.2.3 Oat straw hydrolysates

Oat straw was obtained from a commercial source (Forrajera Marquez Company, San Luis Potosí, México). A farm mill was used to reduce straw particle size to an average length of 2 cm. This material was sequentially acid and enzymatically hydrolyzed in order to

solubilize the hemicellulose and cellulose of the oat straw, respectively. Both hydrolysis procedures were carried out as reported by Gomez-Tovar et al. [23].

The acid hydrolysate had the following composition in g/L: COD 25 ± 3.1; total sugar 20 ± 2.2; glucose 1.5 ± 0.2; xylose 3.7 ± 1.1; arabinose 1.3 ± 0.3; mannose 0.59 ± 0.1; galactose 0.46 ± 0.1. Furfural and phenolic compounds concentrations were (mg/L): hydroxy methyl furfural (HMF) 133.2 ± 23.3; furfural 0.6 ± 0.4; vanillin 3.59 ± 0.9. The enzymatic hydrolysate had the following composition (g/L): COD 30 ± 1; total sugar 7 ± 1; glucose 3.8 ± 0.9; xylose 1.3 ± 0.4; arabinose, mannose, galactose, HMF, furfural and vanillin were not detected in this hydrolysate.

3.2.4 Reactor set-up and operation

Hydrogen production experiments were carried out in a reactor with a 4L working volume (Applikon Technologies). The temperature and the pH were controlled by an Applikon ADJ 1030 Biocontroller. Control of pH was done with automatic additions of 10 N NaOH. Temperature was controlled with an electric jacket and was set at 35 ºC. Mixing was maintained during the filling and reaction phases of the ASBR at 250 rpm. An automatic controller was used to fill and discharge the liquid with Masterflex pumps.

For experiment A the reactor was set at pH 5.5 and was operated in sequencing batch mode with the following parameters: filling time: 10 min, reaction time: 11 h 10 min, settling time: 30 min, and discharge time 10 min. The total cycle time was 12 h, equivalent to a HRT of 24 h; calculated considering a volume exchange ratio of 50% and using equations 1 and 2.

$$Q = \frac{V_{fed}}{t_{cycle}} \qquad (1)$$

$$HRT = \frac{V_r}{Q} \qquad (2)$$

For experiment B the reactor was started-up and operated under selective pressures against methanogens. A pH of 4.5 was set and the reactor was inoculated and operated in batch mode for 24 h. After this time, the reactor was operated as a continuous stirred tank reactor (CSTR) with a 6 h HRT until steady state; onwards, the operation mode was switched to

sequencing batch mode. Total sugar consumption kinetics was followed in the first 3 cycles in order to determine the minimum time for sugar removal. Thus, the following parameters were applied for the rest of the experiment: filling time: 10 min, reaction time: 3h 10 min, settling time: 30 min; and discharge time 10 min for a total cycle time of 4 h, giving a HRT of 8 h; calculated considering a volume exchange ratio of 50% and using equation 1 and 2.

3.2.5 Analytical methods

Gas production was measured using a liquid-displacement device filled with water at pH 2. Gas composition was analyzed by a gas chromatographer (SRI Analyzer 1), equipped with a thermal conductivity detector and two stainless steel columns in series (2 m long; 0.79 mm diameter). The temperature of the injection port, column and detector were 90, 110 and 150 ºC, respectively. Nitrogen was used as the carrier gas at a flow rate of 20 mL/ min.

Liquid samples were taken at the end of the last 3 cycles of every stage and were analyzed for volatile fatty acids (VFA) and solvents as previously described [19]. Type and concentration of hexoses and pentoses in the hydrolysates were determined by capillary electrophoresis as described by Arriaga et al. [17]. Concentration of furfural and phenolic compounds in the hydrolysates was measured by HPLC. A 4.6 x 150 mm 5-micron column was used (Zorbax Eclipse XDB-C18, Agilent Technologies, Santa Clara, CA, USA). A mixture of water/acetronitrile (92/8%) was used as mobile phase at a flow rate of 0.8 mL/min and a temperature of 40 °C. The pH of samples and standards was adjusted to 4.4 before injection. Compounds were detected at 280 nm with a diode array detector.

Total sugar concentration in the hydrolysates and at the end of the cycles of the bioreactor was determined by the phenol-sulphuric acid method, using glucose as standard [24]. COD and VSS concentrations were determined according to standard methods [25]. All samples analysis were carried out in triplicate.

3.2.6 Microbial community analysis by PCR-DGGE of the 16s rDNA

Biomass samples from different stages at steady state were taken to be analyzed by PCR-DGGE. These samples were: experiment A-stage I (EAI), experiment A-stage II (EAII), experiment B-stage II (EBII), experiment B-stage III (EBIII), experiment B-stage VII (EBVII), experiment B-stage IX (EBIX) and inoculum (IN).

3.2.6.1 DNA extraction and PCR amplification

Biomass samples were subjected to DNA extraction following a previously reported protocol [26]. Bacterial specific primers were used for 16s rDNA amplification. Due to methane production in experiment A, archaeal specific primers were also used for amplification from samples of this experiment and from inoculum. Due to its effectiveness, nested PCR technique was used for amplification, using Taq DNA polymerase (Dongsheng, China).

The conditions and primers for bacterial nested PCR were as follows: first round, primers used were 27F (5'-GTTGATCCTGGCTCAG-3') and 1492R (5'-ACGGYTACCTTGTTACGACTT-3'); the reaction conditions were: initial DNA denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 60 s and annealing also 60 s at 45 °C; then, an extension at 72 °C for 1 min. Final extension lasted 10 min at 72 °C. Second round (target sequence): forward primer was 357F (5' CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGC AGCAG-3´) and reverse primer was 907R (5'-CCGTCAATTCMTTTGAGTTT-3'); the 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 to 56 °C; then, an extension at 72 °C for 1 min. Once the temperature reached 56 °C, 20 cycles were performed; final extension lasted 7 min at 72 °C. Primers used were previously reported [27].

For archaea, primers used were reported by Sousa et al. [28]. First round: forward primer was 109KF (5'-ACKGCTCAGTAACAC GT-3') and reverse primer was Uni 1492R (5'-CGGCTACCT TGTTACGAC-3'); reaction conditions were as follows: initial DNA denaturation at 95 °C for 5 min, followed by 25 cycles of denaturation at 95 °C for 30 s and annealing 40 s at 52 °C; then, an extension at 72 °C for 1.5 min. Final extension lasted 5 min at 72 °C. For second round: forward primer was A109F (5'-ACTGCTCAGTAACACGT-3´) and reverse primer was 515R (5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGATCGTATTACCG CGGCTGCTGGCA-3'); reaction conditions were as follows: initial DNA denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s and annealing for 1

min at 52 °C, followed by an extension at 68 °C for 1 min; final extension lasted 7 min at 68 °C.

PCR products were visualized in 1% agarose gels stained with etidium bromide to assess the size and purity of the amplicon.

3.2.6.2 DGGE analysis

Bacterial and archaeal DGGE were performed and stained according to Carrillo-Reyes et al. [26], but using a denaturing gradient for archaea gel of 30 to 50% instead of 30 to 60% used for bacteria. Dominant bands were excised from both gels and eluted in 20 μL of deionized water for three days at 4 °C. The eluted DNA was reamplified by PCR using the following primers: for bacteria 357F without GC-clamp and 907R, for archaea A109F and 515R also without the GC-clamp. Successfully reamplified PCR products were sequenced.

Dendrogram for Bacterial DGGE gel was created according to Carrillo-Reyes et al. [26]. Relative microbial abundances were estimated on bacterial DGGE gel using band intensities by Quantity One analysis software (Bio-Rad, Hercules, California, USA).

Sequences were analyzed with DNA BioEdit software v7.1.3 (Carlsbad, California, USA), and submitted to the nonredundant nucleotide database at GenBank using BLAST (http://www.ncbi.nlm.nih.gov/blast/) and Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp). Finally, a Neighbor-joining phylogenetic tree was constructed for the identified bacteria, using Molecular Evolutionary Genetics Analysis package (MEGA version 4.0) with a bootstrap of 1000 replicates.

3.3 Results and discussion

3.3.1 Performance of the ASBR: Experiment A

The ASBR was operated during 32 days. Table 3.1 shows the features of the two operational stages of the reactor. The performance of the ASBR is shown in Fig. 3.1.

During days 1 to 13 (stage I) the gas composition was almost stable, 56 to 66% H_2 in balance with CO_2. The mean values for HMY and HPR in the steady state were 0.39 mol H_2/mol sugar consumed and 18 mL H_2/L-h, respectively. These values resulted lower than those cited by others using xylose, glucose or a mixture of both as substrate [5, 29], indicating that experimental conditions were not adequate for hydrogen production. The

metabolic by-products obtained in this stage were acetate, butyrate, propionate, valerate and ethanol with mean values of 1228, 1160, 182, 145 and 115 (mg/L), respectively.

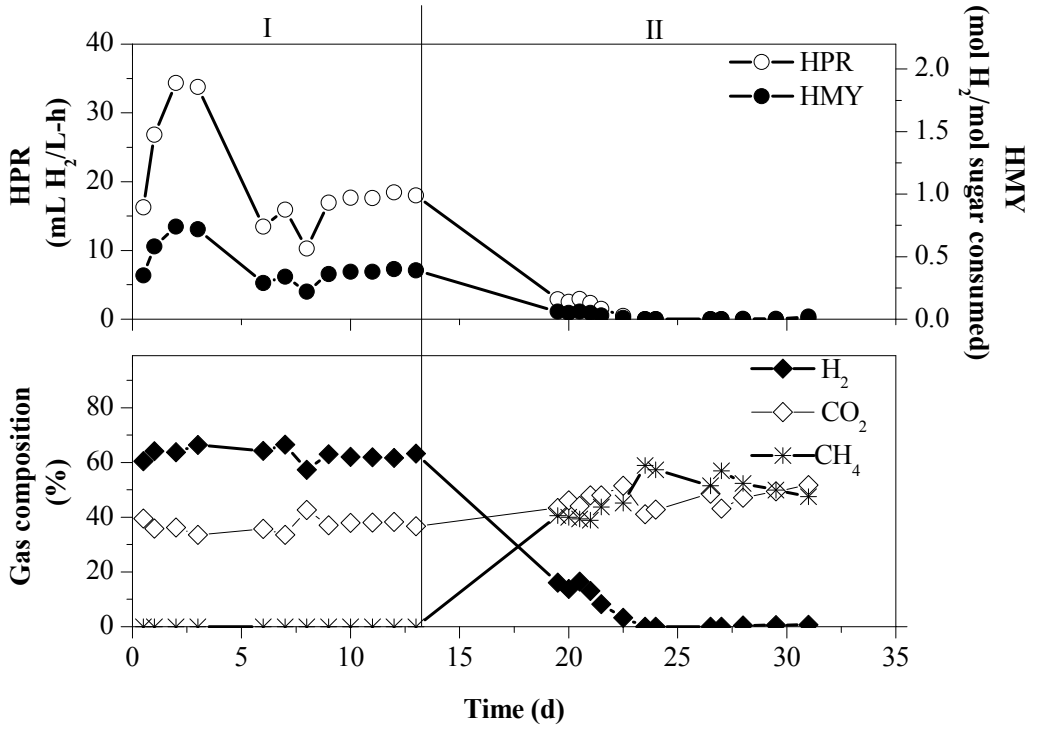

Fig. 3.1 Anaerobic sequencing batch reactor performance during Experiment A. During stage I glucose and xylose (5 g COD/L) were supplied in the feed. During stage II a fraction of the model substrate was replaced by acid hydrolysate (1.25 g COD/L). During stage I and II reactor was operated as an ASBR. HPR: hydrogen production rate. HMY: hydrogen molar yield.

During stage II, the effect of the acid hydrolysate was evaluated (Table 3.1). The model substrate was gradually substituted by hydrolysate and the effect on the ASBR performance was studied. During the first 6 days of stage II, it was not possible to measure the composition of the gas but when it was measured again, the content of hydrogen had decreased from 63% to 17%. Hydrogen content continued decreasing until total suppression. Meanwhile, methane increased its concentration in the gas (Fig. 3.1). Methane production is a frequent problem in hydrogenogenic reactors, which is related to the source

of the inoculum [26] or to the substrate used [19]. In this experiment it is unlikely that methanogens were present in the substrate due to the low pH of the acid hydrolysate (below 2). Therefore, methanogens growth was probably due to their presence in the inoculum. However, it is unclear the reason for methanogens survival after the harsh thermal treatment and why the addition of acid hydrolysate favored their growth.

Thus, this initial experiment could not provide information to evaluate the effect of the hydrolysates on the hydrogen production. Therefore, selective pressures were used for a new experiment in order to avoid the growth of methanogens in the system.

3.3.2 Performance of the ASBR: Experiment B

Selective pressures against methanogens in this experiment included: thermal re-treatment of the biomass, elimination of Mo and Co from the mineral medium (essential elements for methanogens growth [30]), reactor operation at pH 4.5, and start-up of the reactor under a CSTR mode with a short HRT to wash out the methanogens that could survive the thermal treatment. In experiment B the model substrate was substituted by acid hydrolysate using smaller increments than in experiment A, as an attempt to minimize the observed deleterious effect of the acid hydrolysate. The reactor was operated during a total of 56 days and the operational strategy is shown in Table 3.1.

3.3.2.1 Start-up and acclimation: stage I and II

Fig. 3.2 shows the profiles of HPR, HMY and gas composition during these stages. An increase in hydrogen production was observed as compared with experiment A. HPR and HMY mean values obtained during steady state of stage I (operated under continuous feeding mode) were 211 mL H_2/L-h and 1.64 mol H_2/mol sugar consumed, respectively. During the same period, the gas composition was almost constant, 67% H_2 and 33% CO_2. An important observation during this stage was the formation of granules from day 3 of the reactor operation (Fig. 3.3). The characteristics of these granules (spherical shape, cream color and an average diameter of 5 mm) were similar to those reported in the literature for hydrogen production [31]. The low pH used in this experiment (pH of 4.5) was probably an important factor that promoted granulation. It has been reported that acidic pH is a factor that promotes granulation due to surface physicochemical changes on microorganisms [31].

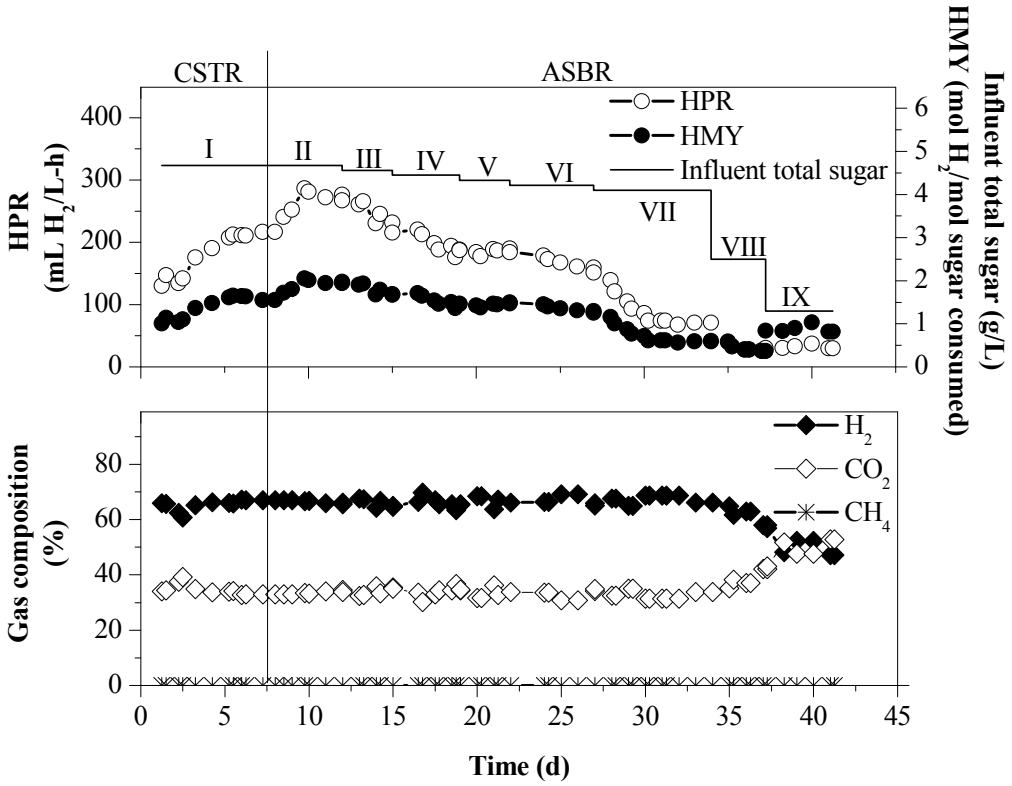

Fig. 3.2 Anaerobic sequencing batch reactor performance during Experiment B. During stage I the reactor was operated as a CSTR and fed with model substrate (glucose/xylose at 5 g COD/L total). From stage II and onwards the reactor was operated as an ASBR. The gradual replacement of model substrate by acid hydrolysate was from period III to VII. During period VIII and IX model substrate and acid hydrolysate were replaced by enzymatic hydrolysate (see Table 3.1). HPR: hydrogen production rate. HMY: hydrogen molar yield.

A system with granules facilitates the syntrophic interactions among microorganisms, which result in a high organic degradation capacity and thus high HPR [32]. This could be the reason for the higher HMY and HPR obtained in experiment B compared with experiment A.

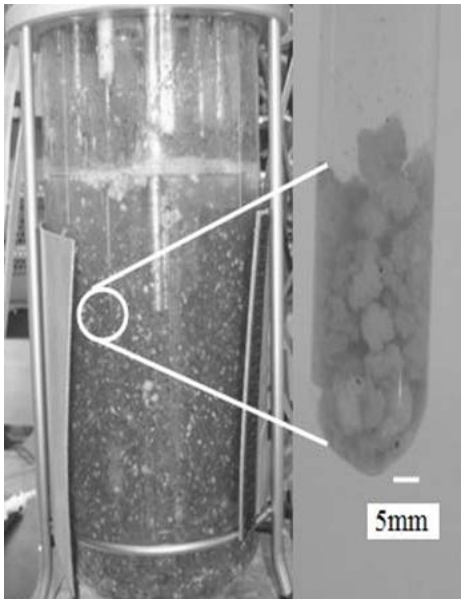

Fig. 3.3 Granules formed during the start-up of the reactor in experiment B (scale bar = 5mm).

In order to establish an ASBR cycle time, the sugar consumption and the hydrogen production were followed in the first three cycles of the stage II. A time of 4 hours was enough to consume most of the substrate and yield high hydrogen production (Fig. 3.4).

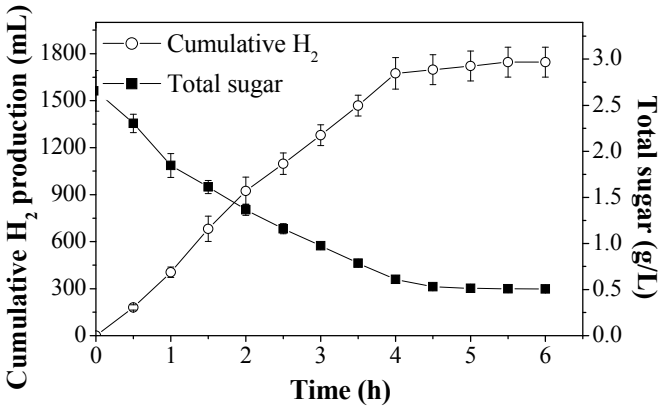

Fig. 3.4 Profile of substrate consumption and hydrogen production during batch experiments at the beginning of stage II (experiment B).

The ASBR resulted to be an efficient system for hydrogen production as can be observed in Fig. 3.2. The steady state mean values for stage II were 2 mol H_2/mol sugar consumed for HMY and 278 mL H_2/L-h for HPR. These values were the highest of the entire experiment and were 5.1 and 15.4 times greater than values of HMY and HPR in experiment A, respectively. Gas composition during stage II was 67% H_2 and 33% CO_2.

Strict anaerobic microorganisms produce mainly hydrogen following the acetate or butyrate pathway [33]. In agreement with that, acetate and butyrate were the main metabolic by-products of the fermentation process during stages I and II. Acetate and butyrate mean concentrations were 531 mg/L and 530 mg/L for stage I and 1071 mg/L and 1091 mg/L for stage II, respectively (Fig. 3.5). The difference in by-product concentrations between stages I and II was due to the fact that when reactor was operated as CSTR mode, only 47% of the sugar was removed; while under the ASBR operation, sugar removal was over 90%.

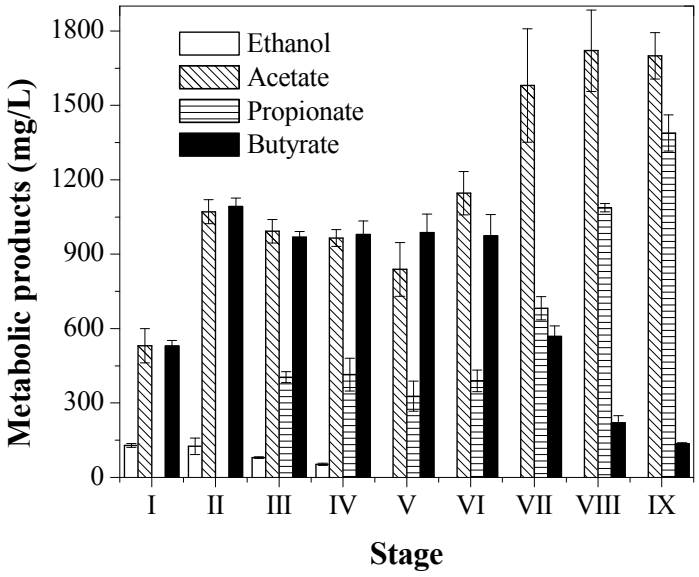

Fig. 3.5 Metabolic by-products produced during the different stages of the Experiment B.

3.3.2.2 Acid hydrolysate effect on hydrogen production: stages: III-VII

An important change observed since the beginning of the stage III was the disaggregation of the granules. This event occurred in spite that the only change in the reactor was the low amount of acid hydrolysate introduced to the system (10% of the feeding, Table 3.1). Although some authors have studied the granulation process [31, 32] there is no study that

reports the effect of lignocellulosic hydrolysates on hydrogenogenic granules. A possible cause for the disaggregation of the granules could be that the bacterial inhibitors (furfural, HMF and vanillin) affected in somehow the activity of some essential bacteria for granule stability. These compounds can affect the cell membrane function, growth and glycolysis of bacteria [34]. However, future studies are needed in order to evaluate their effect on hydrogenogenic granules.

Gas composition mean values during stages III to VII were 66% H_2 and 34% CO_2 (Fig. 3.2). This result indicates that applied selective pressures were effective to avoid methane production; even when higher concentrations of acid hydrolysate were added, comparing with experiment A. Thus, the strategy used in experiment B could be useful for future studies using similar systems and having problems with methane production.

It is also evident from Fig. 3.2 that HPR and HMY decreased with every increase of the acid hydrolysate concentration in the influent. A possible reason for this trend was the lower content of sugar in the influent every time the amount of acid hydrolysate was increased. This is because 5 g COD/L was maintained in the influent at each stage, but the acid hydrolysate had a total sugar concentration equivalent to 85% of its total COD concentration. Based on the previous consideration, the influent total sugar concentration was calculated for the different stages of the experiment B. Total sugar concentration in the influent decreased from 4.68 g/L to 4.11 g/L from stages II to VII (solid line, top panel, Fig. 3.2). However, the small decrease in total sugar concentration neither corresponds to the HPR decrease from 278 to 71.3 mL H_2/L-h nor to the HMY decrease from 2 to 0.59 mol H_2/mol sugar consumed.

Fig. 3.5 shows that concentrations of VFA from stages III to VI were almost constant: 986 ± 126, 977 ± 8 and 384 ± 39 (mg/L) for acetate, butyrate and propionate respectively. However, a major change occurred from stage VI to VII. Acetate increased from 1145 to 1580 (mg/L) and propionate from 389 to 682 (mg/L), while butyrate decreased from 973 to 569 (mg/L). These changes were in agreement with the greatest decrease in HPR and HMY (Fig. 3.2). From stage VI to VII the HPR and HMY decreased from 163 to 71.3 mL H_2/L-h and from 1.32 to 0.59 mol H_2/mol sugar consumed, respectively. It has been reported that propionate formation is unfavorable for hydrogen production [35]. But also high acetate

production could be due to hydrogen-consuming microorganisms such as homoacetogens and others [18, 36, 37]. This issue is further discussed in the COD balance section, 3.3.2.4.

3.3.2.3 Enzymatic hydrolysate effect on hydrogen production: stages: VIII-IX

Enzymatic hydrolysis of lignocellulosic biomass does not produce inhibitory compounds [7]. Therefore, the study of enzymatic hydrolysate effect was divided only in two stages. In stage VIII the remaining model substrate was substituted by enzymatic hydrolysate, and then in stage IX the remaining acid hydrolysate was substituted by enzymatic hydrolysate (Table 3.1).

It is evident from Fig. 3.2 that in stages VIII and IX the ASBR presented a decrease on HPR as compared with stage VII. A possible reason for this behavior is that in stages VIII and IX the influent total sugar concentration represented only 60% and 32%, respectively of that in stage VII. This is due to the fact that the enzymatic hydrolysate had a sugar concentration equivalent to 25% of its total COD concentration, which is lower than the 85% of the acid hydrolysate. Also, a decrease of the hydrogen in the gas composition (Fig. 3.2) and the associated increase in the propionate concentration (Fig. 3.3) contributed to the decrease on hydrogen production in these stages. Nonetheless, it is interesting that in spite of the lower influent sugar concentration in stage IX as compared to stage VIII, the HPR values were similar; which is reflected in a higher HMY in stage IX (Fig. 3.2). This is probably due to the fact that the enzymatic hydrolysate did not contain inhibitory compounds.

HPR and HMY decreased throughout the ASBR operation of this experiment. However, the values obtained in the stages VIII and IX are higher than those obtained in the first stage of experiment A (fed with model substrate), even when those stages in experiment B were fed with hydrolysates. Table 3.2 shows a comparison on hydrogen production performance obtained in the stages VIII and IX of this study and those reported in the literature using lignocellulosic hydrolysates as substrate. It is noticeable that all the reported HPR for hydrolysates, in spite of the type of hydrolysate and type of reactor used, are in a much lower range (4.6-81.4 mL H_2/L-h, Table 3.2) than those HPR reported for the best systems using model substrates (1.5 to 15.6 L H_2/L-h, [1]). This makes evident the relevance to study and improve the HPR from lignocellulosic hydrolysates.

Table 3.2 Comparison on the hydrogen production performance from lignocellulosic hydrolysates with other studies reported in the literature.

Substrate	System	HMY (mol H_2/ mol sugar)	HPR (mL /L-h)	H_2 content in gas (%)	Conditions: temperature (°C), pH and inlet concentration	Reference
Thermal hydrolysate from wheat straw	UASB	1.30	34.2	43	70, 5.2,3.9[a]	[8]
Acid hydrolysate from oat straw	TBR	0.20	81.4	37	28, 5.5, 35[b]	[17]
Acid hydrolysate from sugarcane bagasse	Batch	1.70	67.1	45	37, 5.5, 20[b]	[10]
Acid hydrolysate from sugarcane bagasse	Batch	0.84	4.6	nr	37, 6.5, 11[a]	[11]
Enzymatic hydrolysate from rice straw	Batch	0.76	26.8	26	37, 7.5,9.2[a]	[16]
Thermal hydrolysate from wheat straw	CSTR	1.10	7.7	37	70, 5.5,3.1[a]	[9]
Enzymatic hydrolysate from oat straw	ASBR	0.81	29.6	50	35, 4.5, 5[b]	This study (Experiment B, IX)
Mixture of enzymatic and acid hydrolysates from oat straw	ASBR	0.38	27.0	58	35, 4.5, 5[b]	This study (Experiment B, VIII)

The data correspond to the maximum values reported by all authors at the indicated conditions.
[a]g sugar/L.
[b]g COD/L.
nr-not reported.

A last stage of experiment B was carried out increasing the concentration of the enzymatic hydrolysate to 15 g COD/L (data not shown). However, hydrogen production was totally suppressed at this high concentration and the fermentation was mainly directed to the production of acetate and propionate. Recovery of the reactor by feeding lower substrate concentrations was attempted until day 56 with no success. In spite of the negative results

obtained with the enzymatic hydrolysate at 15g COD/L, further research on the use of enzymatic hydrolysate is encouraged, since performance of the enzymatic hydrolysate resulted higher than performance of the acid/enzymatic hydrolysates mixture when were tested at the same concentration, 5 g COD/L (Table 3.2).

3.3.2.4 COD Balance

The highest HPR and HMY found in stage II of experiment B (Fig. 3.2) are in agreement with the highest percentage of hydrogen obtained in the COD distribution (Table 3.3). In this stage a major proportion of the substrate was used for the production of butyrate 39.7%, followed by acetate 22.9%. Both metabolites are directly related with hydrogen production [33]. On the other hand, the lowest values of HPR and HMY were observed on the last three stages VII, VIII and IX (Fig. 3.2), which also corresponded with the lowest percentages for hydrogen (Table 3.3). In these stages, high percentage of the COD was found in the production of acetate and propionate. The sum of these metabolites in stages VII, VIII and IX amounted from 54.3 to 78.2% of the total COD. In spite of the high production of acetate on the last three stages, a low hydrogen production was found.

Table 3.3 Metabolic products distribution on COD basis during experiment B.

	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**
Remaining sugar	46.2	3.9	2.7	5.2	5.3	4.6	4.2	4.7	2.9
Hydrogen	6.0	15.8	13.2	10.8	10.7	9.1	4.1	1.5	1.7
Acetate	11.3	22.9	21.2	20.6	17.9	24.5	33.7	36.7	36.3
Propionate	0	0	12.2	12.5	9.9	11.8	20.6	32.9	41.9
Butyrate	19.3	39.7	35.2	35.7	35.9	35.4	20.7	8.0	5.0
Ethanol	5.4	5.3	3.4	2.2	0	0	0	0	0
Biomass and others	11.8	12.4	12.1	13.0	20.3	14.6	16.7	16.2	12.2

Generally, acetate production is considered favorable for hydrogen production [33]. However, acetate can also be generated through non-favorable hydrogen pathways by homoacetogenic bacteria [18], syntrophic bacteria [36] and propionibacteria [37]. Regarding propionate, it is well known that its production during hydrogen production by fermentation is undesirable; this is because NADH produced during acidogenesis is used for propionate generation instead of hydrogen production [35, 38].

3.3.3 Microbial analysis

3.3.3.1 Archaea analysis in experiment A

Due to methane production in experiment A, archaea identification became relevant. Fig. 3.6 shows the DGGE profiles for inoculum and stages I and II of experiment A (IN, EAI and EAII samples). Most of the gel bands were subjected to reamplification by PCR; however, only five bands were successfully reamplified and further sequenced. Reamplification problems of archaea 16S rDNA genes have been previously observed [39].

Fig. 3.6 DGGE profiles for archaea in experiment A. EAI: Experiment A stage I; EAII: Experiment A stage II; IN: Inoculum. Arrows and numbers indicate the successfully reamplified and sequenced bands.

Bands 2 and 3 were similar to sequences of *Methanobacterium sp*. and uncultured methanogenic archaeon (98% of similarity for both sequences), respectively. For the case of band 1, sequence was similar to *Methanobrevibacter acididurans* (97% of similarity) (Table 3.4). Both identified microorganisms are hydrogenotrophic methanogens [40, 41] and were found in EAII but not in EAI. This suggests that these microorganisms may have played a key role in the shift from hydrogen to methane production during stage II of the experiment A. Moreover, *M. acididurans* is able to survive at pH 5.5 [41], which was the pH of the ASBR operation in Experiment A. Furthermore, the presence of the Methanobacterium *sp*. and the uncultured methanogenic archaeon in the inoculum as well as during stage II of experiment A may indicate the presence of viable methanogenic microorganism in the heat-treated inoculum.

Table 3.4 Phylogenetic affiliations of the archaea DGGE band sequences

Band	Accession number	Closest relative	Query Coverage (%)	Similiraty (%)	Phylogenetic affiliation (class)
1	NR_028779.1	*Methanobrevibacter acididurans*	68	97	Methanobacteria
2	GU112764.1	*Methanobacterium sp.*	82	98	Methanobacteria
3	GQ453660.1	Uncultured methanogenic archaeon	66	98	Unknown

3.3.3.2 Bacteria analysis during experiments A and B

Bacterial DGGE patterns and dendrogram are shown in Fig. 3.7 (marked bands were successfully reamplified and sequenced). Both stages of the experiment A showed the highest bacterial diversity and were clustered together. Communities from different stages of the experiment B were clustered with the community of the inoculum. Stages EBII and EBIII were identical according to the dendrogram and were also 60% similar to the community in the inoculum, while the communities in stages EBVII and EBIX presented 80% of similarity among them. It is interesting that during stages with the highest hydrogen productions, EBII and EBIII (Fig. 3.2), only two bands were found. Meanwhile, during stages with low hydrogen productions, EAI, EAII, EBVII and EBIX (Fig. 3.2), a higher

number of bands were observed. This observation suggests that a high microbial diversity could have a detrimental effect on the hydrogen production process.

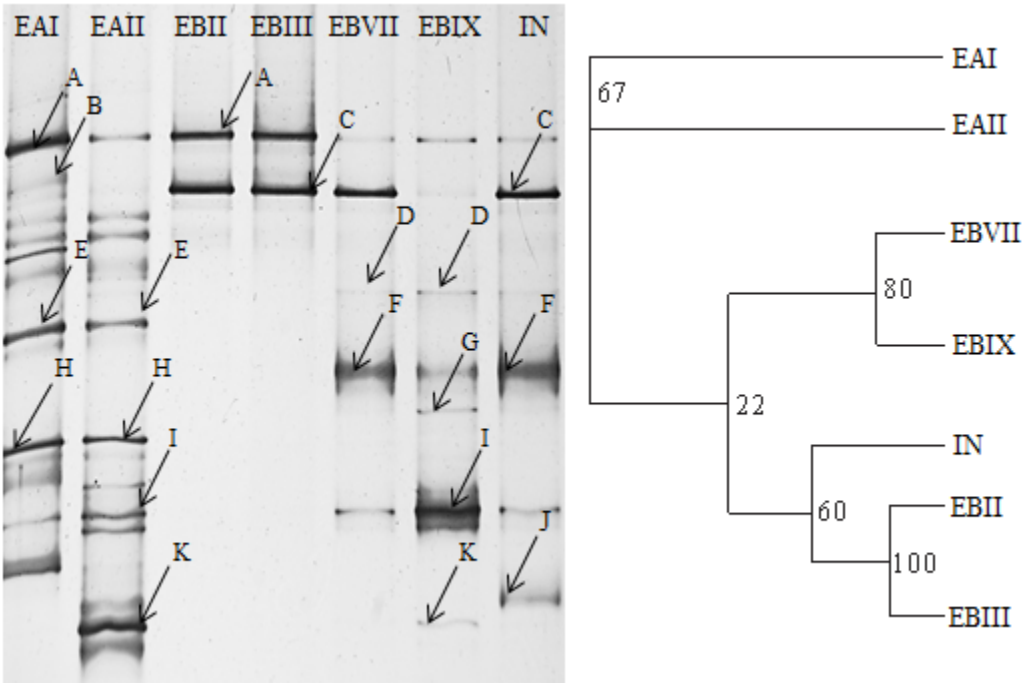

Fig. 3.7 DGGE profiles for bacteria (experiments A and B) and dendrogram with Dice coefficients of similarity. EAI: Experiment A stage I; EAII: Experiment A stage II; EBII: Experiment B stage II; EBIII: Experiment B stage III; EBVII: Experiment B stage VII; EBIX: Experiment B stage IX; IN: Inoculum. Arrows and letters indicate the successfully reamplified and sequenced bands.

In this study, bacterial DGGE patterns and their band intensities were used to estimate relative microbial abundances (Fig. 3.8). For both stages of the experiment A, most of the operational taxonomic units (OTUs) belonged to bands that were not successfully reamplified (Others, Fig. 3.7). On the other hand, Fig. 3.7 shows only two bands (C and A) in EBII and EBIII. According to Table 3.5, band A was similar to an uncultured bacterium, while band C was 99% similar to *Clostridium pasteurianum.* The presence of *C. pasteurianum* is important because this specie has been reported as a hydrogen producer

and granule forming [42], which agrees with the observations already described for stage I and II of the experiment B (Fig. 3.2).

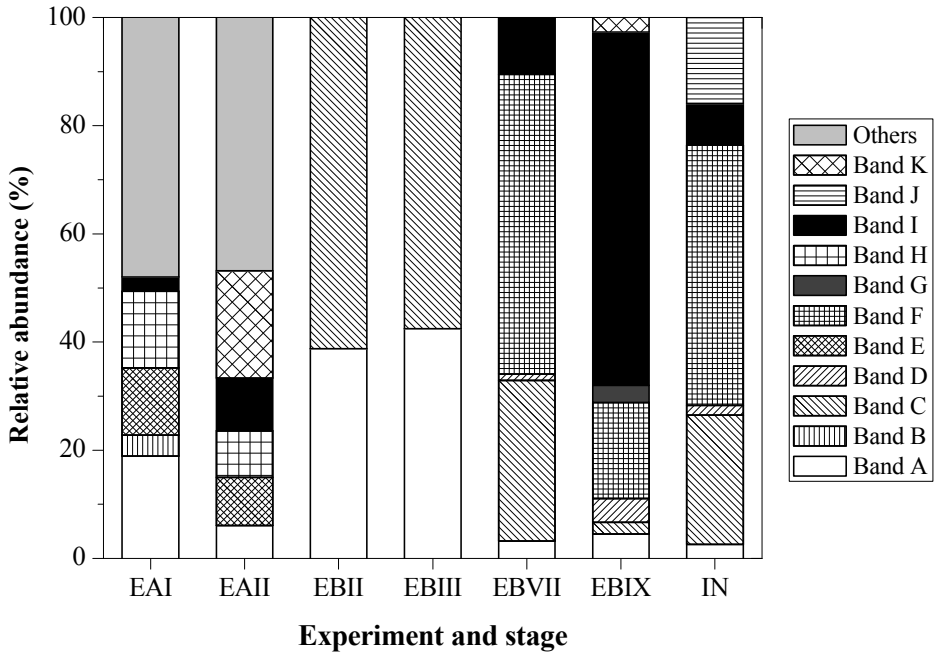

Fig. 3.8 Relative abundance for bacteria DGGE bands. EAI: Experiment A stage I; EAII: Experiment A stage II; EBII: Experiment B stage II; EBIII: Experiment B stage III; EBVII: Experiment B stage VII; EBIX: Experiment B stage IX; IN: Inoculum. Letters A to K indicate the successfully reamplified and sequenced bands as shown in Fig. 3.7.

Fig. 3.8 also shows that bands F and I were dominant during stages EBVII and EBIX, respectively. As shown in Table 3.5, band F was similar to uncultured *Veillonella sp*. and band I to *Clostridium sp* (99 and 97% similarity, respectively). Nonetheless, a wide OTUs diversity was observed in both stages. Thus, it seems that oat straw hydrolysates either negatively affected *C. pasteurianum,* or favored other microorganisms, which could produce fermentation byproducts different to hydrogen, or even consume hydrogen. Further studies are needed in order to clarify the relation among acid hydrolysate addition, granule disaggregation and observed changes in microbial populations.

Table 3.5 Phylogenetic affiliations of the bacteria DGGE band sequences.

Band	Closest relative	Query Coverage (%)	Similiraty (%)	Accession number
A	Uncultured bacterium	63	91	AB219993.2
B	Uncultured bacterium	96	94	DQ325506.1
C	*Clostridium pasteurianum*	83	99	EF656617.1
D	*Lactobacillus concavus*	68	100	NR_043105.1
E	Uncultured bacterium	97	100	GU100497.1
F	Uncultured *Veillonella sp.*	72	99	GQ332226.1
G	*Pectinatus sp.*	98	95	GU586299.1
H	*Megasphaera cerevisiae*	97	99	AB609706.1
I	*Clostridium sp.*	81	97	AY925092.1
J	*Citrobacter freundii*	99	99	JQ781578.1
K	*Propionibacterium cyclohexanicum*	99	98	NR_036827.1

Fig. 3.9 shows the phylogenetic distribution of the identified OTUs. Identified classes were Actinobacteria, Bacilli, Clostridia, Gammaproteobacteria and Negativicutes. Most of the identified bands were phylogenetically related to class Clostridia (4 of 11 bands: A, B, C, and I) and class Negativicutes (3 of 11 bands: F, G and H); both classes belong to phylum Firmicutes. Previous studies have reported that microorganisms belonging to phylum Firmicutes are capable to produce spores, which allow them to survive some adverse conditions, as heat-shock pretreatments or unfavorable reactor conditions [43]. Clostridia are generally fermentative organisms that produce hydrogen along with other byproducts such as acetate, butyrate, ethanol, acetone, succinate, etc. [39]. Negativicutes as *Pectinatus* and *Veillonella* are either, hydrogen producers or hydrogen consumers [44]. On the other hand, microorganisms belonging to classes Bacilli and Actinobacteria as *Lactobacillus concavus* and *Propionibacterium cyclohexanicum* respectively, have shown to be hydrogen competitors [44]; whereas *Citrobacter* species that belongs to class Gammaproteobacteria has been reported as hydrogen producer [45]. Based on the phylogenetic study (Fig. 3.9), it

is evident that during last stages of the experiment B, most of the OTUs were hydrogen consumers, mainly acetate and propionate producers. This observation is supported by data in Fig. 3.5.

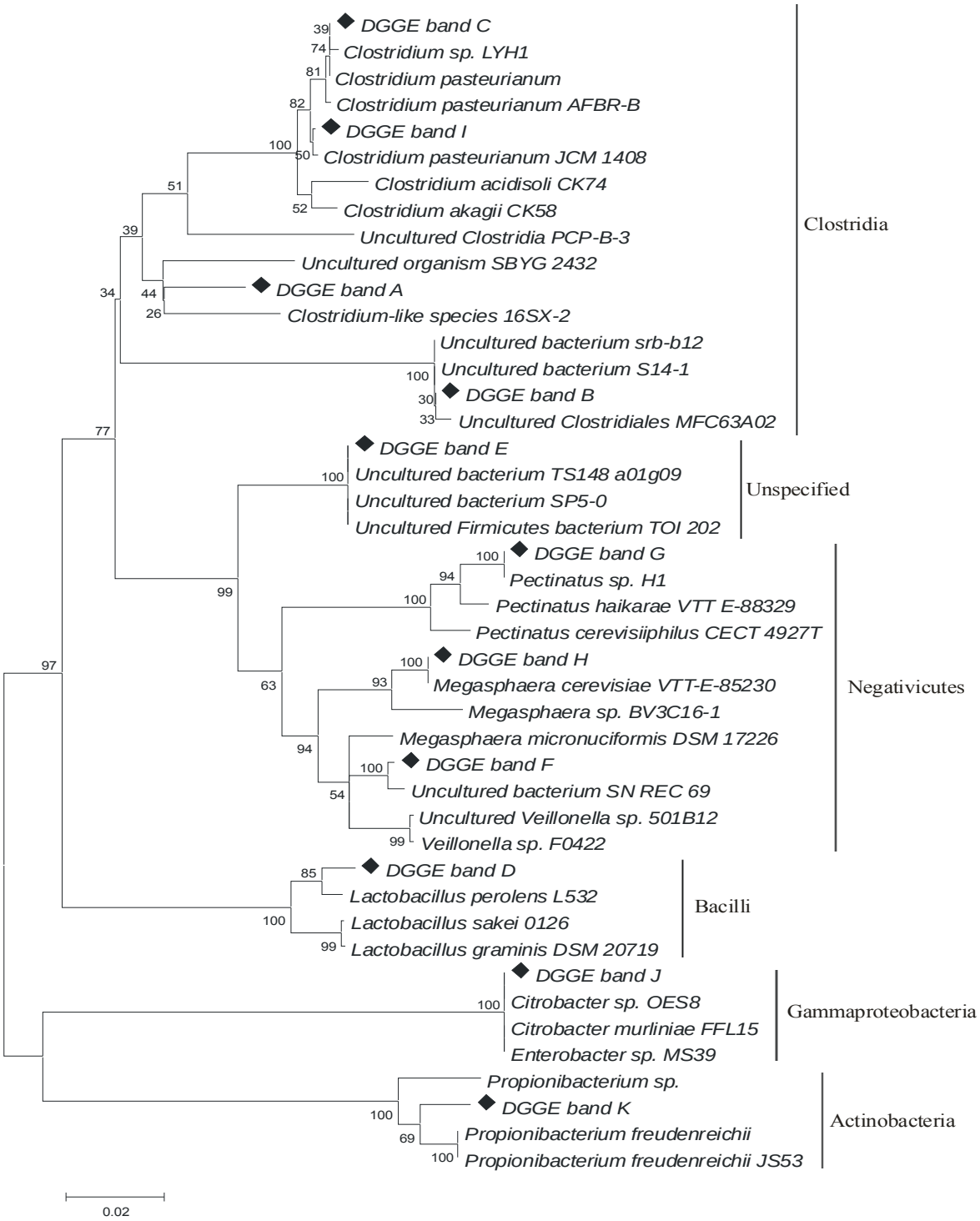

Fig. 3.9 Phylogenetic tree of 16S rDNA sequences from bacteria DGGE profiles.

3.4 Conclusions

Overall results showed that it is feasible to produce hydrogen without co-production of methane from acid and enzymatic oat straw hydrolysates in an ASBR. The application of selection pressure in experiment B prevented methane production. These conditions also promoted granulation of the biomass, high HMY and high HPR when glucose-xylose was used as substrate. However, the use of the acid hydrolysate as substrate promoted the disaggregation of the granules and showed a detrimental effect on the hydrogen production. Decrease of hydrogen production was associated to an increase of acetate and propionate concentrations. The use of the enzymatic hydrolysate as sole substrate increased the HMY, but when enzymatic hydrolysate concentration was increased a detrimental effect on hydrogen production was observed. On the other hand, molecular analysis showed that hydrogenotrophic methanogens were the putative responsible for methane production during experiment A, while *C. pasteurianum* was found as the putative responsible for biomass granulation and high hydrogen production during the first two stages of the experiment B. Moreover, a high bacterial diversity was related to stages with low hydrogen production during experiment B (fed with oat straw hydrolysates).

3.5. References

[1] Davila-Vazquez G, Arriaga S, Alatriste-Mondragon F, De León-Rodríguez A, Rosales Colunga LM, Razo-Flores E. Fermentative biohydrogen production: trends and perspectives. Rev Environ Sci Biotechnol 2008; 7:27-45.

[2] Wang J, Wan W. Factors influencing fermentative hydrogen production: A review. Int J Hydrogen Energy 2009; 34:799-811.

[3] Cheng CL, Lo YC, Lee KS, Lee DJ, Lin CY, Chang JS. Biohydrogen production from lignocellulosic feedstock. Bioresour Technol 2011; 102:8514-23.

[4] Guo XM, Trably E, Latrille E, Carrere H, Steyer JP. Hydrogen production from agricultural waste by dark fermentation: A review. Int J Hydrogen Energy 2010; 35:10660-73.

[5] Ren N, Wang A, Cao G, Xu J, Gao L. Bioconversion of lignocellulosic biomass to hydrogen: Potential and challenges. Biotechnol Adv 2009; 27:1051-60.

[6] Taherzadeh MJ, Karimi K. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review. Int J Mol Sci 2008; 9:1621-51.

[7] Kumar P, Barrett DM, Delwiche MJ, Stroeve P. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind Eng Chem Res 2009; 48:3713-29.

[8] Kongjan P, Angelidaki I. Extreme thermophilic biohydrogen production from wheat straw hydrolysate using mixed culture fermentation: effect of reactor configuration. Bioresour Technol 2010; 101:7789-96.

[9] Kongjan P, O-Thong S, Kotay M, Min B, Angelidaki I. Biohydrogen production from wheat straw hydrolysate by dark fermentation using extreme thermophilic mixed culture. Biotechnol Bioeng 2010; 105:899-908.

[10] Pattra S, Sangyoka S, Boonmee M, Reungsang A. Biohydrogen production from the fermentation of sugarcane bagasse hydrolysate by *Clostridium butyricum*. Int J Hydrogen Energy 2008; 33:5256-65.

[11] Fangkum A, Reungsang A. Biohydrogen production from sugarcane bagasse hydrolysate by elephant dung: Effects of initial pH and substrate concentration. Int J Hydrogen Energy 2011; 36:8687-96.

[12] Datar R, Huang J, Maness PC, Mohagheghi A, Czernik S, Chornet E. Hydrogen production from the fermentation of corn stover biomass pretreated with a steam-explosion process. Int J Hydrogen Energy 2007; 32:932-9.

[13] Pan C, Zhang M, Fan Y, Xing Y, Hou H. Production of cellulosic ethanol and hydrogen from solid-state enzymatic treated cornstalk: a two stage process. J Agric Food Chem 2009; 57:2732-8.

[14] Wang Y, Wang H, Feng X, Wang X, Huang J. Biohydrogen production from cornstalk wastes by anaerobic fermentation with activated sludge. Int J Hydrogen Energy 2010; 35:3092-9.

[15] Ma S, Wang H, Wang Y, Bu H, Bai J. Bio-hydrogen production from cornstalk wastes by orthogonal design method. Renew Energ 2011; 36:709-13.

[16] Lo YC, Lu WC, Chen CY, Chang JS. Dark fermentative hydrogen production from enzymatic hydrolysate of xylan and pretreated rice straw by *Clostridium butyricum CGS5*. Bioresour Technol 2010; 101:5885-91.

[17] Arriaga S, Rosas I, Alatriste-Mondragón F, Razo-Flores E. Continuous production of hydrogen from oat straw hydrolysate in a biotrickling filter. Int J Hydrogen Energy 2011; 36:3442-9.

[18] Arooj MF, Han SK, Kim SH, Kim DH, Shin HS. Effect of HRT on ASBR converting starch into biological hydrogen. Int J Hydrogen Energy 2008; 33:6509-14.

[19] Buitrón G, Carvajal C. Biohydrogen production from tequila vinasses in an anaerobic sequencing batch reactor: Effect of initial substrate concentration, temperature and hydraulic retention time. Bioresour Technol 2010; 101:9071-7.

[20] Sreethawong T, Chatsiriwatana S, Rangsunvigit P, Chavadej S. Hydrogen production from cassava wastewater using an anaerobic sequencing batch reactor: Effects of operational parameters, COD:N ratio, and organic acid composition. Int J Hydrogen Energy 2010; 35:4092-102.

[21] Chen WH, Sung S, Chen SY. Biological hydrogen production in an anaerobic sequencing batch reactor: pH and cyclic duration effects. Int J Hydrogen Energy 2009; 34:227-34.

[22] Wu X, Yao W, Zhu J. Effect of pH on continuous biohydrogen production from liquid swine manure with glucose supplement using an anaerobic sequencing batch reactor. Int J Hydrogen Energy 2010; 35:6592-9.

[23] Gomez-Tovar F, Celis LB, Razo-Flores E, Alatriste-Mondragón F. Chemical and enzymatic sequential pretreatment of oat straw for methane production. Bioresour Technol 2012; 116:372-8.

[24] Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956; 28:350–6.

[25] APHA, AWWA and WPCF. Standard methods for the examination of water and wastewater. In: Eaton, A.D., Clesceri, A.E., Rice E.W., Greenberg, A.E. (Eds.), American Public Health Association, 21st ed. American Water Works Association and Water Environment Federation, Washington D.C. 2005.

[26] Carrillo-Reyes J, Celis LB, Alatriste-Mondragon F, Razo-Flores E. Different start-up strategies to enhance biohydrogen production from cheese whey in UASB reactors. Int J Hydrogen Energy 2012; 37:5591-601.

[27] Kaksonen AH, Plumb JJ, Franzmann PD, Puhakka JA. Simple organic electron donors support diverse sulfate-reducing communities in fluidized-bed reactors treating acidic metal- and sulfate-containing wastewater. FEMS Microbiol Ecol 2004; 47:279-89.

[28] Sousa DZ, Pereira MA, Smidt H, Stams AJM, Alves MM. Molecular assessment of complex microbial communities degrading long chain fatty acids in methanogenic bioreactors. FEMS Microbiol Ecol 2007; 60:252-65.

[29] Prakasham RS, Brahmaiah P, Sathish T, Rao S KRS. Fermentative biohydrogen production by mixed anaerobic consortia: Impact of glucose to xylose ratio. Int J Hydrogen Energy 2009; 34:9354-61.

[30] Schonheit P, Moll J, Thauer RK. Nickel, cobalt, and molybdenum requirement for growth of *Methanobacterium thermoautotrophicum*. Arch Microbiol 1979; 123:105-7.

[31] Zhang ZP, Show KY, Tay JH, Liang DT, Lee DJ, Su A. The role of acid incubation in rapid immobilization of hydrogen-producing culture in anaerobic upflow column reactors. Int J Hydrogen Energy 2008; 33:5151-60.

[32] Fang HHP, Liu H, Zhang T. Characterization of a hydrogen-producing granular sludge. Biotechnol Bioeng 2002; 78:44-52.

[33] Mathews J, Wang G. Metabolic pathway engineering for enhanced biohydrogen production. Int J Hydrogen Energy 2009; 34:7404-16.

[34] Cao GL, Ren NQ, Wang AJ, Guo WQ, Xu JF, Liu BF. Effect of lignocellulose-derived inhibitors on growth and hydrogen production by *Thermoanaerobacterium thermosaccharolyticum* W16. Int J Hydrogen Energy 2010; 35:13475-80.

[35] Wang L, Zhou Q, Li FT. Avoiding propionic acid accumulation in the anaerobic process for biohydrogen production. Biomass Bioenerg 2006; 30:177–82.

[36] Narihiro T, Terada T, Ohashi A, Kamagata Y, Nakamura K, Sekiguchi Y. Quantitative detection of previously characterized syntrophic bacteria in anaerobic wastewater treatment systems by sequence-specific rRNA cleavage method. Water Res 2012; 46:2167-75.

[37] Walker M, Phillips CA. The growth of *Propionibacterium cyclohexanicum* in fruit juices and its survival following elevated temperature treatments. Food microbiol 2007; 24:313-18.

[38] Ren N, Wang B, Huang JC. Ethanol-type fermentation from carbohydrate in high rate acidogenic reactor. Biotechnol Bioeng 1997; 54:428-33.

[39] Mariakakis I, Bischoff P, Krampe J, Meyer C, Steinmetz H. Effect of organic loading rate and solids retention time on microbial population during bio-hydrogen production by dark fermentation in large lab-scale. Int J Hydrogen Energy 2011; 36:10690-700.

[40] Mori K, Harayama S. *Methanobacterium petrolearium sp.* nov. and *Methanobacterium ferruginis sp*. nov., mesophilic methanogens isolated from salty environments. Int J Syst Evol Microbiol 2011; 61:138-43.

[41] Savant DV, Shouche YS, Prakash S, Ranade DR. *Methanobrevibacter acididurans sp* nov., a novel methanogen from a sour anaerobic digester. Int J Syst Evol Microbiol 2002; 52:1081-7.

[42] Zhang Z-P, Adav SS, Show K-Y, Tay J-H, Liang DT, Lee D-J, et al. Characteristics of rapidly formed hydrogen-producing granules and biofilms. Biotechnol Bioeng 2008; 101:926-36.

[43] Goud RK, Raghavulu SV, Mohanakrishna G, Naresh K, Mohan SV. Predominance of *Bacilli* and *Clostridia* in microbial community of biohydrogen producing biofilm sustained under diverse acidogenic operating conditions. Int J Hydrogen Energy 2012; 37:4068-76.

[44] Hung CH, Chang YT, Chang YJ. Roles of microorganisms other than *Clostridium* and *Enterobacter* in anaerobic fermentative biohydrogen production systems - A review. Bioresour Technol 2011; 102:8437-44.

[45] Davila-Vazquez G, de León-Rodríguez A, Alatriste-Mondragón F, Razo-Flores E. The buffer composition impacts the hydrogen production and the microbial community composition in non-axenic cultures. Biomass Bioenerg 2011; 35:3174-81.

Chapter 4

Hydrogen production from glucose and oat straw acid and enzymatic hydrolysates: effect of the inoculum

Summary

The present study evaluated the capability of different inocula (anaerobic granular and flocculent sludge, maize and triticale silage, and aerobic sludge) to use simple and complex substrates (glucose and acid and enzymatic hydrolysates from oat straw) during the fermentative hydrogen production process. The highest volumetric hydrogen production rate, 58 mL H_2/L-h, was achieved by anaerobic granular sludge, using glucose as substrate. However, maize and triticale silages showed higher volumetric hydrogen production rates when acid and enzymatic hydrolysates were used, as compared to the anaerobic granular sludge. Also, the highest hydrogen molar yield (HMY) was achieved by triticale silage, 1.24 mol H_2/mol hexose equivalent, using enzymatic hydrolysate as substrate. These results demonstrate the potential of silages for their use as inoculum in hydrogen production systems; especially when complex substrates are used.

Adapted from: Arreola-Vargas J, Abreu-Sherrer J, Celis LB, Razo-Flores E, Alatriste-Mondragón F. Hydrogen production from oat straw hydrolysates and glucose: effect of the type of substrate and type of inoculum. In: Proc. of the IV International Symposium on Energy from Biomass and Waste. International Waste Working Group. Venice, Italy, 2012. *Cisa Publisher*.

4.1 Introduction

Hydrogen is recognized as an ideal energy carrier and an excellent alternative to fossil fuels [1]. In the last decade, several studies have been conducted on the production of hydrogen through fermentation of organic substrates [2], because it is recognized as an environmental friendly, cost effective, and sustainable process for energy production along with treatment of organic wastes.

Up to now, most of the reported studies on fermentative hydrogen production have used simple model substrates, such as glucose, and only few studies have used complex substrates, as lignocellulosic by-products [1-4]. Thus, the use of lignocellulosic by-products as feedstock for hydrogen production should be investigated in depth, since they are potential substrates in full scale application due to their abundance and low cost [5]. Nonetheless, due to the recalcitrance of the lignocellulosic biomass composition, it cannot be used directly for hydrogen production, therefore a pretreatment is required in order to solubilize sugars from hemicellulose and cellulose fractions. In this regard, sequential pretreatments, such as acid hydrolysis followed by enzymatic hydrolysis, can be applied to solubilize most of the sugars from hemicellulose and cellulose of the lignocellulosic biomass. In this way, is possible to obtain a more suitable substrate for hydrogen production [6, 7].

Regarding the type of inoculum, fermentative hydrogen production depends on the source of microorganisms, which affects directly the development of the community and the hydrogen production potential of the system. Because of this reason, several sources of inocula have been evaluated for hydrogen production, such as pure cultures, compost, caw manure, anaerobic and aerobic sludge, and others [2]. However, to the best of our knowledge, it has not been reported the use of silages as inoculum for hydrogen production. Because of its nature, the silages could be an excellent source of fermentative microorganisms.

Therefore, the objective of this work was to compare the capability of different inocula for hydrogen production: anaerobic granular sludge (AGS), anaerobic flocculent sludge (AFS), maize silage (MS), triticale silage (TS) and aerobic sludge (AS). Furthermore, the capability of each inoculum to use simple (glucose) or complex substrates (acid and

enzymatic oat straw hydrolysates) was compared. Oat straw was used as a lignocellulosic by-product model.

4.2 Material and methods

4.2.1 Substrates

Glucose used was reagent grade and the oat straw was obtained from a commercial source (Forrajera Marquez Company, San Luis Potosí, México). A farm mill was used to reduce straw particle size to an average length of 2 cm. This material was sequentially acid and enzymatically hydrolyzed in order to solubilize the hemicellulose and cellulose of the oat straw. Both hydrolysis procedures were carried out as reported by Arreola-Vargas et al. [6]. The acid hydrolysate had the following composition in g/L: COD 25 ± 3.1; total sugar 20 ± 2.2; glucose 1.5 ± 0.2; xylose 3.7 ± 1.1; arabinose 1.3 ± 0.3; mannose 0.59 ± 0.1; galactose 0.46 ± 0.1. Furfural and phenolic compounds concentrations were (mg/L): hydroxy methyl furfural (HMF) 133.2 ± 23.3; furfural 0.6 ± 0.4; vanillin 3.59 ± 0.9. The enzymatic hydrolysate had the following composition (g/L): COD 30 ± 1; total sugar 7 ± 1; glucose 3.8 ± 0.9; xylose 1.3 ± 0.4; arabinose, mannose, galactose, HMF, furfural and vanillin were not detected in this hydrolysate. All chemicals used for the hydrolysis were reagent grade.

4.2.2 Inocula

The inocula used and their sources were: AGS and AFS from different operational periods of a full-scale up-flow anaerobic sludge blanket reactor treating wastewater from a confectionery industry, AS from a full-scale sequential batch reactor treating municipal wastewater and MS and TS from a dairy farm. Prior to inoculation, the inocula were thermally treated at 104 °C during 24 h in order to inhibit the activity of hydrogen consumers and to induce the formation of hydrogen producing spores; then, the dried inocula were powdered in a mortar and finally stored in a container at room temperature.

4.2.3 Experimental procedure

The effect of five inocula was evaluated for hydrogen production, using the substrates indicated in section 4.2.1 for each inoculum. Batch experiments were carried out in 120 mL serum vials with a working volume of 80 mL. In order to compare results, all the vials

contained 4.5 g volatile suspended solids (VSS)/L of inoculum, mineral medium and substrate at a concentration of 5 g COD/L. The mineral medium used in the experiments was adjusted to an initial pH of 7.5 and its composition was as follows (g/L): $NH_4H_2PO_4$, 4.5; Na_2HPO_4, 11.9; K_2HPO_4, 0.125; $MgCl_2 \cdot 6H_2O$, 0.1; $MnSO_4 \cdot 6H_2O$, 0.015; $FeSO_4 \cdot 5H_2O$, 0.025; $CuSO_4 \cdot 5H_2O$, 0.005; $ZnCl_2$, 0.075. All chemicals used were reagent grade. After sealing the vials with rubber stoppers and aluminum crimps, the headspace was purged with nitrogen gas for 15 seconds. Vials were placed in a horizontal shaker at 150 rpm and 35ºC. Gas production and composition in the headspace were measured periodically and metabolic by-products were measured at the end of the experiments. All the batch assays in this work were carried out by triplicate.

4.2.4 Kinetic Analysis

The cumulative H_2 production during batch experiments were fitted to a modified Gompertz model, using equation (1) and KaleidaGraph 4.0 (Synergy software). This equation has been widely used to model gas production data [6, 7].

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

Where H (t) (mL) is the total amount of H_2 produced at culture time t (h), H_{max} (mL) is the maximum cumulative amount of H_2 produced, R_{max} (mL/h) is the maximum H_2 production rate, and λ (h) is the lag time before exponential H_2 production. H_2 produced is reported at standard conditions (0 ºC and 1 atm).

4.2.5 Analytical methods

For hydrogen production, the volume of gas produced was measured by a liquid displacement device, filled with acidified water (pH 2). The gas composition (measured by a GC-TCD), the type and concentration of hexoses, pentoses and volatile fatty acids (measured by capillary electrophoresis) and the concentration of furfural and phenolic compounds (measured by HPLC) were determined as described by Arreola-Vargas et al. [6]. Protein content on the enzyme was determined as described by Bradford [8]. The filter paper activity of the cellulase was determined by the IUPAC method [9]. Reducing sugars

were determined by the dinitrosalicylic acid (DNS) method, using glucose as substrate [10]. COD and VSS concentrations were determined according to standard methods [11].

4.3 Results and discussion

4.3.1 Hydrogen production kinetics

According to Table 4.1, all the experiments obtained high reducing sugar removals (above 98%), which implies that almost all the sugars were metabolized by the microbial communities present in each assay. Table 4.1 also shows that hydrogen represented 58 to 74% of the gas (except for AS). Furthermore, silages presented the shortest acclimation time, 12 to 16 h; whereas AS presented the largest one, 33 to 36 h. These results may indicate that AS was not as adequate as the rest of the inocula for hydrogen production.

Table 4.1 Sugar removal, gas composition and lag phase during the batch assays

*Inoculum/Substrate	Sugar removal (%)	Gas composition (%H_2/%CO_2)	Lag phase (h)
*Anaerobic flocculent sludge			
Glucose	100	70/30	29
Enzymatic hydrolysate	99	54/56	31
Acid hydrolysate	98	63/37	20
*Anaerobic granular sludge			
Glucose	99	74/26	28
Enzymatic hydrolysate	98	70/30	15
Acid hydrolysate	98	65/35	14
*Aerobic sludge			
Glucose	98	40/60	33
Enzymatic hydrolysate	98	32/68	36
Acid hydrolysate	98	30/70	36
*Maize silage			
Glucose	100	71/29	14
Enzymatic hydrolysate	99	65/35	16
Acid hydrolysate	100	66/34	16
*Triticale silage			
Glucose	100	73/27	15
Enzymatic hydrolysate	100	70/30	12
Acid hydrolysate	99	70/30	13

Fig. 4.1 shows that the highest cumulative hydrogen production was obtained by silages, using glucose as substrate. MS produced 47 mL of hydrogen and was followed by TS with 43 mL. On the other hand, when the acid and enzymatic hydrolysates were evaluated as substrates, both showed similar cumulative hydrogen productions, which were around half of the cumulative hydrogen produced by glucose (except for AS). This is understandable because both hydrolysates contained other substances besides sugars, which may not produce hydrogen or may even inhibit its production [12]. The maximum cumulative hydrogen production, using hydrolysates as substrate (around 20 mL), was observed when MS or TS were used as inocula. These cumulative hydrogen productions are similar to those obtained by Cui et al. [7], which used poplar leaves hydrolysates as substrate.

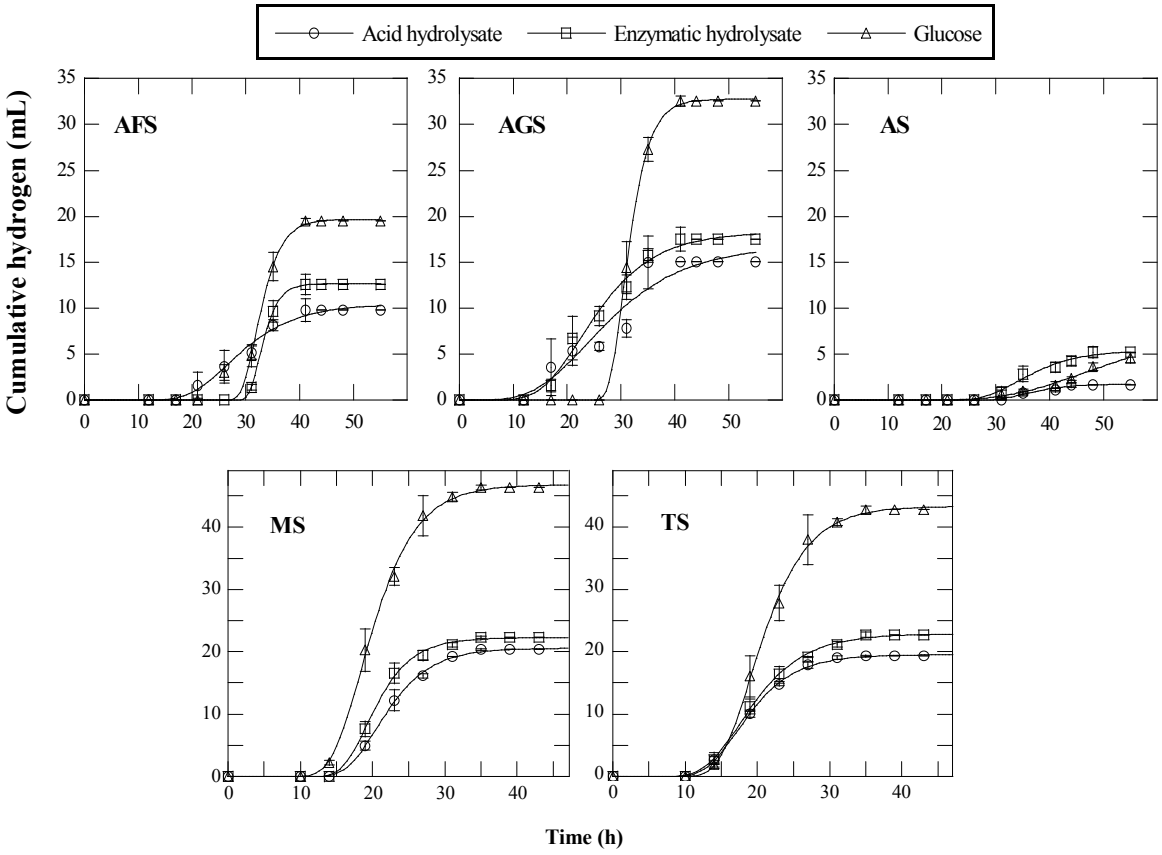

Fig. 4.1 Hydrogen production profiles obtained during the batch experiments for the different inocula and substrates. AFS (anaerobic flocculent sludge); AGS (anaerobic granular sludge); AS (aerobic sludge); MS (maize silage), TS (triticale silage). Symbols: experimental data; line: Gompertz fitting; standard deviation is represented by error bars.

On the other hand, AS produced the lowest amount of hydrogen, independently of the substrate used (Fig. 4.1). Besides, and as already mentioned, AS also obtained the lowest percentage of hydrogen in gas and the longest lag phase (Table 4.1). Even though this type of inoculum have already being used for hydrogen production, low content of hydrogen in gas and low performance has been reported [13].

4.3.2 Hydrogen molar yield (HMY) and volumetric hydrogen production rate (VHPR)

The VHPR is an important parameter in hydrogen production processes because it shows the potential for practical applications; besides, it has not theoretical limitation [2]. Therefore, VHPR was determined in order to assess the hydrogen potential of the different inocula, using Gompertz equation (1). Fig. 4.2 shows that the highest VHPR were obtained when glucose was used as substrate. The highest VHPR was obtained by AGS, 58 mL H_2/L-h. However, when the acid and enzymatic hydrolysates were used as substrate, in general, the highest values were obtained by both silages (Fig. 4.2).

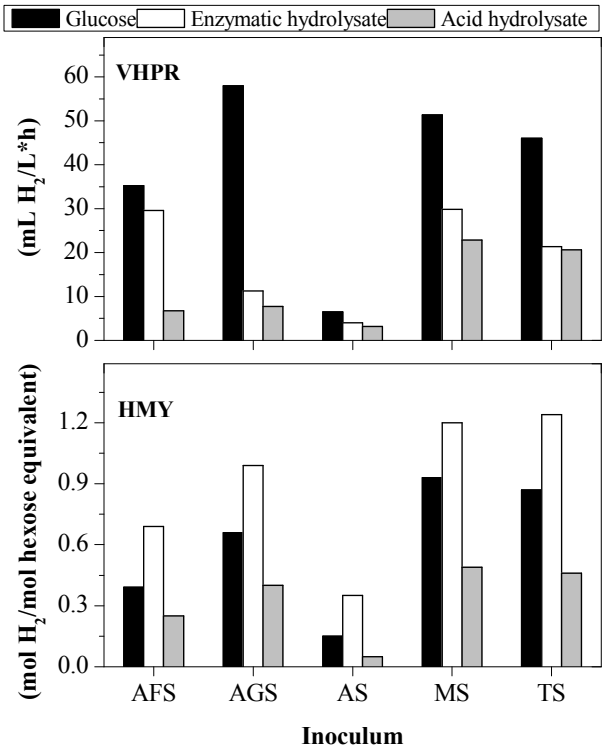

Fig. 4.2 Volumetric hydrogen production rates (VHPR) and hydrogen molar yields (HMY) obtained during the batch experiments. AFS (anaerobic flocculent sludge); AGS (anaerobic granular sludge); AS (aerobic sludge). MS (maize silage), TS (triticale silage).

Even though the HMY has a theoretical limitation, it is important to evaluate the amount of electron equivalents from the substrate that are used to produce hydrogen. Fig. 4.2 shows that the highest HMY were obtained with silages, independently of the substrate used. The highest HMY was obtained by TS (1.24 mol H_2/mol hexose equivalent). In general, HMY values obtained during the different experiments, independently of the type of substrate or inoculum, are much lower than the maximum theoretical (4 mol H_2/mol glucose) [14]. This behavior suggests that electrons obtained from the substrate were directed to alternative metabolic pathways instead to the acetate pathway (2).

$$(2) \quad C_6H_{12}O_6 + 2H_2O = 2CH_3COOH + 2CO_2 + 4H_2$$

4.3.3 Metabolic by-products

Main metabolic pathways followed in fermentative systems, aiming hydrogen production, are the acetate pathway (2), butyrate pathway (3) and propionate pathway (4) [14]. The metabolic by-products of these pathways were determined in the batch assays.

$$(3) \quad C_6H_{12}O_6 = CH_3(CH_2)_2COOH + 2H_2 + 2CO_2$$

$$(4) \quad C_6H_{12}O_6 + 2H_2 = 2CH_3CH_2COOH + 2H_2O$$

Fig. 4.3 shows that acetate was the main metabolic by-product in all the experiments (787-1954 mg/L). According to equation (2), high acetate production should generate high HMY; however, as shown in Fig. 4.2, the highest HMY was 1.24 mol H_2/mol hexose equivalent, which suggests that acetate was produced through other metabolic pathway. A possible explanation is that acetate was produced by homoacetogenism (equation (5)), a hydrogen consumer pathway [15].

$$(5) \quad 4H_2 + 2CO_2 = CH_3COOH + 2H_2O$$

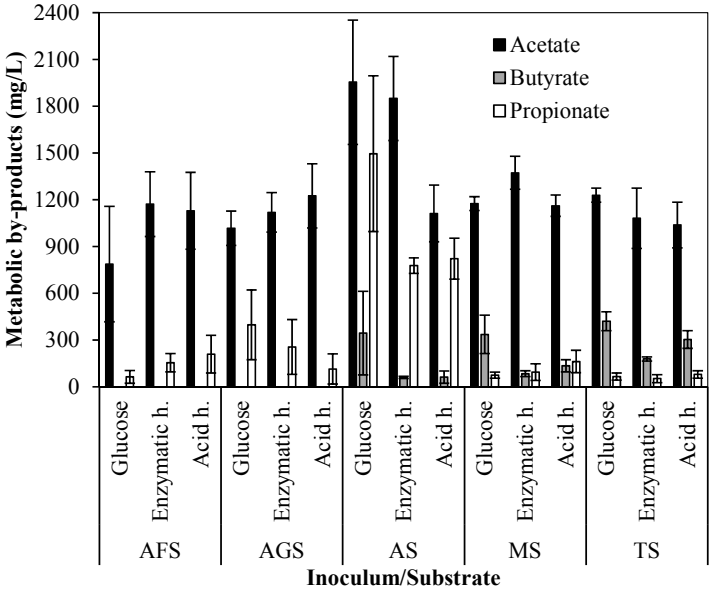

Fig. 4.3 Metabolic by-products obtained during the batch assays. AFS (anaerobic flocculent sludge); AGS (anaerobic granular sludge); AS (aerobic sludge). MS (maize silage), TS (triticale silage).

Fig. 4.3 also shows that butyrate was produced only by silages and aerobic sludge. Thus, the differences found in hydrogen production by sludges and silages were probably due to differences in the metabolic pathways followed. On the other hand, the experiments with AS presented also high propionate concentrations, which can explains the lowest hydrogen productions, since hydrogen is consumed for the formation of propionate (equation 4).

4.4 Conclusions

Feasibility to produce hydrogen from different inocula, using glucose and oat straw acid and enzymatic hydrolysates was demonstrated; nonetheless, differences in hydrogen production performance were observed. The highest VHPR was achieved by anaerobic granular sludge, when glucose was used as substrate. However, when the acid and enzymatic oat straw hydrolysates were used as substrate, the highest VHPR and HMY were achieved by silages. Also, differences in metabolic by-products among the inocula were observed, which suggest that microorganisms present in silages and sludges are different and/or follow different pathways. This is the first study that reports the use of silages as

inoculum for hydrogen production; due to the positive results with complex substrates, future studies are encouraged to evaluate their capacity in continuous systems.

4.5 References

[1] Lee HS, Rittmann B. Evaluation of metabolism using stoichiometry in fermentative biohydrogen. Biotechnol Bioeng 2009; 102:749-58.

[2] Davila-Vazquez G, Arriaga S, Alatriste-Mondragon F, De León-Rodríguez A, Rosales-Colunga L, Razo-Flores E. Fermentative biohydrogen production: trends and perspectives. Rev Environ Sci Biotechnol 2008; 7:27-45.

[3] Guo XM, Trably E, Latrille E, Carrere H, Steyer JP. Hydrogen production from agricultural waste by dark fermentation: A review. Int J Hydrogen Energy 2010; 35:10660-73.

[4] Wang J, Wan W. Factors influencing fermentative hydrogen production: A review. Int J Hydrogen Energy 2009; 34:799-811.

[5] Ren N, Wang A, Cao G, Xu J, Gao L. Bioconversion of lignocellulosic biomass to hydrogen: Potential and challenges. Biotechnol Adv 2009; 27:1051-60.

[6] Arreola-Vargas J, Celis LB, Buitrón G, Razo-Flores E, Alatriste-Mondragón F. Hydrogen production from acid and enzymatic oat straw hydrolysates in an anaerobic sequencing batch reactor: Performance and microbial population analysis. Int J Hydrogen Energy 2013; 38:13884-94.

[7] Cui MJ, Yuan ZL, Zhi XH, Wei LL, Shen JQ. Biohydrogen production from poplar leaves pretreated by different methods using anaerobic mixed bacteria. Int J Hydrogen Energy 2010; 35:4041-7.

[8] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248-54.

[9] Ghose TK. Measurement of cellulase activities. Pure Appl Chem 1987; 59:257-68.

[10] Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal Chem 1959; 31:426-8.

[11] APHA. Standard methods for the examination of water and wastewater, 20th ed. American Public Health Association/American Water Works Association/Water Environmental Federation, Washington, DC. 1998.

[12] Arriaga S, Rosas I, Alatriste-Mondragón F, Razo-Flores E. Continuous production of hydrogen from oat straw hydrolysate in a biotrickling filter. Int J Hydrogen Energy 2011; 36:3442-9.

[13] Lin CY, Jo CH. Hydrogen production from sucrose using aan anaerobic sequencing batch reactor process. J Chem Tech Biot 2003; 78:678-84.

[14] Fangkum A, Reungsang A. Biohydrogen production from sugarcane bagasse hydrolysate by elephant dung: Effects of initial pH and substrate concentration. Int J Hydrogen Energy 2011; 36:8687-96.

[15] Arooj MF, Han SK, Kim SH, Kim DH, Shin HS. Effect of HRT on ASBR converting starch into biological hydrogen. Int J Hydrogen Energy 2008; 33:6509-14.

Chapter 5

Continuous hydrogen production in a trickling bed reactor by using triticale silage as inoculum: comparison between simple and complex substrates

Summary

The effect of simple (glucose, xylose and sucrose) and complex substrates (acid and enzymatic oat straw hydrolysates) was for first time evaluated over the hydrogen production in a continuous system, a trickling bed reactor. Novel inoculum (triticale silage) and biofilm support (vertically organized PET tubing) were used in the experiment. Results showed that enzymatic hydrolysate is a suitable substrate for hydrogen production, since its hydrogen molar yield was similar to the obtained with glucose/xylose, 1.6 mol H_2/mol sugar consumed and 1.7 mol H_2/mol sugar consumed, respectively. By contrast, hydrogen was not produced from the acid hydrolysate, which was presumably due to the high oligosaccharides concentration and presence of inhibitory compounds. On the other hand, the highest hydrogen production rate (840 mL H_2/L-h) was obtained at an organic loading rate of 160 g COD/L-d by means of using glucose as substrate. In spite of the high organic loading rate, clogging due to excessive biomass growth was not observed. PCR-DGGE analysis revealed a low bacterial diversity throughout the reactor operation and bacteria from *Clostridium* genus as the putative responsible for the hydrogen production. This work demonstrates that hydrogen production is affected by complexity of the substrates.

Adapted from: Arreola-Vargas J, Alatriste-Mondragón F, Celis LB, Razo-Flores E, López-López A, Méndez-Acosta HO. Continuous hydrogen production in a trickling bed reactor by using triticale silage as inoculum: comparison between simple and complex substrates. To be submitted to Journal of Chemical Technology and Biotechnology.

5.1 Introduction

Hydrogen production through fermentation has energetic and environmental advantages that make it an attractive process for fuel production from biomass [1, 2]. Nonetheless, some basic aspects such as the type of substrate, inoculum and reactor configuration need to be further investigated in order to improve the hydrogen production process [2, 3].

Regarding the type of substrate, lignocellulosic biomass has been recognized as an attractive feedstock for the hydrogen production, since it is abundant, inexpensive and current techniques are capable to hydrolyze the hemicellulose and cellulose fractions [4-7]. However, reported hydrogen production rates (HPR) from lignocellulosic hydrolysates (up to 81 mL H_2/L-h, [8]) are much lower than those reported for simple substrates, such as sucrose (up to 15 L H_2/L-h [9]), using different reactor configurations and process conditions. To our knowledge, in the current literature there is no report that compares the performance of these complex substrates with their model substrates (i.e lignocellulosic hydrolysates vs. glucose/xylose) in a continuous system, operated under the same experimental conditions. This comparison is important to gain further understanding of the reasons for the lower hydrolysates performance and also for the further improvement of the process.

Concerning the type of inoculum, most of the studies available in the current literature have reported the use of mixed cultures [2, 3]. The advantages of mixed cultures over pure cultures include the use of a wide range of potential substrates, such as the lignocellulosic hydrolysates, under non-sterile conditions. Up to now, most of the reported studies with mixed cultures have used anaerobic sludge as inoculum [2, 3]; however, this issue had led to problems with methane production in fixed biomass reactors [10]. Thus, a possibility to overcome this problem is the use of fermentative inocula from different sources.

On the other hand, different reactor configurations have been used for the hydrogen production process [2-4]. Up to now, the highest HPR, using simple model substrates, have been obtained in a continuous stirred tank reactor (CSTR) and in a trickling bed reactor (TBR), 15 L H_2/L-h and 10.5 L H_2/L-h, respectively [9, 11]. Nonetheless, during operation of the CSTR, the stirring process could detach microorganisms from immobilization carriers; thus, the biomass may be washed out if the reactor operates at low hydraulic retention times (HRT). By contrast, the TBR is a fixed biomass reactor; where the

microorganisms form a biofilm on a packing material, while a thin substrate fluid layer is trickled over the biofilm which is surrounded by a gaseous phase [11-14]. Thus, this configuration promotes cell immobilization and easy hydrogen release, avoiding the inhibition by high hydrogen pressures in the biofilm [3, 11-14].

In spite of the TBR advantages, only a few reports regarding hydrogen production in TBRs have been published [8, 11-14]. The main TBR concern is related with the excessive growth of biomass, which may cause clogging of the reactor. This phenomenon has been reported on TBRs for different purposes [8, 12, 15], but some preventive and/or corrective actions can be followed in order to avoid clogging and improve the TBR stability [15]. Among preventive actions, the selection of the support for the biofilm growth is a key factor.

Therefore, the main objective of this work was to compare the effect of complex substrates (lignocellulosic hydrolysates) and model substrates (glucose/xylose/sucrose) over the hydrogen production in a TBR operated continuously under the same experimental conditions for both types of substrates. Novel biofilm support configuration for TBRs (vertically organized PET tubing) and inoculum (triticale silage) were tested.

5.2 Materials and methods

5.2.1 Reactor configuration and experimental strategy

The reactor was made up of acrylic; Fig. 5.1 shows schematically the reactor layout. Multiple PET tubing pieces (1 cm inner diameter; 27 cm length) were vertically organized in three modules as support for the biofilm growth. The effective TBR volume was 1.6 L (initial bed void fraction). The temperature was controlled at 35 ± 1 °C with a water jacket and the pH was kept at 5 with the addition of NaOH solution at influent. Recirculation flow was maintained during all the reactor operation at 180 mL/min in order to guarantee an appropriate substrate medium flow along the reactor. Feeding flow was adjusted from 2.2 to 4.4 and 8.8 (mL/min) for the different HRTs tested of 12 h, 6 h and 3 h, respectively (Table 5.1).

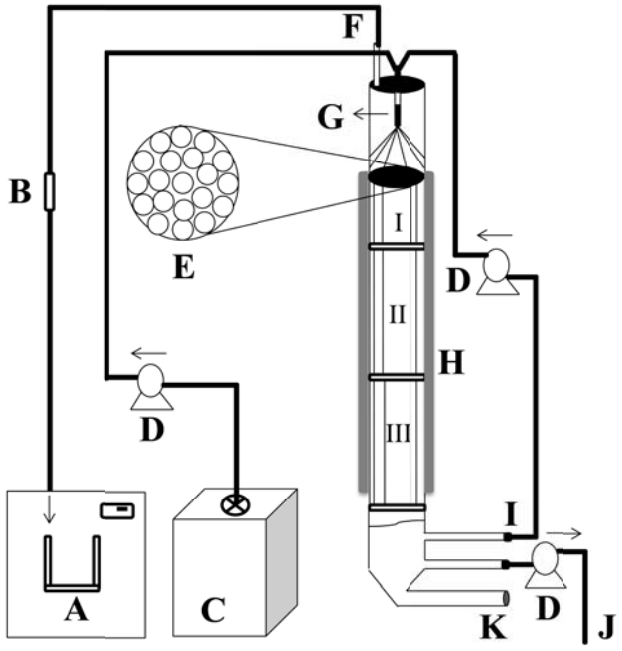

Fig. 5.1 Trickling bed reactor scheme. A: Wet gas meter; B: Gas sampling port; C: Feeding tank; D: Peristaltic pumps; E: Vertically organized PET tubing, superior view; F: Gas outlet; G: Spray nozzle; H: Water jacket; I: Recirculation; J: Effluent; K: Biomass purge port. Modules of the reactor are indicated by numbers I, II and III.

Table 5.1 shows the experimental strategy that was followed to evaluate the performance of the TBR. From period I to VIII, it was evaluated the effect of model (glucose/xylose/sucrose) and complex substrates (lignocellulosic hydrolysates) over the hydrogen production at a constant organic loading rate (OLR), except for period III. During periods IX to XIII, the effect of OLR increments over the hydrogen production was also evaluated. Additionally, biomass formation and clogging were assessed at the end of the TBR operation.

Reactor start up: In order to develop the biofilm on the PET tubing, the reactor was filled with the substrate medium and it was operated under continuous mode with an HRT of 24 h until day 20; afterwards, the liquid was drained from the reactor and operation as a TBR mode started. The criterion for testing a new period was when the reactor was operated for at least 20 HRTs and three similar values of hydrogen production and sugar removal were achieved.

Table 5.1 Experimental strategy during the operation of the TBR

Period	Substrate	Operation time (d)	Influent substrate concentration (g COD/L)	HRT (h)	OLR (g COD/L-d)
I	Glucose:xylose (1:1)	1-32	5	12	10
II	Acid oat straw hydrolysate	32-41	5	12	10
III[a]	Acid oat straw hydrolysate + glucose:xylose (1:1)	41-54	10	12	20
IV	Acid oat straw hydrolysate	54-63	5	12	10
V	Glucose	63-72	5	12	10
VI	Enzymatic oat straw hydrolysate	72-88	5	12	10
VII	Glucose	88-107	5	12	10
VIII	Sucrose	107-123	5	12	10
IX	Glucose	123-133	5	12	10
X	Glucose	133-139	5	6	20
XI	Glucose	139-144	5	3	40
XII	Glucose	144-149	10	3	80
XIII	Glucose	149-158	20	3	160

[a] This period was fed with 5g COD/L of oat straw acid hydrolysate + 5g COD/L of glucose:xylose (1:1)

5.2.2 Inoculum and mineral medium

Triticale silage from a dairy farm was used as a source of fermentative microorganisms. The triticale silage was thermally treated at 104 °C during 24 h in order to eliminate hydrogen consumers and to favor hydrogen spores-formers; then, the dried product was powdered in a mortar. For spore extraction, 2 L of mineral medium with 100 g of dried triticale silage were stirred overnight; the liquid was filtered in a 1μm membrane and the flow through was used as inoculum in the start-up phase of the reactor (1.6 L). The mineral medium composition was modified from Arriaga et al. [8] and was as follows (g/L): $NH_4H_2PO_4$, 4.5; Na_2HPO_4, 11.9; K_2HPO_4, 0.125; $MgCl_2 \cdot 6H_2O$, 0.1; $MnSO_4 \cdot 6H_2O$, 0.015; $FeSO_4 \cdot 5H_2O$, 0.025; $CuSO_4 \cdot 5H_2O$, 0.005; $ZnCl_2$, 0.075.

5.2.3 Substrates

Simple model substrates (glucose, xylose and sucrose) were reagent grade. Oat straw was used as a model lignocellulosic biomass and was obtained from a local commercial source (Forrajera Marquez Company, San Luis Potosí, México). A farm mill was used to reduce straw particle size to an average length of 2 cm. This material was sequentially acid and enzymatically hydrolyzed in order to solubilize the hemicellulose and cellulose of the oat straw, respectively. Both hydrolysis procedures were carried out as reported by Gomez-Tovar et al. [7].

The composition of the acid hydrolysate expressed in g/L was as follows: Chemical oxygen demand (COD) 25 ± 3.1; total sugar 20 ± 2.2; glucose 1.5 ± 0.2; xylose 3.7 ± 1.1; arabinose 1.3 ± 0.3; mannose 0.6 ± 0.1; galactose 0.5 ± 0.1. Furfural and phenolic compounds concentrations in mg/L: hydroxy methyl furfural (HMF) 133.2 ± 23.3; furfural 0.6 ± 0.4; vanillin 3.6 ± 0.9. The composition of the enzymatic hydrolysate was as follows (g/L): COD 30 ± 1; total sugar 7 ± 1; glucose 3.8 ± 0.9; xylose 1.3 ± 0.4; arabinose, mannose, galactose, HMF, furfural and vanillin were not detected in this hydrolysate.

5.2.4 Molecular analysis

5.2.4.1 DNA extraction and PCR amplification

Biomass samples were taken at day 3 (suspended biomass), day 88 (biofilm from tubing of the middle module) and day 158 (biofilm from tubing of the top, middle and bottom modules). These samples were subjected to DNA extraction following a previously described methodology [10].

Bacteria specific primers [16] were used for 16s rDNA amplification, using nested PCR technique and Taq DNA polymerase (Dongsheng, China). First round: 27F (5'-GTTGATCCTGGCTCAG-3') and 1492R (5'-ACGGYTACCTTGTTACGACTT-3'); the reaction conditions were: initial DNA denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 60 s and annealing also 60 s at 45 °C; then, an extension at 72 °C for 1 min. Final extension lasted 10 min at 72 °C. For the amplification of the target sequence, the second round primers and conditions were as follows: 357F-GC (5' CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGC AGCAG-3′) and 907R (5'-CCGTCAATTCMTTTGAGTTT-3'); 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 to 56 °C; then, 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% agarose gels stained with ethidium bromide to assess the size and purity of the amplicon.

5.2.4.2 DGGE and sequencing

DGGE was performed and stained according to Carrillo-Reyes et al. [10]. Dominant bands were excised from the gel and eluted in 20 μL of sterile deionized water for three days at 4 °C. The eluted DNA was re-amplified by PCR using the following primers: 357F without GC-clamp (40 nucleotides attached to the 5' end) and 907R. Successfully re-amplified PCR products were sequenced and data was analyzed with DNA BioEdit software v7.1.3 (Carlsbad, California, USA), and submitted to the non-redundant nucleotide database at GenBank, using BLAST (http://www.ncbi.nlm.nih.gov/blast/) and Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp). Finally, a dendrogram for the DGGE gel was constructed according to Carrillo-Reyes et al. [10].

5.2.5 Analytical methods

Gas production was measured by a liquid-displacement device and gas composition was measured by GC-TCD, as previously described [10]. Liquid samples from the effluent of the reactor and from hydrolysates were analyzed for sugars and volatile fatty acids by capillary electrophoresis, as described by Arriaga et al. [8]. Concentration of furfural and phenolic compounds in the hydrolysates was measured by HPLC. A 4.6 x 150 mm 5-micron column was used (Zorbax Eclipse XDB-C18, Agilent Technologies, Santa Clara, CA, USA). A mixture of water/acetronitrile (92/8%) was used as mobile phase at a flow rate of 0.8 mL/min and a temperature of 40 °C. The pH of samples and standards was adjusted to 4.4 before injection. Compounds were detected at 280 nm using a diode array detector.

In order to assess the potential clogging of the reactor by the excessive growth of biomass, the bed void fraction was determined at the beginning and at the end of the TBR operation, according to Arriaga et al. [8]. Total sugar concentration in the hydrolysates and in the TBR

effluent was measured by the phenol-sulphuric acid method, using glucose as standard [17]. COD was determined according to standard methods [18]. The volume of hydrogen produced throughout this study is reported at standard temperature and pressure conditions (0 ºC and 1 atm).

5.3 Results and discussion

5.3.1 Hydrogen production from simple and complex substrates

The TBR was operated during 158 days. Periods I to VIII (day 1 to 123) evaluated the effect of simple (monosaccharides and a disaccharide) and complex substrates (acid and enzymatic oat straw hydrolysates) over the hydrogen production (Table 5.1). During period I the reactor was fed with a mixture of glucose and xylose because these sugars were the main components of the acid hydrolysate. Fig. 5.2A shows that under the studied conditions, hydrogen was produced by bacteria from triticale silage inoculum. The mean HPR value during period I (simple sugars) was 70 mL H_2/L-h. Methane was not detected throughout the experiment and hydrogen was produced in balance with CO_2.

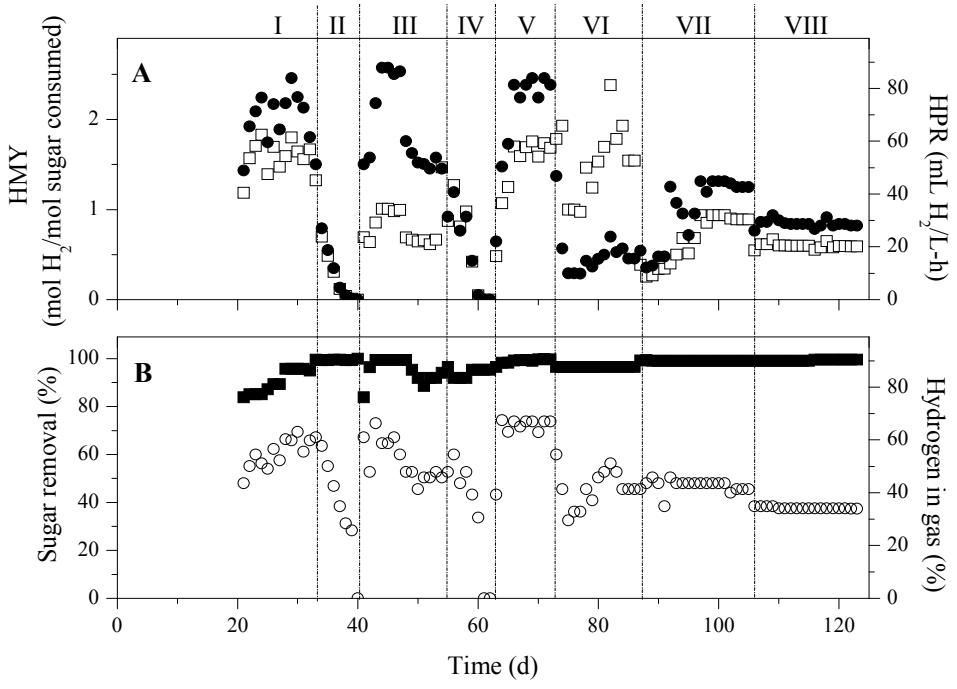

Fig. 5.2 Performance of the trickling bed reactor from days 1 to 123 (periods I to VIII). A) Hydrogen production rate, HPR (●); hydrogen molar yield, HMY (□). B) Sugar removal (■); hydrogen in gas (○).

Another key parameter for evaluating the hydrogen production performance is the hydrogen molar yield (HMY). The mean HMY value during period I was 1.6 mol H_2/mol sugar consumed (Fig. 5.2A). This value resulted similar to previous studies [4, 5], which used similar substrates but different reactor configurations and inocula.

The effect of the acid hydrolysate was evaluated during period II (days 32 to 41). Fig. 5.2 shows that hydrogen production decreased gradually during this period, until being totally suppressed. The total suppression of the hydrogen production was an unexpected result. Due to the acid hydrolysate complexity, a lower hydrogen production performance as compared to model substrates (glucose/xylose) was expected, but not total hydrogen suppression, since previous studies have reported hydrogen production from hemicellulose hydrolysates in continuous systems [8, 19, 20].

Due to the fact that sugar removal during period II was maintained above 90% (Fig. 5.2B), the hydrogen suppression observed during this period could be linked to microbial population or metabolic pathway changes rather than a total microbial inhibition. Recent studies have demonstrated that furfural and/or phenolic compounds, which are released during the pretreatment of lignocellulosic materials, have negative impacts over the hydrogen producer microorganisms [21, 22]. Therefore, hydrogen suppression due to the presence of furfural and/or phenolic compounds in the acid hydrolysate was hypothesized. In an attempt to confirm this hypothesis, model substrates at the same concentration than in period I were added to the acid hydrolysate and fed in period III (Table 5.1). Hydrogen production was reassumed (Fig. 5.2A). Lower mean values of HPR and HMY, as compared to period I, were obtained during the last 8 days of the period III (54 mL H_2/L-h and 0.7 mol H_2/mol sugar consumed, respectively). This result pointed out that negative effect of the acid hydrolysate might be not only related to the presence of furfurals and/or phenolic compounds, since the hydrogen production was partially recovered even though these compounds were present in the feeding. During period IV the negative effect of the acid hydrolysate was corroborated, i.e. model substrate was removed from the feeding and only acid hydrolysate was fed to the reactor; the same behavior than in period II was observed.

Period V was fed with glucose (Table 5.1) in order to recover the hydrogen production and also because this is the main sugar in the enzymatic hydrolysate. Fig. 5.2 shows that during this period the hydrogen production performance was immediately recovered and similar

HMY and HPR than in period I were achieved. Also, high hydrogen percentage in gas was obtained, 68% (mean value). On period VI the effect of the enzymatic hydrolysate (which is a complex substrate without potential inhibitory compounds) was evaluated (Table 5.1), mean values for HPR and HMY were 18 mL H_2/L-h and 1.6 mol H_2/mol sugar consumed, respectively (Fig. 5.2A). It is interesting that the HMY reached in period VI was similar to the values obtained in periods I and V, even though these two latter periods were fed with simple model substrates. The lower HPR than in periods I and V was likely due to the fact that sugars represented only 23% of the enzymatic hydrolysate COD.

On the other hand, another noticeable difference between the acid and enzymatic hydrolysates was the putative concentration of oligosaccharides (considered as the gap between the sum of monosaccharides and total sugar concentrations, see section 5.2.3). In the case of the acid hydrolysate this difference accounted 12.4 g/L, whereas in the enzymatic hydrolysate accounted only 1.9 g/L. According to Quéméneur et al. [23], the hydrolysis of oligosaccharides negatively affects the hydrogen production. Therefore, during period VII of the present study, the reactor was fed again with a monosaccharide (glucose), while period VIII was fed with a disaccharide, sucrose (Table 5.1). In period VII, the hydrogen performance was lower than in period V, even though during both periods the reactor was fed with glucose (Fig. 5.2). This behavior was probably due to a negative effect caused by a brief exposure to air, since the reactor was opened to take a biofilm sample at the end of period VI. In spite of this event, it is clear that during period VIII a lower hydrogen performance was obtained as compared to period VII. The mean HMY and HPR values at steady state were: 45 mL H_2/L-h and 0.9 mol H_2/mol sugar consumed for period VII, and 29 mL H_2/L-h and 0.6 mol H_2/mol sugar consumed for period VIII (Fig. 5.2A). This result was similar to the observed by Quéméneur et al. [23], and indicates that oligosaccharides present in the acid hydrolysate negatively affect the hydrogen production. Further studies are encouraged in order to clarify the effect of oligosaccharides, furfurals and phenolic compounds over the hydrogen production in continuous systems.

5.3.2 OLR effect over the hydrogen production

Second part of this study, periods IX to XIII, was devoted to evaluate the effect of incrementing the OLR over the hydrogen production (Table 5.1, Fig. 5.3C). Furthermore,

the effectiveness of the TBR support configuration as an adequate system for preventing clogging at high OLRs was also evaluated. Due to the better performance of glucose during the periods I to VIII, it was selected as substrate for the periods IX to XIII. Period IX was carried out at the same OLR than previous periods in order to recover the hydrogen production performance, during this period the mean HPR was 40 mL H_2/L-h and the mean HMY was 0.8 mol H_2/mol sugar consumed (Fig. 5.3A), which were higher than in period VIII.

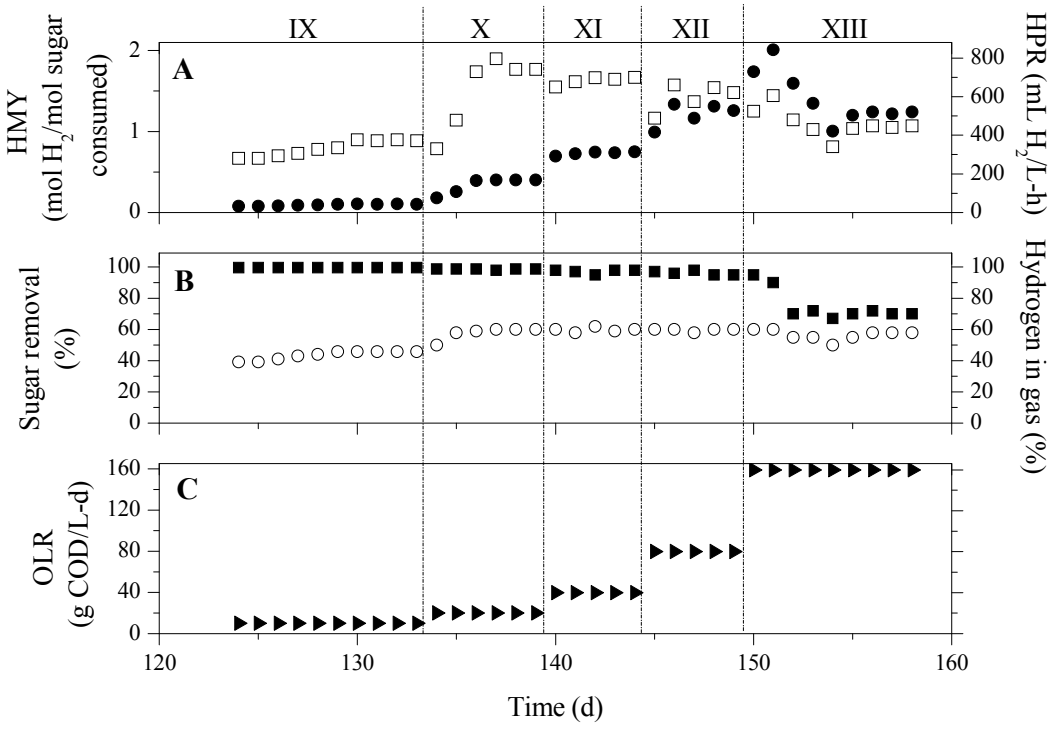

Fig. 5.3 Performance of the trickling bed reactor from days 124 to 158 (periods IX to XIII). A) Hydrogen production rate, HPR (●); hydrogen molar yield, HMY (□). B) Sugar removal (■); hydrogen in gas (○). C) Organic loading rate, OLR (►).

Fig. 5.3A also shows that during period X, where the hydraulic retention time (HRT) was decreased from 12 to 6 h (Table 5.1), the highest HMY mean value of all the experimental periods was achieved, 1.7 mol H_2/mol sugar consumed. The mean value of the HPR in this period was 168 mL H_2/L-h, which represented a 4-fold increment as compared to period IX. These results agree with previous studies in different systems which obtained a better hydrogen performance at short HRT [8, 24, 25]. An advantage of fixed biomass reactors, as

the TBR, is the possible operation at very short HRT without experimenting biomass washed out; generally, hydrogen producing CSTRs experience biomass washed out at HRT shorter than 6 hours [24]. Due to this reason, in period XI the HRT was decreased to 3 h (Table 5.1). Fig. 5.3A shows that the HMY resulted similar to the obtained in period X, 1.6 mol H_2/mol sugar consumed; nonetheless, the HPR increased to 308 mL H_2/L-h, almost a 2 fold increment as compared to period X.

The OLR was increased in periods XII and XIII by maintaining the same HRT (3 h) and increasing the substrate concentration (Table 5.1). Period XII was operated at an OLR of 80 g COD/L-d and the mean values obtained were 1.5 mol H_2/mol sugar consumed and 498 mL H_2/L-h for HMY and HPR, respectively (Fig. 5.3A). By contrast, the period XIII was operated at an OLR of 160 g COD/L-d; at day 151 the highest HPR of all the experiment was achieved, 840 mL H_2/L-h (Fig. 5.3A). However, since that day, an excessive foam accumulation was observed and the HPR decreased until reach similar values as in period XII. A probable explanation for this behavior is that the foam hindered the hydrogen release and the substrate uptake (sugar removal decreased from 90 to 70%, Fig. 5.3B).

Even though high cell density is an advantage of TBRs [12], it also may cause clogging, which affects the hydrogen production performance [8, 12, 15]. In the present study, the support configuration (vertical organized PET tubing) was tested as a clogging preventive method. During all the TBR operation no decrement on hydrogen performance was related to clogging, and the bed void fraction at the end of the TBR operation was 96% of the initial bed void fraction. This means that in spite of the high OLRs applied, the PET tubing just supported a thin biofilm, which was corroborated when the interior of the reactor was observed (Fig. 5.4). A previous study, which was operated with similar OLRs and for a shorter period of time (102 days) than the present study (158 days), presented clogging by excessive biomass growth as the main drawback (using perlite as support) [8]. Further studies evaluating reactor performances at the same conditions for comparing effectiveness of different TBR support configurations are encouraged. The use of antifoam is recommended.

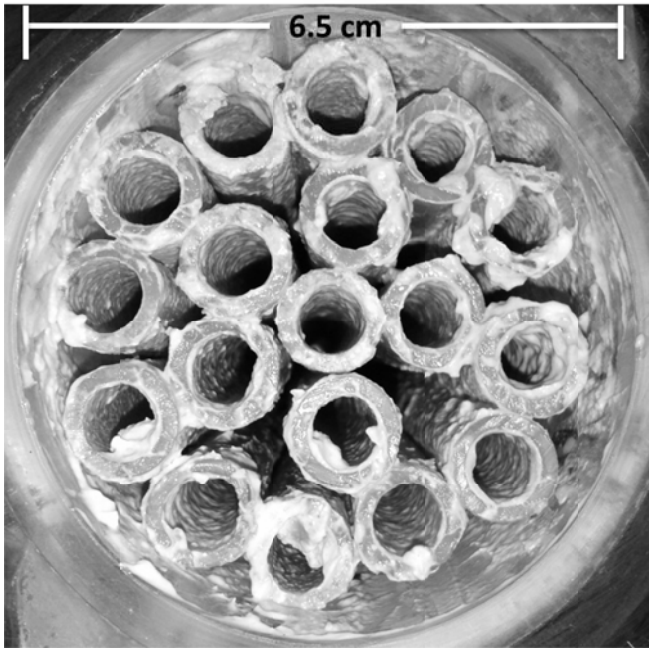

Fig. 5.4 Transversal view of the thin biofilm formed over the middle module PET tubing at the end of the TBR operation, scale bar indicates the inner diameter of the module.

5.3.3 Metabolic by-products and COD Balance

Fig. 5.5 shows the metabolic by-products concentration and the COD balance from period III onwards, since data from periods I and II were accidentally lost. Nonetheless, periods IV and V were similar to periods II and I, respectively (Table 5.1, Fig. 5.2). Thus, these periods may have produced similar metabolites.

In fermentative systems the main metabolic pathways that produce hydrogen, are acetate and butyrate pathways [26, 27, 28]. According to Fig. 5.5A, both metabolites were the main metabolic byproducts during all the experiment. Minimum variable amounts of ethanol, propionate and lactate, which are common by-products [29, 30, 31], were also present. An interesting observation in period IV was the acetate production, 770 mg/L (Fig. 5.3A), despite that hydrogen was not produced in this period (Fig. 5.2), suggesting that acetate could be produced through hydrogen-consuming pathways, such as homoacetogenesis [10, 32]. In general, metabolic by-products were similar throughout the experiment, with butyrate as the dominant metabolite in most of the periods. The highest concentration of metabolic by-products in the last two periods (Fig. 5.5A) is explained by the increment on the substrate concentration (Table 5.1).

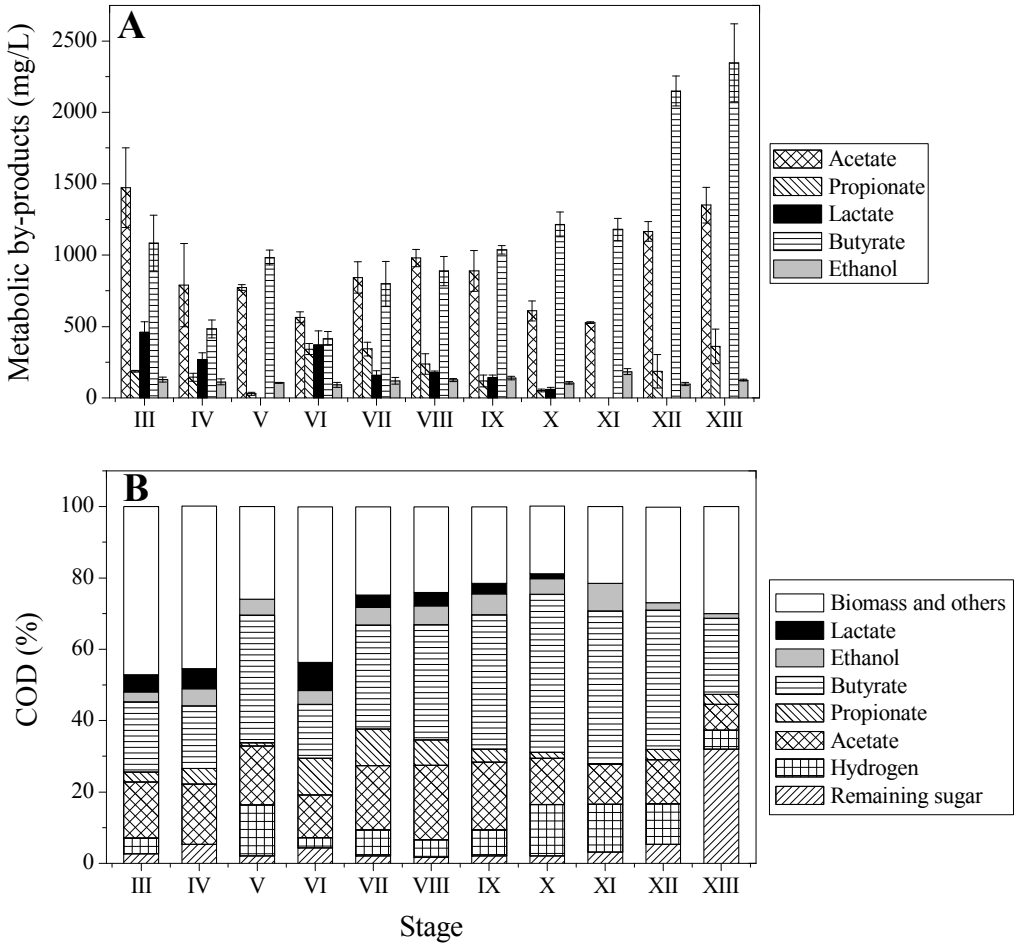

Fig. 5.5 By-products and COD balance during periods III to XIII. A) Metabolic by-products concentration; B) COD balance.

COD balances from period III to XIII are presented in Fig. 5.5B. Approximately 70% of the COD was identified in the periods where model substrates were fed (V, VII-XIII); the rest of the COD was due to non-determined metabolites and biomass. In contrast, a lower COD percentage, approximately 50%, was identified on periods where hydrolysates were fed (III, IV, VI). This could be due to the presence of compounds in the hydrolysates (other than sugars) that were not fermented. Fig. 5.5B also shows that in periods V, X and XI, where the highest HMY values were obtained (Fig. 5.2 and Fig. 5.3), most of the substrate was used to produce butyrate. This result suggests that during these periods most of the hydrogen was produced through the butyrate pathway; which is in agreement with the observations of other studies [23, 29].

5.3.4 Microbial population analysis

PCR-DGGE is a widely used technique for the study of microbial communities on hydrogenogenic systems [24, 33]. In the present study, biomass samples taken from days 3, 88 and 158, were analyzed by PCR-DGGE. Samples at different heights in the TBR were taken at day 158 in order to evaluate microbial population homogeneity along the reactor. Fig 5.6 shows that samples at the end of the TBR operation (day 158) were clustered together and the band patterns from the bottom (B) and middle (M) modules of tubing where identical, while the band pattern from the top module (T) was 90% similar. This result suggests that microbial population along the reactor was practically the same. In contrast, the band pattern from period VI (day 88) was 64% similar to the band patterns at the end of the TBR operation, and 91% similar to the band pattern in period I.

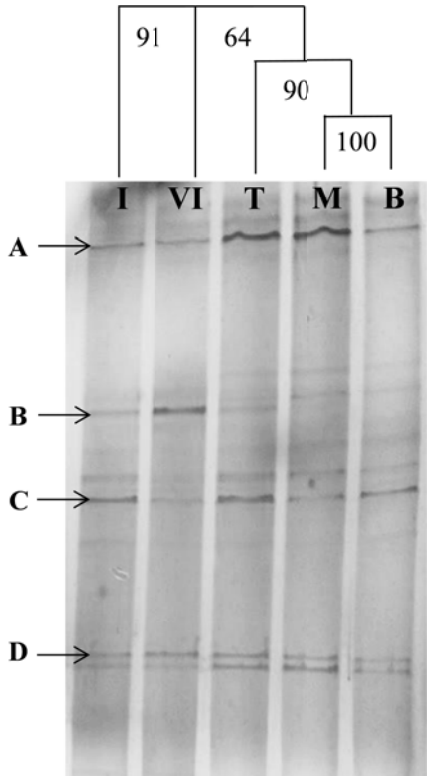

Fig. 5.6. DGGE profiles and dendrogram with Dice coefficients of similarity obtained from the analysis of the biomass. I: Period I (day 3); VI: Period VI (day 88); T: Top module tubing at the end of operation (day 158); M: Middle module tubing at the end of operation (day 158); B: Bottom module tubing at the end of operation (day 158). Marked bands were successfully re-amplified and sequenced.

Representative bands were re-amplified and sequenced. Table 5.2 shows the phylogenetic affiliations of the bands sequences, band A that was present in all the samples (Fig. 5.6) was 98% similar to *Lactobacillus plantarum*, while band B that predominated on period VI (Fig. 5.6) was 99% similar to *Lactobacillus paracasei*. Both microorganisms belong to bacillus class. According to Hung et al. [34] some lactobacillus have shown negative effect over the hydrogen production. By contrast, Bands C and D, which were present in all the samples (Fig. 5.6), were similar to *Clostridium sp* (97%) and *Clostridium butyricum* (96%), respectively. Clostridia class is widely reported as the main responsible for the hydrogen production [34]; nonetheless, some clostridia can also act as homoacetogens [35]. In general, low bacterial diversities were found in all the samples (Fig. 5.6), indicating a low bacterial diversity on the triticale silage and demonstrating the microbial population stability along the TBR operation.

Table 5.2 Phylogenetic affiliations of the DGGE bands sequences

Band	Accession number	Closest relative	Query Coverage (%)	Similarity (%)	Phylogenetic affiliation (Phylum/Class)
A	JN560925.1	*Lactobacillus plantarum*	96	98	Firmicutes/Bacilli
B	AJ272010.1	*Lactobacillus paracasei*	81	99	Firmicutes/Bacilli
C	AB678389.1	*Clostridium sp.*	91	97	Firmicutes/Clostridia
D	KC195777.1	*Clostridium butyricum*	90	96	Firmicutes/Clostridia

5.4 Conclusions

Under the studied conditions, the enzymatic oat straw hydrolysate demonstrated to be an adequate substrate for the hydrogen production, since its hydrogen molar yield was similar to those obtained with model substrates. In contrast, the acid hydrolysate promoted hydrogen suppression, presumably due to its high oligosaccharides content and to the presence of phenolic and furfural compounds. On the other hand, the tested support configuration in the trickling bed reactor resulted effective for operating the reactor at high

organic loading rates, achieving high hydrogen production rates and preventing clogging. Molecular analysis showed homogeneity and low bacterial diversity along the reactor operation. Furthermore, microorganisms from *Clostridium* genus were recognized as the putative responsible for the hydrogen production.

Finally, it is important to remark that this is the first study that provides experimental evidence of the differences in hydrogen production from lignocellulosic hydrolysates (acid and enzymatic) and its main model sugars (glucose/xylose) in a continuous system (trickling bed reactor).

5.5 References

[1] Chu CY, Sen B, Lay CH, Lin YC, Lin CY. Direct fermentation of sweet potato to produce maximal hydrogen and ethanol. Appl Energy 2012; 100:10-18.

[2] Davila-Vazquez G, Arriaga S, Alatriste-Mondragon F, De León-Rodríguez A, Rosales Colunga LM, Razo-Flores E. Fermentative biohydrogen production: trends and perspectives. Rev Environ Sci Biotechnol 2008; 7:27-45.

[3] Wang J, Wan W. Factors influencing fermentative hydrogen production: A review. Int J Hydrogen Energy 2009; 34:799-811.

[4] Guo XM, Trably E, Latrille E, Carrere H, Steyer JP. Hydrogen production from agricultural waste by dark fermentation: A review. Int J Hydrogen Energy 2010; 35:10660-73.

[5] Ren N, Wang A, Cao G, Xu J, Gao L. Bioconversion of lignocellulosic biomass to hydrogen: Potential and challenges. Biotechnol Adv 2009; 27:1051-60.

[6] Lloyd TA, Wyman CE. Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids. Bioresour Technol 2005; 96:1967-77.

[7] Gomez-Tovar F, Celis LB, Razo-Flores E, Alatriste-Mondragón F. Chemical and enzymatic sequential pretreatment of oat straw for methane production. Bioresour Technol 2012; 116:372-8.

[8] Arriaga S, Rosas I, Alatriste-Mondragón F, Razo-Flores E. Continuous production of hydrogen from oat straw hydrolysate in a biotrickling filter. Int J Hydrogen Energy 2011; 36:3442-9.

[9] Wu SY, Hung CH, Lin CN, Chen HW, Lee AS, and Chang JS. Fermentative hydrogen production and bacterial community structure in high-rate anaerobic bioreactors containing silicone-immobilized and self-flocculated sludge. Biotechnol Bioeng 2006; 93:934-46.

[10] Carrillo-Reyes J, Celis LB, Alatriste-Mondragon F, Razo-Flores E. Different start-up strategies to enhance biohydrogen production from cheese whey in UASB reactors. Int J Hydrogen Energy 2012; 37:5591-601.

[11] Jeon BS, Um YS, Lee SM, Lee SY, Kim HJ, Kim YH, Gu MB, Sang BI. Performance analysis of a proton exchange membrane fuel cell (PEMFC) integrated with a trickling bed bioreactor for biological high-rate hydrogen production. Energ Fuel 2008; 22:83-6.

[12] Oh YK, Kim SH, Kim MS, Park S. Thermophilic biohydrogen production from glucose with trickling biofilter. Biotechnol Bioeng 2004, 88:690-8.

[13] Van Groenestijn JW, Geelhoed JS, Goorissen HP, Meesters KPM, Stams AJM, Claassen PAM. Performance and population analysis of a non-sterile trickle bed reactor inoculated with Caldicellulosiruptor saccharolyticus, a thermophilic hydrogen producer. Biotechnol Bioeng 2009; 102:1361-67.

[14] Zhang H, Bruns MA, Logan BE. Biological hydrogen production by *Clostridium acetobutylicum* in an unsaturated flow reactor. Water Res 2006; 40:728-34.

[15] Yang C, Chen H, Zeng G, Yu G, Luo S. Biomass accumulation and control strategies in gas biofiltration. Biotechnol Adv 2010; 28:531-40.

[16] Kaksonen AH, Plumb JJ, Franzmann PD, Puhakka JA. Simple organic electron donors support diverse sulfate-reducing communities in fluidized-bed reactors treating acidic metal- and sulfate-containing wastewater. FEMS Microbiol Ecol 2004; 47:279-89.

[17] Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956; 28:350–6.

[18] APHA, AWWA and WPCF. Standard methods for the examination of water and wastewater. In: Eaton, A.D., Clesceri, A.E., Rice E.W., Greenberg, A.E. (Eds.), American Public Health Association, 21st ed. American Water Works Association and Water Environment Federation, Washington D.C. 2005.

[19] Kongjan P, Angelidaki I. Extreme thermophilic biohydrogen production from wheat straw hydrolysate using mixed culture fermentation: effect of reactor configuration. Bioresour Technol 2010; 101:7789-96.

[20] Kongjan P, O-Thong S, Kotay M, Min B, Angelidaki I. Biohydrogen production from wheat straw hydrolysate by dark fermentation using extreme thermophilic mixed culture. Biotechnol Bioeng 2010; 105:899-908.

[21] Cao GL, Ren NQ, Wang AJ, Guo WQ, Xu JF, Liu BF. Effect of lignocellulose-derived inhibitors on growth and hydrogen production by *Thermoanaerobacterium thermosaccharolyticum* W16. Int J Hydrogen Energy 2010; 35:13475-80.

[22] Quéméneur M, Hamelin J, Barakat A, Steyer JP, Carrére H, Trably E. Inhibition of fermentative hydrogen production by lignocellulose-derived compounds in mixed cultures. Int J Hydrogen Energy 2012; 37:3150-9.

[23] Quéméneur M, Hamelin J, Benomar S, Guidici-Orticoni MT, Latrille E, Steyer JP, Trably E. Changes in hydrogenase genetic diversity and proteomic patterns in mixed-culture dark fermentation of mono-, di- and tri-saccharides. Int J Hydrogen Energy 2011; 36:11654-65.

[24] Davila-Vazquez G, Cota-Navarro CB, Rosales-Colunga LM, De león-Rodriguez A, Razo-Flores E. Continuous biohydrogen production using cheese whey: Improving the hydrogen production rate. Int J Hydrogen Energy 2009; 34:4296-304.

[25] Buitrón G, Carvajal C. Biohydrogen production from tequila vinasses in an anaerobic sequencing batch reactor: Effect of initial substrate concentration, temperature and hydraulic retention time. Bioresour Technol 2010; 101:9071-7.

[26] Mohanakrishna G, Mohan SV. Multiple process integrations for broad perspective analysis of fermentative H_2 production from wastewater treatment: Technical and environmental considerations. Appl energy 2013; 107:244-54.

[27] Fangkum A, Reungsang A. Biohydrogen production from sugarcane bagasse hydrolysate by elephant dung: Effects of initial pH and substrate concentration. Int J Hydrogen Energy 2011; 36:8687-96.

[28] Mathews J, Wang G. Metabolic pathway engineering for enhanced biohydrogen production. Int J Hydrogen Energy 2009; 34:7404-16.

[29] Luo G, Xie L, Zou Z, Zhou Q, Wang JY. Fermentative hydrogen production from cassava stillage by mixed anaerobic microflora: Effects of temperature and pH. Appl Energy 2010; 87:3710-7.

[30] Yang Z, Guo R, Xu X, Fan X, Luo S. Fermentative hydrogen production from lipid-extracted microalgal biomass residues. Appl Energy 2011; 88:3468-72.

[31] Sigurbjornsdottir MA, Orlygsson J. Combined hydrogen and ethanol production from sugars and lignocellulosic biomass by *Thermoanaerobacterium* AK54, isolated from hot spring. Appl Energy 2012; 97:785-91.

[32] Arooj MF, Han SK, Kim SH, Kim DH, Shin HS. Effect of HRT on ASBR converting starch into biological hydrogen. Int J Hydrogen Energy 2008; 33:6509-14.
[33] Nissilä ME, Li YC, Wu SY, Lin CY, Puhakka JA. Hydrogenic and methanogenic fermentation of birch and conifer pulps. Appl Energy 2012; 100:58-65.
[34] Hung CH, Chang YT, Chang YJ. Roles of microorganisms other than Clostridium and Enterobacter in anaerobic fermentative biohydrogen production systems - A review. Bioresour Technol 2011; 102:8437-44.
[35] Köpke M, Held C, Hujer S, Liesegang H, Wiezer A, Wollherr A, et al. Clostridium ljungdahlii represents a microbial production platform based on syngas. P Natl Acad Sci 2010; 107:13087-92.

Chapter 6

General discussion, conclusions and final remarks

6.1 General discussion

Lignocellulosic biomass is recognized as an excellent source of sugars for a sustainable biofuel production. Therefore, studies on hydrogen production via hydrolysis of agricultural residues and downstream fermentation have increased in the last years [1]. The present thesis evaluated the fermentative hydrogen production from acid and enzymatic oat straw hydrolysates. The hydrolysates were obtained sequentially in order to use the hemicellulose and cellulose fractions of the oat straw. Results demonstrated that it is possible to produce hydrogen from fermentation of these substrates in batch, semi-continuous and continuous systems. Nonetheless, different hydrogen production performances were obtained among these systems (Table 6.1), confirming the general hypothesis of this work.

Table 6.1 Comparison on hydrogen production performance among the different systems used in the present study (using oat straw hydrolysates as substrate).

Substrate	HMY (mol H_2/ mol sugar)	HPR (mL H_2/L-h)	System
Acid hydrolysate	1.1	70.4	Batch[a]
Enzymatic hydrolysate	2.4	110.9	Batch[a]
Mixture of acid and enzymatic hydrolysates	0.4	27	ASBR[b]
Enzymatic hydrolysate	0.8	29.6	ASBR[b]
Acid hydrolysate	0	0	TBR[c]
Enzymatic hydrolysate	1.6	18	TBR[c]

Data correspond to the maximum values obtained in each system. [a]Chapter 2, Table 2.3; [b]Chapter 3, Fig. 3.2; [c]Chapter 5, Fig. 5.2.

Table 6.1 shows that the highest hydrogen production performance was achieved in batch systems. This result is interesting because even though different hydrogen production performances among batch, semi-continuous and continuous systems were hypothesized, these differences were expected to be in favor of semi-continuous and continuous systems. This is due to the fact that ASBRs and TBRs present some advantages over batch reactors, such as the formation of granules or biofilms, which considerably increases the biomass concentration inside the reactors [2, 3]. Both structures, granules and biofilms, are formed by exopolymeric substances and microbial communities; the difference between them is that granules consist on microbial aggregates, while biofilms consist on microorganisms immobilized onto a solid surface. Their formation promotes the retention of high biomass concentrations and the capacity to degrade complex substrates at high organic loading rates [4]. Even though formation of granules and biofilms were observed during the present study, in the ASBR and TBR respectively, the highest hydrogen production performances were obtained in batch assays in which biomass was not aggregated or forming biofilms. A possible explanation for this result is that in spite of the high biomass concentrations reached during ASBR and TBR experiments (due to granules or biofilm formation), the constant uptake of the hydrolysates constituents by microorganisms present in these reactors could promote changes on metabolic pathways or microbial population; as it was corroborated during ASBR operation.

During this experiment (Chapter 3), gradual substitution of glucose/xylose by acid hydrolysates negatively affected the hydrogen production performance and promoted disaggregation of previously formed granules. Furthermore, the microbial community became more diverse when only hydrolysates were fed. In this regard, a recent study reported that acid hydrolysates from sunflower stalks inhibit the fermentative hydrogen production by means of shifting the dominant microbial population [5]. Furthermore, another study carried out by the same research group [6], demonstrated that lignocellulosic hydrolysates components such as furfurals and phenolic compounds, greatly affect the growth of *Clostridium pasteurianum*, a microorganism that has been reported as a high hydrogen producer and granule former [7]. In agreement with this report, *C. pasteurianum* (99% of similarity) was the most abundant specie during the startup of the ASBR and was almost completely replaced by other species when the hydrolysates were fed. This shift in

the microbial community could explain the observed disaggregation of the granules and the low hydrogen production performance in the ASBR.

Up to now, the general consensus regarding the type of reactor is that semi-continuous and continuous reactors that allow retaining high concentrations of biomass, such as the ASBR and TBR, are the more suitable reactors for obtaining high hydrogen production performances. However, this consensus was reached taking into account that most of the reported works have used substrates different to lignocellulosic hydrolysates [8-10], since reported studies with this type of substrates are limited [1-11]. Due to the results obtained in the present work, when acid hydrolysates are used as substrate for fermentative hydrogen production, this consensus should be reconsidered.

On the other hand, Table 6.1 shows that regardless of the system used, the enzymatic oat straw hydrolysate was a more suitable substrate for the hydrogen production, as compared to the acid hydrolysate. This trend could be due to different factors. First, arabinose, a constituent of the acid hydrolysate, produces only 40% of the hydrogen produced by glucose, the main sugar of the enzymatic hydrolysate (Chapter 2). According to Li et al [12] and Mangayil et al [13], the poor performance of arabinose as substrate for hydrogen production could be due to the energy intensive arabinose utilization route, which involves complex enzymatic reactions before entering the pentose phosphate and subsequent glycolysis pathways. Second, even though furfural and phenolic compounds showed no inhibitory effect in the batch assays (Fig 2.2), their constant feeding in the ASBR and/or TBR could have induced an inhibitory effect over some microbial communities, as it was observed during the ASBR operation (Chapter 3). Furfural and phenolic compounds, which are formed by dehydratation of hexoses/pentoses and by lignin hydrolysis, are released during acid hydrolysis and could affect some types of bacteria by interfering with glycolytic enzymes or damaging the microbial cellular membranes [1, 6]. Third, the higher concentration of oligosaccharides in the acid hydrolysate as compared to the enzymatic hydrolysate could have also negatively affected the hydrogen production from the acid hydrolysate, as it was demonstrated during the TBR operation (Chapter 5). According to Quéméneur et al [14], the hydrolysis of oligosaccharides negatively affects the hydrogen production because electronic carriers are invested in high energy yield routes for allowing the production of hydrolytic enzymes instead of the hydrogen production routes. Finally, as

it was demonstrated in batch assays (Chapter 2), the best performance of the enzymatic hydrolysate was also due to fermentation of the commercial enzymatic preparation (Fig. 2.3), which greatly contributed to the hydrogen production. This positive effect of the enzymatic preparation over the hydrogen production is for first time experimentally evidenced.

Due to the characteristics of the enzymatic oat straw hydrolysate, the HPR from this substrate could be very likely improved by establishing an adequate experimental strategy. For example, feeding this substrate from the startup of the process and increasing the organic loading rate in different semi-continuous or continuous reactors. By contrast, in order to increase the feasibility of using acid oat straw hydrolysates in semi-continuous or continuous reactors is necessary to effectively remove the microbial inhibitors from this substrate. Reported methods to overcome microbial inhibitors in acid hydrolysates include physical (evaporation), physicochemical (solvent extraction, activated charcoal adsorption, use of ion exchange resins or overlime procedures) and biological (laccases, genetic engineering or microbial adaptation) treatments [15]. Even though several studies have reported the use of acid hydrolysates for the fermentative hydrogen production [1, 16-18], in the available literature only one report has used a detoxified acid hydrolysate [19]. During that study, the fermentative hydrogen production from sugarcane bagasse hydrolysate was investigated. The sugarcane bagasse was hydrolyzed by sulphuric acid and the hydrolysate was detoxified by passing it through an adsorbent exchange resin. Results showed that this hydrolysate produced 35% more hydrogen than the undetoxified hydrolysate. Thus, further studies are required in depth to study the feasibility of using this type of treatments.

During the mentioned study [19], the authors also evaluated the feasibility to integrate dark-fermentation with photo-fermentation to produce more hydrogen. This is an interesting approach because during fermentation great part of the substrate is converted to VFAs, which represent a high waste of energy when downstream processes are not applied. As it is shown in Table 6.1, regardless of the system used, the HMY represent less than 20% of the available electron equivalents in the substrate (considering 24 electron equivalents for glucose and 2 for hydrogen [20]). This was due to the fact that most of the electron equivalents were directed to VFAs production; indeed, even though maximum theoretical

yields were achieved during the hydrogen production process, most of the electron equivalents (66%) would be directed to VFAs production [8-10]. Therefore, in recent years some works have been evaluating the feasibility of using VFAs as substrates in different processes, such as: anaerobic digestion for methane production; microbial electrolysis cells for hydrogen production; photo fermentation for hydrogen production; microbial fuel cells for electricity production; acetone- butanol- ethanol fermentation for butanol production; microbial lipid accumulation for biodiesel production; polyhydroxyalkanoates accumulation for bioplastics production and VFAs utilization as carbon source for nitrogen removal [21]. The integration of any of these processes with the fermentative hydrogen production from lignocellulosic biomass in a biorefinery concept (Fig. 6.1) should increase the economic feasibility of the whole process.

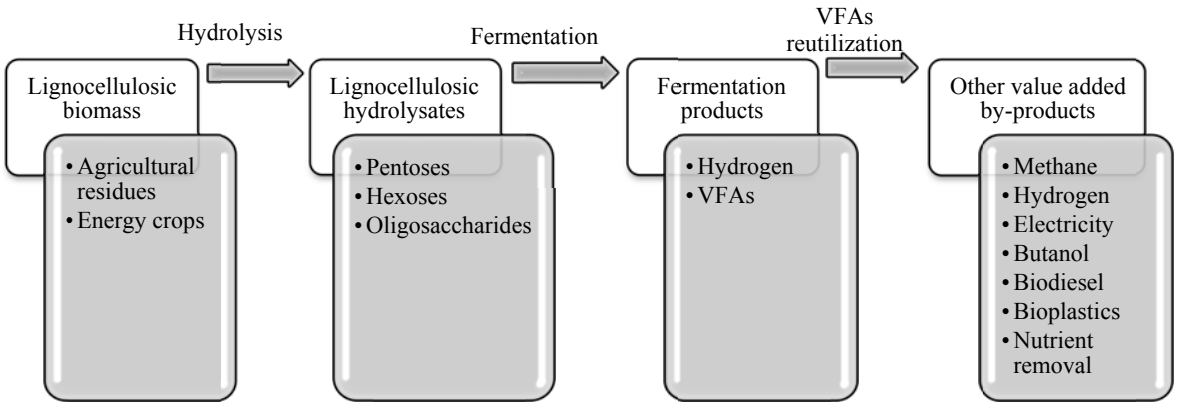

Fig. 6.1 Flowsheet for the production of hydrogen and other value added by-products from lignocellulosic biomass in a biorefinery concept.

6.2 Conclusions and final remarks

Overall results of this thesis demonstrate the feasibility to produce hydrogen from fermentation of oat straw hydrolysates in batch, semi-continuous and continuous systems. Nonetheless, results also showed that at the studied conditions, the batch reactors are more adequate than ASBRs and TBRs for hydrogen production from oat straw hydrolysates. Furthermore, it was also found that the enzymatic oat straw hydrolysate is a more suitable substrate for hydrogen production, as compared to the acid oat straw hydrolysate. This

finding was related to the content of arabinose, microbial inhibitors and oligosaccharides in the acid hydrolysate.

During ASBR operation, it was demonstrated that model substrates (glucose-xylose) achieve a better performance than oat straw hydrolysates. This performance differences were associated to a microbial population change. On the other hand, results of the TBR operation demonstrated that the enzymatic oat straw hydrolysate is an adequate substrate for continuous hydrogen production, since the HMY was similar to the value obtained with glucose. Nonetheless, it is important to remark that part of the hydrogen produced from the enzymatic hydrolysate was due to the fermentation and hydrogen contribution of Celluclast 1.5L, as it was demonstrated in batch assays.

Even though the use of lignocellulosic biomass is attractive for the hydrogen production process, problems addressed in this research need to be further investigated. Possible approaches to improve the hydrogen performance from acid hydrolysates include: 1) the reduction of the oligosaccharides content by a harsher hydrolysis and further detoxification of the hydrolysate by means of either activated charcoal adsorption, use of ion exchange resins or enzymatic (with laccases) treatments and 2) the adaptation of high hydrogen producers strains to hydrolysate composition and their further application to semi-continuous or continuous systems. On the other hand, it is recommended to optimize the hydrogen production from enzymatic hydrolysates, which may be addressed by means of hydraulic retention time decrements and substrate concentration increments in different reactor configurations. Moreover, in order to increase the economic viability of the process, it is also recommended to study the feasibility of using the fermentation by-products as substrates in any of the downstream processes shown in Fig. 6.1.

6.3 References

[1] Chen R, Wang YZ, Liao Q, Zhu X, Xu TF. Hydrolysates of lignocellulosic materials for biohydrogen production. BMB Rep 2013; 46:244-51.

[2] Wirtz RA, Dague RR. Laboratory studies on enhancement of granulation in the anaerobic sequencing batch reactor. Water Sci Technol 1997; 36:279-86.

[3] Van Groenestijn JW, Geelhoed JS, Goorissen HP, Meesters KPM, Stams AJM, Claassen PAM. Performance and population analysis of a non-sterile trickle bed reactor inoculated with Caldicellulosiruptor saccharolyticus, a thermophilic hydrogen producer. Biotechnol Bioeng 2009;102:1361-67.

[4] Liu Y, Tay JH. The essential role of hydrodynamic shear force in the formation of biofilm and granular sludge. Water res 2002;36:1653-65.

[5] Monlau F, Aemig Q, Trably E, Hamelin J, Steyer JP, Carrere H. Specific inhibition of biohydrogen-producing Clostridium sp. after dilute-acid pretreatment of sunflower stalks. Int J Hydrogen Energy 2013; 38:12273-82.

[6] Quéméneur M, Hamelin J, Barakat A, Steyer JP, Carrére H, Trably E. Inhibition of fermentative hydrogen production by lignocellulose-derived compounds in mixed cultures. Int J Hydrogen Energy 2012; 37:3150-9.

[7] Zhang Z-P, Adav SS, Show K-Y, Tay J-H, Liang DT, Lee D-J, et al. Characteristics of rapidly formed hydrogen-producing granules and biofilms. Biotechnol Bioeng 2008; 101:926-36.

[8] Davila-Vazquez G, Arriaga S, Alatriste-Mondragon F, De León-Rodríguez A, Rosales Colunga LM, Razo-Flores E. Fermentative biohydrogen production: trends and perspectives. Rev Environ Sci Biotechnol 2008; 7:27-45.

[9] Wang J, Wan W. Factors influencing fermentative hydrogen production: A review. Int J Hydrogen Energy 2009; 34:799-811.

[10] Rittman S, Herwig C. A comprehensive and quantitative review of dark fermentative biohydrogen production. Microb Cell Fact 2012; 11:115.

[11] Cheng CL, Lo YC, Lee KS, Lee DJ, Lin CY, Chang JS. Biohydrogen production from lignocellulosic feedstock. Bioresour Technol 2011; 102:8514-23.

[12] Li J, Ren N, Li B, Qin Z, He J. Anaerobic biohydrogen production from monosaccharides by a mixed microbial community culture. Bioresour Technol 2008; 99:6528-37.

[13] Mangayil R, Santala V, Karp M. Fermentative hydrogen production from different sugars by *Citrobacter sp.* CMC-1 in batch culture. Int J Hydrogen Energy 2011; 36:15187-94.

[14] Quéméneur M, Hamelin J, Benomar S, Guidici-Orticoni MT, Latrille E, Steyer JP, Trably E. Changes in hydrogenase genetic diversity and proteomic patterns in mixed-culture dark fermentation of mono-, di- and tri-saccharides. Int J Hydrogen Energy 2011; 36:11654-65.

[15] Parawira W, Tekere M. Biotechnological strategies to overcome inhibitors in lignocellulose hydrolysates for ethanol production: review. Cr Rev Biotechn 2011; 31:20-31.

[16] Arriaga S, Rosas I, Alatriste-Mondragón F, Razo-Flores E. Continuous production of hydrogen from oat straw hydrolysate in a biotrickling filter. Int J Hydrogen Energy 2011; 36:3442-9.

[17] Pattra S, Sangyoka S, Boonmee M, Reungsang A. Biohydrogen production from the fermentation of sugarcane bagasse hydrolysate by *Clostridium butyricum*. Int J Hydrogen Energy 2008; 33:5256-65.

[18] Fangkum A, Reungsang A. Biohydrogen production from sugarcane bagasse hydrolysate by elephant dung: Effects of initial pH and substrate concentration. Int J Hydrogen Energy 2011; 36:8687-96.

[19] Rai PK, Singh SP, Asthana RK, Singh S. Biohydrogen production from sugarcane bagasse by integrating dark and photo-fermentation. Bioresour Technol 2014; 99:140-46.

[20] Rittmann BE, McCarty PL. Environmental biotechnology: principles and applications. McGraw-Hill: New York, NY, USA. 2001.

[21] Lee WS, Chua ASM, Yeoh HK, Ngoh CG. A review of the production and applications of waste-derived volatile fatty acids. Chem. Eng. J 2014; 235:83-99.

List of publications

List of publications in the science citation index

- Arreola-Vargas, J., Celis L.B., Buitrón, G., Razo-Flores, E., Alatriste-Mondragón, F. Hydrogen production from acid and enzymatic oat straw hydrolysates in an anaerobic sequencing batch reactor: Performance and microbial population analysis. *International Journal of Hydrogen Energy*. 2013; 38:13884-13894.
- Arreola-Vargas J, Razo-Flores E, Celis LB, Alatriste-Mondragón F. Oat straw sugar solubilization and hydrogen production from hydrolysates: role of hydrolysates constituents. Submitted to *Renewable Energy.*
- Arreola-Vargas, J., Alatriste-Mondragón, F., Celis L.B., Razo-Flores, E., López-López A., Méndez-Acosta HO. Continuous hydrogen production in a trickling bed reactor by using triticale silage as inoculum: comparison between simple and complex substrates. To be submitted to *Journal of Chemical Technology and Biotechnology.*

List of publications in refereed conference proceedings

- Arreola-Vargas, J., Celis, L.B., Razo-Flores, E., Alatriste-Mondragón, F. Effect of different sequential chemical pretreatments of oat straw on sugar solubilization and biohydrogen production. In: Proc. of the *X Latin American Workshop and Symposium on Anaerobic Digestion (X DAAL).* International Water Association. Ouro Preto, MG, Brazil. October 2011.
- Arreola-Vargas, J., Abreu-Sherrer J., Celis, L.B., Razo-Flores, E., Alatriste-Mondragón, F. Hydrogen production from oat straw hydrolysates and glucose: effect of the type of substrate and type of inoculum. In: Proc. of the *IV International Symposium on Energy from Biomass and Waste.* International Waste Working Group. Venice, Italy, 2012. *CISA publisher.*