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

POSGRADO EN CIENCIAS APLICADAS

Optimización de las condiciones de fermentación para la producción de hidrógeno en cultivos en lote y continuo

Tesis que presenta

Gustavo Dávila Vázquez

Para obtener el grado de

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Director de la Tesis:

Dr. Elías Razo Flores

Co-director: **Dr. Antonio de León Rodríguez**

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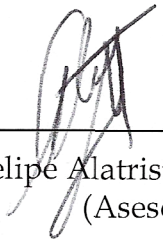
La tesis **"Optimización de las condiciones de fermentación para la producción de hidrógeno en cultivos en lote y continuo"** presentada para obtener el Grado de Doctor en Ciencias Aplicadas en la opción de Ciencias Ambientales fue elaborada por **Gustavo Dávila Vázquez** y aprobada el **28 de noviembre de 2008** 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.

Dr. Elías Razo Flores
(Director)

Dr. Antonio de León Rodríguez
(Co-director)

Dra. Sonia Lorena Arriaga García
(Asesor)



Dr. Felipe Alatriste Mondragón
(Asesor)

Dr. Germán Buitrón Méndez
(Asesor)

Esta tesis fue elaborada en los Laboratorios de la División de Ciencias Ambientales, además del Laboratorio de Biotecnología Molecular de la División de Biología Molecular del Instituto Potosino de Investigación Científica y Tecnológica, A.C., bajo la dirección del Dr. Elías Razo Flores y la co-dirección del Dr. Antonio de León Rodríguez

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EN LA OPCIÓN DE CIENCIAS AMBIENTALES

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Optimización de las condiciones de fermentación para la producción de hidrógeno en cultivos en lote y continuo

que se desarrolló bajo la dirección de

Dr. Antonio De León Rodríguez
Dr. Elías Razo Flores

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SECRETARIA ACADEMICA

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Resumen

Optimización de las condiciones de fermentación para la producción de hidrógeno en cultivos en lote y continuo

Palabras clave: bio-hidrógeno, cultivos mixtos, lodo anaerobio, PCR-DGGE

El hidrógeno (H_2) es considerado como el vector energético del futuro. Esto porque comparado con los hidrocarburos, contiene gravimétricamente 2.75 veces más energía, además de que su combustión solo genera vapor de agua como subproducto. El H_2 también puede ser utilizado para la producción de energía eléctrica mediante celdas de combustible, sin la generación de gases de efecto invernadero. Alrededor del 95% del H_2 utilizado en la industria se produce a partir de hidrocarburos, lo que lo convierte en un proceso no sustentable. Por el contrario, el bio-hidrógeno (Bio-H_2) generado a partir de biomasa, es una alternativa energética sustentable y neutra en emisiones de gases de efecto invernadero. Algunos microorganismos son capaces de producir Bio-H_2 bajo condiciones anaerobias, a partir de un amplio rango de sustratos orgánicos. En esta tesis se estudió la producción de Bio-H_2 en experimentos en lote y continuo utilizando sustratos tales como la glucosa y lactosa, además de lactosuero (LS), y lodo anaerobio granular tratado térmicamente como inoculo. En los experimentos en lote se estudió el efecto del pH inicial y de la concentración inicial de sustrato sobre el rendimiento molar (RMH) y la velocidad volumétrica de producción de Bio-H_2 (VVPH). El RMH y la VVPH obtenidas en los experimentos en lote para los tres sustratos, se encontraron a pH iniciales superiores a los óptimos comúnmente reportados en la literatura. Posteriormente, por ser un subproducto industrial y mostrar valores altos tanto de RMH y VVPH, se seleccionó al LS como sustrato, para evaluar la producción de hidrógeno utilizando dos medios minerales diferentes (A y B). El análisis de la comunidad microbiana mostró diferencias en los microorganismos presentes utilizando ambos medios, predominando para el medio B la presencia de especies de *Clostridium* y *Enterobacter,* mientras que en los experimentos con medio A solo se identificó a una proteobacteria. Consecuentemente, el uso de medio mineral B produjo alrededor del doble de la VVPH que fue lograda con el medio A, aunque el RMH tuvo solo un ligero incremento con la utilización del medio B. Finalmente, se operó un tanque agitado continuo durante 65.6 días utilizando LS como sustrato, suplementado con medio mineral B. Se probaron tres tiempos de residencia hidráulico (TRH: 10, 6 y 4h) obteniendo la VVPH más alta con 6h. Además, se probaron cuatro cargas orgánicas (CO) incrementales a un TRH fijo de 6h. La VVPH más alta, 46.61 mmol H_2/l/h y el RMH de 2.8 mol H_2/mol lactosa se obtuvieron a una CO de 138.6 g lactosa/l/d. Las especies bacterianas dominantes a TRH de 10 y 6 h fueron las del género *Clostridium*. La VVPH obtenida en este estudio, en experimentos en continuo, es la más alta reportada para un sistema de tanque agitado inoculado con lodo anaerobio granular utilizando LS como sustrato. El aumento en los valores tanto de RMH como de la VVPH es crítico para determinar la aplicabilidad a mayor escala de estos procesos fermentativos, considerados potenciales generadores primarios de energía sustentable en el futuro cercano.

Abstract

Optimization of fermentation conditions in batch and continuous hydrogen production

Keywords: biohydrogen, mixed cultures, anaerobic sludge, PCR-DGGE

Hydrogen (H_2) is seen as a future energy carrier. Because compared to hydrocarbons, it contains 2.75-times more energy by weight, besides the fact that its combustion only generates steam as by-product. H_2 can be also used for electricity generation with the aid of fuel cells, without the production of greenhouse gases. Around the 95% of the H_2 used in industry is now produced from hydrocarbons, which makes this process unsustainable. On the other hand, biohydrogen (Bio-H_2) generated from biomass, is a sustainable energetic alternative which is also neutral in greenhouse gases emission. Some microorganisms are capable of produce Bio-H_2 under anaerobic conditions, from a wide range of organic substrates. In this thesis, the production of Bio-H_2 was studied with glucose, lactose and cheese whey (CW) as substrates, in batch and continuous experiments and heat-treated anaerobic granular sludge as inoculum. The effect of initial pH and initial substrate concentration on both the hydrogen molar yield (HMY) and volumetric Bio-H_2 production rate (VHPR) was studied for the three substrates in batch experiments. Both, higher HMY and VHPR obtained in batch experiments were found at higher initial pH than commonly reported as optima in the literature. Furthermore, CW was selected to assess Bio-H_2 production with two different media (A and B) due to the high HMY and VHPR obtained, and because CW is an industrial by-product. The microbial community analysis showed differences in the microorganisms present for the experiments with each medium. *Clostridium* and *Enterobacter* species dominated with the use of medium B, while experiments with medium A only showed the presence of a proteobacterium. Consequently, the use of mineral medium B yielded around twice the VHPR than the obtained with medium A, while HMY had a slight increase with the use of medium B. Finally, a continuous stirred tank reactor was operated for 65.6 days using CW as substrate, supplemented with mineral medium B. Three hydraulic retention times (HRT: 10, 6 and 4h) were tested attaining the highest VHPR at 6h. Moreover, four organic loading rates (OLR) were evaluated at a fixed HRT of 6h. The highest VHPR, 46.61 mmol H_2/l/h and HMY of 2.8 mol H_2/mol lactose were attained at an OLR of 138.6 g lactose/l/d. The dominant bacterial species at HRT of 10 and 6h belonged to the *Clostridium* genus. The VHPR obtained here in continuous experiments is the highest reported for a stirred tank reactor inoculated with anaerobic granular sludge using LS as substrate. The enhancements of both HMY and VHPR are critical to assess the full-scale practical application of fermentative processes, which are now considered as potential primary generators of sustainable energy in the near future.

Chapter 1

Biological hydrogen production: State of the art

Summary

Biologically produced hydrogen (Bio-H_2) is a valuable gas that is seen as a future energy carrier since its utilization via combustion or fuel cells produce pure water. Heterotrophic (dark) fermentations for biohydrogen production are driven by a wide variety of microorganisms such as strict anaerobes, facultative anaerobes and aerobes kept under anoxic conditions. Substrates such as simple sugars, starch, cellulose, as well as diverse organic waste materials can be used for biohydrogen production. Various bioreactor types have been used and operated under batch and continuous conditions; substantial increases in hydrogen yields are been achieved through optimum design of the bioreactor and fermentation conditions. This chapter explores the research work carried out in fermentative hydrogen production using biomass as substrates.

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1.1 Introduction

A large proportion of the world energy needs are being covered by fossil fuels, which have led to an accelerated consumption of these non-renewable resources. This has resulted in both, the increase in CO_2 concentration in the atmosphere and the rapid depletion of fossil resources. The former is considered the main cause of global warming and associated climate change, whereas the latter will lead to an energy crisis in the near future (Kapdan and Kargi 2006). For these reasons, large efforts are being conducted worldwide in order to explore new sustainable energy sources that could substitute fossil fuels. Processes, which produce energy from biomass, are typical examples of environmentally friendly technologies as biomass is included in the global carbon cycle of the biosphere. Large amounts of biomass are available in the form of organic residues, such as solid municipal wastes, manure, forest and agricultural residues. Some of these residues can be used after minor steps of pre-treatment (usually dilution and maceration), while others may require extensive chemical transformations prior to being utilized as a raw material for biological energy production (Claassen *et al*. 1999). Biological processes such as methane and hydrogen production under anaerobic conditions, and ethanol fermentation are future oriented technologies that will play a major role in the exploitation of energy from biomass.

Using the appropriate microbial mechanisms of anaerobic digestion, hydrogen (biohydrogen) would be the desired product of the digestion process while the organic acids would be the by-products. The major advantage of energy from hydrogen is the absence of polluting emissions since the utilization of hydrogen, either via combustion or via fuel cells, results in pure water (Claassen *et al*. 1999). This chapter provides an overview of the state of the art of fermentative hydrogen production by microorganisms. For a full overview of previous work on this topic the reader is referred to excellent reviews published elsewhere (Nandi and Sengupta 1998; Claassen *et al.* 1999; Hallenbeck and Benemann 2002; Hawkes *et al.* 2002; Nath and Das 2004; Kapdan and Kargi 2006; Hawkes *et al.* 2007; Redwood *et al.* 2008).

1.2 Biohydrogen producing microorganisms

Biohydrogen can be produced by strict and facultative anaerobes (*Clostridia, Micrococci, Methanobacteria, Enterobacteria*, etc), aerobes (*Alcaligenes and Bacillus*) and also by photosynthetic bacteria (Nandi and Sengupta 1998). Table 1.1 shows that during the time period covered by this review, most of the studies were conducted using mixed cultures, and just a few of them were pure cultures. Different sources of inocula were reported (soil, sediment, compost, aerobic and anaerobic sludges, etc.) and most of them were heat or acid treated before being used. These treatments have been used in previous studies as methods for increasing hydrogen production by altering the microbial communities present in the starting mixed population (Cheong and Hansen 2006). The reason for this is that unlike H_2-consuming methanogens, H_2- producing bacteria are commonly tolerant to harsher environmental conditions (Kawagoshi *et al*. 2005). For example *Clostridium* and *Bacillus* species tolerate higher temperatures than H_2-consuming methanogens due to the formation of endospores (Setlow 2000). Also, H_2-producing bacteria can grow at lower pH than H_2-consuming methanogens (Cheong and Hansen 2006).

Various studies have been carried out to identify the microbial communities present in mixed cultures used for H_2 production (Ueno *et al.* 2001; Fang *et al.* 2002; Ueno *et al.* 2004; Kawagoshi *et al.* 2005; Kim *et al.* 2006; Koskinen *et al.* 2007; Yang *et al*. 2007; Chang *et al.* 2008; Cheng *et al.* 2008). Fang *et al*. (2002) identified the microbial species in a granular sludge used for H_2 production from sucrose. They found that 69.1% of the microorganisms were *Clostridium* species and 13.5% were *Bacillus/Staphylococcus* species. Kawagoshi *et al*. (2005) studied the effect of both pH and heat conditioning on different inoculums. In their study they concluded that the highest hydrogen production was obtained with heat-conditioned anaerobic sludge. They also found DNA bands with high similarity (>95%) to *Clostridium tyrobutyricum, Lactobacillus ferintoshensis, L. paracasei,* and *Coprothermobacter* spp. Kim *et al*. (2006a) indicated that heat-treatment (90 ^{o}C for 20 min) caused a change in the microbial community composition of a fresh culture used to produce H_2 from glucose in a membrane bioreactor. They reported that most of the species

found in the fresh sludge were affiliated to the *Lactobacillus* sp. and *Bifidobacterium* sp.; in contrast a *Clostridium perfringens* band was observed in the heat-treated sludge. When mixed cultures are used as inocula the predominant species in a bioreactor depends on operational conditions such as temperature, pH, substrate, inoculum type, inoculum pre-treatment, hydrogen partial pressure, etc. Kotay and Das (2006) studied the H_2 production with glucose and sewage sludge as substrates using a defined microbial consortium consisting of three facultative anaerobes, *Enterobacter cloacae*, *Citrobacter freundii* and *Bacillus coagulans*. They carried out experiments with the consortium (three species) and the individual species. *E. cloacae* produced a higher yield than the other strains, but similar to the consortium suggesting that *E. cloacae* dominated the consortium. Koskinen *et al*. (2007) studied the dynamics of the microbial community present in a fluidized-bed reactor (FBR). They found that the fast growth of *Clostridium butyricum* in the FBR was related to high Bio-H_2 and butyrate production. *Escherichia coli* strains were also detected at high glucose loading rates. In a work by Yang *et al*. (2007) mixed anaerobic cultures were used to produce Bio-H_2 from cheese whey powder. For the periods with the highest hydrogen production, the authors found that more than 50% of the bacteria present were *Lactobacillus* strains and about 5% were members of the *Clostridium* genus. Recently, Chang *et al*. (2008) produced Bio-H_2 from condensed molasses and sewage sludge in a continuous reactor. Molecular monitoring of the process revealed the presence of *Clostridium, Corynebacterium, Bifidobacterium, Neisseria, Alcaligenes* and *Acidaminococcus* strains depending on the operating conditions. Some studies using pure strains have also been carried out for H_2 production. *Escherichia coli* (genetically modified strains), *Clostridium butyricum*, *C. saccharoperbutylacetonicum*, *C. thermolacticum*, and *C. acetobutylicum* are among the microorganisms used (Table 1.1).

Table 1.1. Hydrogen production rates and yield coefficients from pure and complex substrates under batch, semi-continuous and continuous operation.

System	Inoculum	Substrate[a]	Volumetric H_2 production rate (mmol $H_2/l_{culture}/h$)[f]	H_2 yield	Culture conditions[a] [HRT (h), OLR, pH, Temperature (°C), H_2 in biogas (%v/v)]	Reference
Batch	*Clostridium butyricum* CGS5	Sucrose (20 g COD/l)	8.2	2.78 mol H_2/mol sucrose	--, --, 5.5-6.0[c], 37, 64	Chen *et al*. 2005
	Clostridium saccharoperbutylacetonicum ATCC 27021	Crude cheese whey (*ca*. 41.4 g lactose/l)	9.4	2.7 mol H_2/mol lactose	--, --, 6.0[d], 30, NR[b]	Ferchichi *et al.* 2005
	Escherichia coli strains	Glucose (4 g/l)	NR[b]	~2 mol H_2/mol glucose	--, --, 7.0, 37, NR	Bisaillon *et al.* 2006
	Escherichia coli strains	Formic acid (25 mM)	11795	1 mol H_2/mol formiate	--, --, 6.5 [d], 37, NR	Yoshida *et al*. 2005
	Thermotoga maritima DSM 3109	Glucose (7.5 g/l)	8.2	1.67 mol H_2/mol glucose	--, --, 6.5 – 7.0 [d], 80, 30-33	Nguyen *et al.* 2008
	Thermotoga neapolitana DSM 4359		8.7	1.84 mol H_2/mol glucose	--, --, 6.5 – 7.5 [d], 75, 30-33	
	Defined consortium (1:1:1, and separately tested): *Enterobacter cloacae* IIT-BT 08, *Citrobacter freundii* IIT-BT L139, *Bacillus coagulans* IIT-BT S1	Glucose (10 g/l)	NR	41.23 ml H_2/ g $COD_{removed}$	--, --, 6.0[d], 37, NR	Kotay and Das 2006

Mesophilic bacterium HN001	Starch (20 g/l)	59	2 mol H_2/mol glucose	--, --, 6.0[c], 37, NR	Yasuda and Tanisho 2006
Aerobic and anaerobic sludges, soil and lake sediment (acid and heat conditioned)	Glucose (20 g/l)	NR	1.4 mol H_2/mol glucose	--, --, 6.0[c], 35, NR	Kawagoshi *et al*. 2005
Aerobic sludge (heat conditioned)	Glucose (2 g/l)	NR	2.0 mol H_2/mol glucose	--, --, 6.2,[d] 30, 87.4	Park *et al*. 2005
Soil (heat conditioned)	Organic matter present in four carbohydrate-rich wastewaters.	6.2	100 ml H_2/g $COD_{removed}$	--, --, 6.1,[d] 23, 60	Van Ginkel *et al*. 2005
Anaerobic sludge (acid treatment and acclimated in a CSTR)	Sucrose (20 g COD/l)	96	1.74 mol H_2/mol sucrose	--, --, 6.1,[d] 40, 45	Wu *et al*. 2005
Anaerobic sludge (heat conditioned)	Glucose (10 g/l)	27.2 mmol/g_{VSS}-$l_{culture}$/h	1.75 mol H_2/mol glucose	--, --, 6.0,[d] 37, 40	Zheng and Yu 2005
Anaerobic sludge (acid treatment)	Glucose (~21.3 g/l)	4.9-8.6	0.8-1.0 mol H_2/mol hexose	--, --, 5.7,[c] 34.5, 59-66	Cheong and Hansen 2006
Microflora from a cow dung compost (heat treatment)	Wheat straw wastes (25 g/l)	2.7 mmol H_2/g_{TVS} $l_{culture}$/h	2.7 mmol H_2/g_{TVS}	--, --, 7.0,[d] 36, 52	Fan *et al*. 2006
Anaerobic sludge (heat treated)	Sucrose (10 g/l)	8	1.9 mol H_2/mol sucrose	--, --, 5.5,[c] 35, NR	Mu *et al*. 2006a
Anaerobic sludge (heat treated)	Sucrose (24.8 g/l)	20	3.4 mol H_2/mol sucrose	--, --, 5.5,[c] 34.8, 64	Mu *et al*. 2006b
Anaerobic sludge (heat treated)	Glucose (3.76 g/l)	9	1.0 mol H_2/mol glucose	--, --, 6.2,[d] 30, 66	Salerno *et al*. 2006
Anaerobic sludge (heat treated)	Glucose (2.82 g/l)	NR	0.968 mol H_2/mol glucose	--, --, 6.2,[d] 25, 57-72	Oh *et al*. 2003

	Microflora from soil (heat treated)	Glucose, sucrose, molasses, lactate, potato starch, cellulose (each: 4 g COD/l)	NR	0.92 mol H_2/mol glucose, 1.8 mol H_2/mol sucrose, 0.59 mol H_2/mol potato starch,[e] 0.01 mol H_2/mol lactate, 0.003 mol H_2/mol cellulose[e]	--, --, 6.0,[d] 26, 62	Logan *et al*. 2002
	Anaerobic digested sludge	Cheese whey powder	NR	10 mM/g COD	--, --, 7.3, [d] 35, 49	Yang *et al.* 2007
	Microflora from compost	Vegetable kitchen wastes (75 g COD/l)	0.44 mmol H_2/g VSS/h	0.49 mmol H_2/g COD	--, --, 7.0, [c] 55, 73	Lee *et al.* 2008
Fed Batch	Mixed culture	OFMSW-Semisolid substrate	14.7 mmol/$g_{VSdestroyed}$	NR[b]	504, 11 g_{VS}/Kg_{wmr} d, 6.4, 55, 58	Valdez-Vazquez *et al*. 2005
	POME sludge	Palm oil mill effluent (2.5% w/v)	17.82	NR	24, NR, 5.5, 60, 66	Atif *et al*. 2005
	Windrow yard waste compost	Glucose (2 g/l)	7.44	1.75 mol H_2/mol glucose	76, NR, 5.4, 55, NR	Calli *et al*. 2006
	Compost	Lactose (2 g/l)	1.61	3.7 mol H_2/mol lactose	22, 2.2 g/l/d, 5.0,[c] 55, NR	Calli *et al.* 2008
			2.55	3.2 mol H_2/mol lactose	22, 2.2 g/l/d, 5.3,[c] 55, NR	
CSTR	Mixed culture	Sucrose (20 g COD/l)	17	3.5 mol H_2/mol sucrose	12, 1.7 g COD/l/h, 6.8, 35, 45.9	Lin *et al*. 2006b
	Mixed culture	Sucrose (40 g/l)	20	1.15 mol H_2/mol hexose	12, 80 g/l/d, 5.2, 35, 60	Kyazze *et al.* 2006

Mixed culture	Cheese whey powder	1000 ml H_2/l/h	1.98 mM/ g COD	24, 14 g/l/d, 5.2, 35, 30	Yang *et al*. (2007)
Mixed culture immobilized in silicone gel	Sucrose (30 g COD/l)	612.5	3.86 mol H_2/mol sucrose	0.5, 1440 g/l/d, 6.5, 40, 44	Wu *et al*. 2006a
Mixed culture	Xylose (20 g COD/l)	5	1.1 mol H_2/mol xylose	12, 1.7 g COD/l/h, 7.1, 35, 32	Lin and Cheng 2006
Mixed culture	Broken kitchen wastes (10 Kg COD/m^3/d) and corn starch (10 Kg COD/m^3/d)	1.7	NR	96, NR, 5.3-5.6, 35, NR	Cheng *et al*. 2006
Mixed culture	Glucose (15 g COD/l)	13.23	1.93 mol H_2/mol glucose	4.5, 80 g COD/l/d, 5.5, 37, 67	Zhang *et al*. 2004
Dewatered and thickened sludge	Glucose (4 g COD/l)	3.47	1.9 mol H_2/mol glucose	10, 0.4 g COD/l/h, 5.5, 35, 67	Salerno *et al*. 2006
Mixed culture	Sucrose (20 g COD/l)	15.6	3.6 mol H_2/mol sucrose	12, 1.7 g COD/l/h, 5.5, 35, 50	Lin and Chen 2006
Mixed culture	Organic wastewater (4 g COD/l)	4.96	NR	12, 333 mg COD/l/h, 4.4, 8 Kg_{COD}/m^3 d, 30, NR	Wang *et al*. 2006b
Sewage sludge	Sucrose (20 g COD/l)	52.6	3.43 mol H_2/mol sucrose	12, 1.7 g COD/l/h, 6.8, 35, 50.9	Lin and Lay 2005
Mixed culture	Sucrose and sugarbeet	5.15	1.9 mol H_2/mol hexose	15, 16 Kg sugar/m^3/d, 5.2, 32, NR	Hussy *et al*. 2005
Mixed culture	Glucose (15 g/l)	0.115 g $_{H2\text{-}COD}$/g $_{Feed\ COD}$	1.38 mol H_2/mol hexose	10, 1.5 g/l/h, 5.5, 35, 45	Kraemer and Bagley 2005

	C. thermolacticum (DSM 2910)	Lactose (10 g/l)	2.58	2.1-3 mol H_2/mol lactose	17.2, NR, 7.0, 58, 55	Collet *et al*. 2004
	Seed sludge	Molasses (3 g COD/l)	26.13 mol H_2/Kg COD removed	NR	11.4,27.98 Kg COD/m^3 reactor-d, 4.5, 35, 45	Ren *et al*. 2006
	Mixed culture	Glucose (10 g/l)	2.18	2.47 mol H_2/mol glucose	26.7, 0.37 g/l/h, 4.8 – 5.5, 70, NR	Kotsopoulos *et al*. 2006
	Granular sludge	Glucose (2.5 – 40 g/l)	*ca*. 135	1.84 mol H_2/mol glucose	0.5, 20 g glucose/l/h, 5.5, 37, NR	Show *et al*. 2007
	Anaerobic sludge (suspended)	Glucose (20 g COD/l)	24.5	1.53 mol H_2/mol glucose	6, 3.33 g COD/l/h, 6.4, 40, 46	Wu *et al*. 2008
	Immobilized-cell-seeded anaerobic bioreactor: (ICSAB)		25	0.87 mol H_2/mol glucose	4, 5 g COD/l/h, 6.3, 40, 36	
	(agitated granular sludge bed: AGSB)		39.7	1.57 mol H_2/mol glucose	4, 5 g COD/l/h, 6.6, 40, 39	
UASB	Mixed culture	Sucrose rich waste water	5.93	1.61 mol H_2/mol glucose	12, NR, 7, 39, NR	Mu and Yu 2006
	Mixed culture	Citric acid waste water (18 kg COD/l)	1.23	0.84 mol H_2/mol hexose	12, 38.4 kg COD/m^3/d, 7, 35, NR	Yang *et al*. 2006
	Mixed culture	Sucrose (20 g COD/l)	11.3	1.5 mmol H_2/mol sucrose	8, 175 mmol sucrose/l/d, 6.7, 35, 42.4	Chang and Lin 2004
	Mixed culture	Glucose (7.7 g/l)	18.4	1.7 mol H_2/mol glucose	2, 3.85 g/l/h, 6.4, 55, 36.8	Gavala *et al*. 2006
		(1.3 g/l)	19	0.7 mol H_2/mol glucose	2, 0.65 g/l/h, 4.4, 35, 29.4	

CSTR and UASB	Mixed culture	Starch (10 g /l) and xylose (1:1 w/w)	4.5 4.76 2.54		32.9, NR, 7, 35, 68 6.7, NR, 7, 35, 68 20.5, NR, 7, 35, 68	Camilli and Pedroni 2005
CSTR UASB UFBR	Mixed culture	Glucose (6.86 g/l)	37.5	1.6 molH_2/mol glucose	12, NR, 5.5, 60, 48	Oh *et al*. 2004
TBR	*Clostridium acetobutylicum* (ATCC 824)	Glucose (10.5 g/l)	8.9	0.9 mol H_2/mol glucose	0.035, 8.3 g/l/h, 4.9, 30, 74	Zhang *et al*. 2006
	Mixed culture	Glucose (2 g/l)	NR	2.48 mol H_2/mol glucose	0.5, 96 kg/m^3/d, 7.7, 30, NR	Leite *et al*. 2006
PBR	Mixed culture	Sucrose (17.8 g/l)	0.298	3.88 mol H_2/mol sucrose	0.5, NR, 6.7, 40, 42	Lee *et al*. 2006
CIGSB	Cow dung	Palm oil mill effluent (5-60 g DQO/l)	0.42 l/g $_{COD\ destroyed}$	NR	3-7, NR, 5, NR, 53-56	Vijayaraghavan and Ahmad 2006
UACF	Cow dung	Jackfruit peel (22.5 g VS/l)	0.72 l biogas/g $_{VS\ destroyed}$	NR	288, NR, 5, NR, 56	Vijayaraghavan *et al*. 2006
MBR	Mixed culture	Glucose (10 g/l)	71.4	1.1 mol H_2/mol glucose	0.79, 12.7 g/l/h, 5.5, 37, 70	Kim *et al*. 2006
MBR	Mixed culture	Glucose (20 g COD/l)	60.5	1.27 mol H_2/mol hexose	1, 20 g COD/l/h, 6.7, 35, 41	Lee *et al*. 2007
		Sucrose (20 g COD/l)	84.6	1.39 mol H_2/mol hexose	1, 20 g COD/l/h, 6.7, 35, 43	
		Fructose (20 g COD/l)	112	1.36 mol H_2/mol hexose	1, 20 g COD/l/h, 6.7, 35, 40	

FBR DTFBR	Mixed culture	Sucrose (20 g COD/l)	50.27 95.23	2.10 mol H_2/mol sucrose 1.22 mol H_2/mol sucrose	2, 10 g COD/l/h, 6.9, 4, 40 0.5, 40 g COD/l/h, 7, 40, 35	Wu *et al.* 2006b
FBR	Anaerobic sludge	Glucose (10 g/l)	28	1.9	1.8, 2.8 g glucose/l/h, 6, 35, 20	Koskinen *et al.* 2007
EGSB	Activated sludge	Molasses (10 g COD/l)	29	3.47 mol H_2/mol sucrose	2, 120 kg COD/m^3/d, 4.36, 35, 30-53	Guo *et al.* 2008

Notes: [a]When optimization trials were carried out, optimum values are reported. [b]NR: Not reported. [c]Controlled value. [d]Initial, not controlled. [e]starch, celulose: $[(C_6H_{10}O_5)_n]$. [f] In some cases unit conversions were made according to the conditions reported by the authors. POME: Anaerobic pond of a palm oil mill effluent. COD: Chemical oxygen demand. Kg_{wmr}: Kilograms of wet mass in the reactor. CSTR: Continuous stirred tank reactor. TBR: Trickling biofilter. OFMSW: Organic fraction of municipal solid wastes. PBR: Packed bed reactor. MBR: Membrane bioreactor. FBR: Fluidized bed bioreactor. DTFBR: Draft tube fluidized bed reactor. UFBR: Up-flow fixed bed reactor. CIGSB: Carrier induced granular sludge bed. UASB: Upflow anaerobic sludge blanket. UACF: Up-flow anaerobic contact filter. EGSB: Expanded granular sludge bed reactor.

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1.3 Substrates used for biohydrogen production

Glucose, sucrose and to a lesser extent starch and cellulose, have been extensively studied as carbon substrates for biohydrogen production (Table 1.1). They have been used as *model* substrates for research purposes due to their easy biodegradability and because some of them can be either present in some wastewaters or obtained from different organic wastes. Other substrates suitable for biohydrogen production are protein- and fat-rich wastes. Although they are less available than carbohydrate-rich wastes, them represent potential feeds for the biological conversion of organic wastes to hydrogen (Svensson and Karlsson 2005). As Kapdan & Kargi (2006) pointed out, the main criteria for substrate selection are: availability, cost, carbohydrate content and biodegradability. Consequently, some other substrates (e.g. agricultural residues and wastewaters) that better comply with these criteria are being used instead. Actual yields in metabolisms that lead to H_2 production are low compared to theoretical stoichiometric conversion:

$$\text{Glucose} + 6\,H_2O \rightarrow 12\,H_2 + 6\,CO_2 \quad \Delta G^{0'} = -25\ \text{kJ} \qquad (1.1)$$

One can see that complete conversion of 1 mol of hexose yields 12 mol of H_2. Also it can be noticed that there is a small free energy change available from this reaction. This figure is too little to drive ATP synthesis and makes this solo reaction unsuitable to support bacterial life (Hallenbeck 2005). Recent works (Table 1.1) show that even when substrate consumptions are high, hydrogen molar yields (HMY) do not exceed 4 mol of H_2 per mol of monosaccharide or 8 mol of H_2 per mol of disaccharide. This so called *fermentation barrier* is maintained regardless of the fermentation system used for H_2 production e.g. batch, semi-continuous or continuous one step-processes (Logan 2004). Besides molar hydrogen yield, another important feature of hydrogen fermentation is volumetric H_2 production rate (VHPR). According to Levin *et al*. (2004) it would be useful to express VHPR in units that allow comparison between different hydrogen producing systems. For this reason one can consider that the effort to standardize units well worth it.

1.4 Biohydrogen production in batch, continuous and semi-continuous systems

Biohydrogen production by dark fermentation is highly dependent on the process conditions such as temperature, pH, mineral medium formulation, type of organic acids produced, hydraulic residence time (HRT), type of substrate and concentration (gas partial pressure) and reactor configuration (Table 1.1).

Fermentation reactions can be operated at mesophilic (25-40°C), thermophilic (40-65°C), extreme thermophilic (65-80°C), or hyperthermophilic (>80°C) temperatures.

Operation temperature affects the growth rate and metabolic activity of microorganism. Most of the results presented on Table 1.1 have been carried out at mesophilic and thermophilic conditions. When mixed cultures were used operation at thermophilic conditions were favorable. Oh *et al*. (2004) showed that thermophilic (60°C) conditions suppress lactate-forming bacteria and increase VHPR. These results can be explained thermodynamically; the theoretical yield per mole of glucose, the free energy and standard enthalpy are described in the following reaction (Vazquez-Duhalt 2002), a maximum of 4 mol H_2 is produced per mole glucose when the end product is acetic acid:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2 \qquad (1.2)$$

$\Delta G°$=-176.1 (kJ/mol)

$\Delta H°$=+90.69 (kJ/mol)

The Gibbs free energy of the reaction has negative sign which indicates that the reaction can occur spontaneously. The van´t Hoff equation (Smith *et al.* 2000) explains the effect of temperature on the equilibrium constant and in consequence on the yield coefficient:

$$\ln\frac{K_1}{K_2} = -\frac{\Delta H°}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \qquad (1.3)$$

$$\frac{K_1}{K_2} = \frac{[H_2]_1^4 [CO_2]_1^2 [CH_3COOH]_1^2}{[H_2]_2^4 [CO_2]_2^2 [CH_3COOH]_2^2} \quad (1.4)$$

If temperature increases the kinetic constant also increase because the reaction is endothermic (ΔH° has positive sign, Eq. 1.3). So, increments of temperature in the fermentation of glucose enhance VHPR as shown in Table 1.1. Valdez-Vazquez *et al*. (2005) studied the semi-continuous H_2 production at mesophilic and thermophilic conditions, they found that VHPR was 60% greater at thermophilic than mesophilic conditions. They suggested that this behavior is related with the optimal temperature of the enzyme hydrogenase that was between 50 and 70°C and with the optimal conditions of clostridia which is heat resistant and thermophilic bacteria. Wu *et al*. (2005) showed that VHPR was greater at 40°C than at 30°C in batch tests using immobilized sludge in vinyl acetate copolymer. Moreover, fermentation at high temperatures inhibited the activity of hydrogen consumers and destroyed pathogens for residues coming out from anaerobic digestion process, consequently this residues can be used as fertilizer for application on agricultural soil.

A recent work by Nguyen *et al*. (2008) showed the feasibility of using two hyperthermophilic bacteria namely *Thermotoga maritima* and *T. neapolitana.* These anaerobic eubacteria were able to produce hydrogen at temperatures up to 75 – 80°C and were capable to use a broad range of substrates such as glucose and cellulose. The reported HMY and VHPR are below the obtained for most mesophilic fermentations, nevertheless the hyperthermophilic microorganisms are very attractive for biotechnology due to their highly thermostable enzymes which make them of enormous potential application (Table 1.1).

On the other hand, too high temperatures induce proteins thermal denaturation affecting the microorganism activity. Lee *et al*. (2006) studied the effect of temperature on hydrogen production in a carrier induced granular sludge bed bioreactor (CIGSB) and demonstrated that temperatures around 45°C affected the

biomass growth of granular sludge (Table 1.1). Thus, although thermophilic processes can increase energy costs, in some particular cases it could be considered that them could prevent contamination (e.g. pathogens) and could represent cost savings due to the lack of cooling (Nguyen *et al.* 2008).

The maximum VHPR has been obtained at pH between 5.0 and 6.0 (Table 1.1). However, some researchers reported the optimum pH around 7.0 (Lee *et al.* 2006; Lin and Cheng 2006; Mu and Yu 2006). Various studies have recently pointed out that in order to inhibit methanogenesis and to increase VHPR and stability of continuous systems, moderate acid pH and high temperatures should be applied (Oh *et al.* 2004; Atif *et al.* 2005; Kotsopoulos *et al.* 2006). For the operation of batch systems an optimum initial pH of 5.5 has been reported (Fan *et al.* 2006; Fang *et al.* 2006; Mu *et al.* 2006b; Mu *et al.* 2006c) but there are also some studies reporting optima pH above 7 (Cai *et al.* 2004; Wang *et al.* 2006a; Wang *et al.* 2007; Nguyen *et al.* 2008). Commonly, final pH in anaerobic hydrogen production is around 4-5 regardless of initial pH, this is due to the production of organic acids which diminishes the buffering capacity of the medium resulting in low final pH. Mu *et al.* (2006c) found that VFA (volatile fatty acids) formation was pH dependant and when pH was decreased from 4.2 to a lower level or increased to a higher level, the fermentative pathway would shift from butyrate to caporate or ethanol. It is well documented that high VHPR is associated with butyrate and acetate production and inhibition of hydrogen production has been demonstrated with propionic acid formation (Oh *et al.* 2004; Wang *et al.* 2006b). Therefore, control of pH at the optimum level is required. Initial pH also influences the extent of lag phase in batch hydrogen production. Some studies reported that low pH of 4-4.5 causes longer lag periods and high initial pH levels such as 9 decrease lag time; however, lower the yield of hydrogen production (Cai *et al.* 2004).

Taking into account the mineral salt composition (MSC) effects on hydrogen production, Lin and Lay (2005) found an optimal MSC by using the Taguchi fractional design method. The VHPR obtained with the optimal MSC was 66% greater than the value obtained with conventional acidogenic nutrient formulation.

Also, they found that magnesium, sodium, zinc and iron were important trace metals affecting VHPR with magnesium being the most important nutrient factor that produced a notorious effect on VHPR. Other authors studied the effect of sulfate and ammonia concentrations on VHPR in continuous stirred tank reactor (CSTR) systems with sucrose and glucose as substrate (Lin and Chen 2006; Salerno *et al.* 2006). Increasing sulfate concentration from 0 to 3000 mg/l at pH 6.7 reduces the activity of H_2 producing microorganism and can stimulate changes on metabolic pathway from butyrate to ethanol fermentation, but at pH 5.5 with sulfate concentration of 3000 mg/l VHPR increased 40% (Lin and Chen 2006). A decrease of 40% on VHPR and hydrogen yield was obtained at ammonia concentrations of 7.8 g N/l comparing with the value obtained at 0.8 g N/l which was the optimal ammonia concentration for hydrogen production (Salerno *et al.* 2006).

As it is well know, hydrogen and VFA can be produced during exponential and stationary growth phases. Various authors demonstrated that VFA and hydrogen production are maximal during the exponential growth phase, and alcohols production occurs in stationary phase decreasing hydrogen production (Lay 2000; Levin *et al.* 2004). Hydrogen production in continuous and discontinuous systems is dependant on both biomass and substrate concentrations. Yoshida *et al*. (2005) studied the effect of biomass concentration on hydrogen production; they found that increasing cell density from 0.41 g/l to 74 g/l the specific hydrogen production rate (SHPR) increased 67 %.

The maximum HMY of 4 mol/mol has not been reached because in nature fermentation serves to produce biomass and not hydrogen. Also, hydrogen production by fermenting cells "*is considered*" as wasted energy by the bacteria and therefore elaborated machineries exist to recycle the evolved hydrogen in these cells. Additionally, the HMY is negatively affected by the partial pressure of the product. Theoretically, up to 33% of the electrons in hexose sugars can go to hydrogen when growth is neglected and at least 66% of the substrate electrons remain on VFA production.

When continuous systems are revised the most appropriate parameter to analyze is the organic loading rate (OLR) which is function of substrate concentration ([S]) and the hydraulic retention time (HRT):

$$OLR = \frac{[S]}{HRT} \qquad (1.5)$$

VHPR increase when substrate concentration increase and HRT diminishes. However, at low HRT microbial wash-out might be greater than microbial growth. Thus, the low concentration of biomass in a CSTR led to the decrease of VFA production and the increase of pH. High substrate concentration would result in the accumulation of VFA and a fall of pH in the reactor, and even inhibit the growth of hydrogen producing bacteria. In addition, when substrate concentration increases in batch systems the partial pressure of hydrogen rises and the microorganism would switch to alcohol production, thus inhibiting hydrogen production (Fan *et al.* 2006). Park *et al*. (2005) showed that chemical scavenging of the CO_2 increased hydrogen production by 43% in batch glucose fermentation. It has been demonstrated that applying vacuum, gas sparging or CO_2 scavenging may all be effective methods of increase hydrogen production (Levin *et al.* 2004; Valdez-Vazquez *et al.* 2006).

For most of the results presented on Table 1.1, optimal HRT between 0.5 to 12 h and substrate concentrations around 20 g/l can be found. Chang and Lin (2004) studied the effect of HRT on HMY, VHPR and SHPR in an upflow anaerobic sludge blanket (UASB) reactor fed with sucrose. They found that HMY was independent on HRT between 8-20 h and VHPR and SHPR were dependent on HRT. Oh *et al*. (2004) showed that decreasing HRT at 4 h and increasing substrate concentration from 6.86 to 20.6 g/l resulted in an increase of lactate concentration reducing VHPR. Show *et al*. (2007) used a CSTR seeded with anaerobic granular sludge to produce Bio-H_2 from glucose at different HRTs (0.25 – 2h) and OLRs (2.5 – 20 g glucose/l/h); best VHPR and HMY were attained at the shortest HRT and

highest OLR. With their results, the authors proposed the use of granular sludge based-CSTR as a promising biosystem for efficient Bio-H_2 production.

The reactor configuration is another parameter that affects VHPR as is shown on Table 1.1. The VHPR varied from different reactor configurations, having the best performance with immobilized cell bioreactors. High cell densities are needed to maximize hydrogen production rates. Therefore, major improvements are expected in systems with biomass retention, e.g. by immobilized cells, under nutrient limitations operating in a continuous mode. Oh *et al*. (2004) studied hydrogen production in a trickling biofilter (TBR) with glucose as substrate and found a maximum VHPR of 37.5 mmol/l/h. TBR could maintain a high density of 18-24 g VSS/l which is higher than other immobilized systems and significantly higher than most suspension reactors (CSTR). More else, packed bed reactors maintain a lower gas hold up since biogas is removed more efficiently. This alleviates the inhibition by hydrogen and severe channeling of liquid and gas flows in the reactor. A fluidized bed reactor (FBR) and a draft tube bed reactor (DTFBR) systems with effluent recycle and immobilized cell were studied for the production of hydrogen using sucrose as substrate (Lin *et al.* 2006a; Wu *et al.* 2006a). A VHPR of 95.23 mmol/l/h was obtained with DTFBR which was 50% greater than the one obtained with FBR. However, when using suspended immobilized-cell systems it could be important to consider the biogas accumulation and excessive gas hold up produced. Other work with a FBR was reported by Koskinen *et al*. (2007). During the continuous operation of the FBR, the VHPR peaked at 28 mmol/l/h with a HMY of 1.9 mol H_2/mol glucose. In their work the authors studied the dynamics of the microflora and concluded that changes in the community structure were closely related to the productivity and efficiency of the process.

The maximum VHPR that have been obtained up to now is 612.5 mmol/l/h by using a CSTR containing silicone immobilized sludge (10% v/v) and sucrose as substrate (Wu *et al.* 2006a). This VHPR is at least six times greater than any other VHPR published (Table 1.1). This work demonstrated that an appropriate process design containing simultaneously granular, immobilized and freely suspended

sludge had a major contribution on hydrogen production than other studies presented on Table 1.1. Also, in that study HMY was 3.86 mol/mol which is similar to the highest yield of 3.88 mol/mol obtained in a carrier induced granular sludge (CIGSB) system for fermentation of sucrose (Lee *et al.* 2006). Ren *et al*. (2006) showed the adequate performance of a pilot scale CSTR to produce hydrogen from molasses. However, CSTR problems could be present when high dilution rates are used and the system is unstable and wash-out of the cells is often experienced. To prevent wash-out, many strategies could be implemented. Lee *et al*. (2007) attached a hollow-fiber microfiltration module to a CSTR to retain cells in the system even at a HRT of 1h, with efficient Bio-H_2 production from fructose, sucrose and glucose (Table 1.1).

Gavala *et al*. (2006) obtained similar VHPR in CSTR and UASB reactors for glucose fermentation. But the HMY obtained in the CSTR was greater than the obtained with UASB. Overall, analogous VHPR are obtained by using UASB and CSTR systems (Table 1.1) (Chang and Lin 2004; Gavala *et al.* 2006; Lin and Chen 2006; Zhang *et al.* 2006).

Kim *et al*. (2006a) showed that the use of a membrane bioreactor (MBR) for hydrogen production allows advantages such as high cell density, high organic removal rates, and high quality effluent by the membrane and easy control of pH and temperature. They used a MBR system with glucose as substrate and found a maximum VHPR of 71.4 mmol/l/h which is greater than the values obtained with CSTR and UASB and comparable to immobilized cell systems. A MBR was also used to achieve a maximum VHPR of 112 mmol/l/h with fructose as substrate (Lee *et al.* 2007). However, the use of MBR system has been limited at laboratory scale because high capital cost and this technology has not been demonstrated at full-scale. One can point out that whereas immobilized cells and MBR systems shows the highest VHPR, it is not easy to compare various reactor configurations and draw a conclusion that a specific one is better than the others, even under a specific set of conditions.

1.5 Scope and structure of the thesis

The aim of this thesis was to develop a Bio-H_2 producing system using mixed cultures at lab scale. First of all, a survey in the literature regarding fermentative Bio-H_2 production was carried out and presented in this chapter. Subsequent chapters are introduced as follows:

In Chapter 2 a response surface methodology was used in batch experiments to study the effect of initial pH (3.88 – 8.12) and initial substrate concentration (0.86 – 29.14 g/l) on both HMY and VHPR. Lactose, cheese whey powder (CWP) and glucose were used as substrates. Best conditions (initial substrate concentration and initial pH) for each substrate are presented with the potential application of the findings.

In Chapter 3, batch experiments with CWP as substrate were performed to assess the total hydrogen production (H_{max}), VHPR, maximum lactose consumption (S_{max}), maximum lactose consumption rate ($R_{max,S}$), HMY and microbial community present using two mineral media formulation.

In Chapter 4, continuous Bio-H_2 production from CWP was performed in order to improve the published VHPR using this substrate, by an appropriate selection of operation parameters such as HRT and OLR.

Finally, in Chapter 5 some strategies such as novel metabolic engineering in clostridia, along with two-stage systems to increase overall hydrogen yields are discussed and presented as trends to follow in the Bio-H_2 field. Furthermore, the potential impacts from the results of this work together with final recommendations are mentioned as well.

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Chapter 2

Fermentative hydrogen production in batch experiments using lactose, cheese whey and glucose: Influence of initial substrate concentration and pH

Summary

Biologically-produced hydrogen using biomass and mixed bacterial cultures is one approach to generate renewable H_2. Response surface methodology (RSM) was used to study the effect of initial pH (3.88 – 8.12) and initial substrate concentration (0.86 – 29.14 g/l) on both hydrogen molar yield (HMY) and volumetric H_2 production rate (VHPR). Lactose, cheese whey powder (CWP) and glucose were used as substrates and heat-treated anaerobic granular sludge as inoculum. For lactose, 3.6 mol H_2/mol lactose and 5.6 mmol H_2/l/h were found at pH 7.5 and 5 g lactose/l. CWP yielded 3.1 mol H_2/mol lactose at pH 6 and 15 g CWP/l while 8.1 mmol H_2/l/h were attained at pH 7.5 and 25 g CWP/l. Glucose yielded 1.46 mol H_2/mol substrate (pH 7.5, 5 g glucose/l), with a VHPR of 8.9 mmol H_2/l/h, at pH 8.12 and 15 g glucose/l. Acetic and butyric acids were the main organic metabolites detected. HMY and VHPR obtained in this study were found at initial pH above the reported optimum pH value for hydrogen production. These findings could be of significance when alkaline pretreatments are performed on organic feedstock by eliminating the need to lower the pH to acidic levels before fermentation start-up.

Davila-Vazquez G., Alatriste-Mondragón F., de León-Rodriguez A. & Razo-Flores E. (2008). Fermentative hydrogen production in batch experiments using lactose, cheese whey and glucose: Influence of initial substrate concentration and pH. *Int. J. Hydrogen Energy.* **33**, 4989-4997.

2.1 Introduction

Hydrogen gas (H_2) is considered a valuable energy carrier, and an alternative to fossil fuels, since its combustion or utilization in fuel cells to produce electricity only yield water and heat as by-products (Claassen *et al.* 1999). Under anaerobic conditions, a wide variety of microorganisms evolve H_2 (Bio-H_2) from organic matter (Nandi and Sengupta 1998). Among the known biochemical routes to produce H_2, fermentative hydrogen production is a promising one (Redwood and Macaskie 2006).

Mixed anaerobic microbial populations from different sources (soil, sediment, compost, aerobic and anaerobic sludges) have been studied as inocula for H_2 production (Davila-Vazquez *et al*. 2008). In these processes, most of the microbial populations were treated, before inoculation, with heat or acid to select for biohydrogen producing communities. According to a review by Kraemer and Bagley (2007), heat treatment has been a common method for killing methanogens (hydrogen-consuming microorganisms), leaving behind sporogenic bacteria such as *Clostridium*, *Bacillus* and *Thermoanaerobacterium*. However, in some cases heat treatment was not effective in selecting only H_2-producing microorganisms because few hydrogen-consuming bacteria, such as lactic or propionic acid producers and acetogens, could survive.

There are a number of reports in which pure cultures or microbial populations have been used for biohydrogen production using sugars or complex substrates such as organic wastes (Kapdan and Kargi 2006). Among the sugars used extensively are glucose, sucrose and to a lesser extent lactose. Due to thermodynamic constraints, a maximum of 4 mol of H_2 can be produced from 1 mol of glucose when acetic acid is the main organic product. This yield is lower ($\leq$ 2 mol H_2/mol glucose) when more reduced metabolites, such as butyric acid, are also produced (Hallenbeck and Benemann 2002; Angenent *et al.* 2004).

Glucose and sucrose are of interest as model substrates due to their easy biodegradability. On the other hand lactose is also an interesting model substrate because it is present in wastes or by-products from the dairy industry. One lactose-

containing by-product is cheese whey, that represents around 85-90% of the total volume of processed milk and it is a potential substrate for fermentative processes (De León-Rodríguez *et al.* 2006). Dry cheese whey powder (CWP) is obtained from cheese whey by spray or drum drying with a cost of around 0.30 USD/kg CWP (Ozmihci and Kargi 2007). Therefore, CWP represents a cheap concentrated source of lactose (> 61 % w/w).

One of the approaches used to study the effect of parameters such as temperature, pH, substrate concentration and others, as independent variables, is by using an *a priori* statistical experimental design along with the analysis of the results using response surface methodology (RSM). There are few reports in the literature in which this approach has been used to find optimal conditions for Bio-H_2 production using starch (Lay 2000; Wang *et al.* 2007), sucrose (Van Ginkel *et al.* 2001; Fan *et al.* 2004; Mu *et al.* 2006b) and food waste (Kim *et al.* 2004).

Thus, the aim of this work was to study the kinetics of hydrogen production using an enriched mixed population and glucose, lactose or CWP as carbon substrates in batch experiments. The effect of different levels of initial substrate concentration ($[S_0]$) and initial pH on both, the volumetric hydrogen production rate (VHPR) and hydrogen molar yield (HMY), was evaluated using a central composite experimental design and RSM. The concentration of fermentation end products was also measured.

2.2 Materials and methods

2.2.1 Inoculum and substrate

Anaerobic granular sludge from a full-scale up-flow anaerobic sludge blanket (UASB) reactor was used as inoculum for biohydrogen production. The UASB reactor treats wastewater from a candy factory in San Luis Potosí, México. The granular sludge was washed with three volumes of tap water and then boiled for 40 minutes to inactivate methanogenic microflora and stored at 4ºC before use. Glucose and lactose were obtained from Sigma-Aldrich (Minnesota, USA), and CWP was purchased from Land O´Lakes Inc. (Minnesota, USA). The lactose

content of CWP was 77% with 11% protein (w/w). All chemicals were purchased as reagent grade.

2.2.2 Biohydrogen production experiments

Batch experiments were conducted in 120 ml serum vials with a working volume of 80 ml. Calculated masses of substrate: 4.5 g volatile suspended solids (VSS)/l of inoculum and 1 ml of mineral medium modified from Van Ginkel *et al*. (2001) were added to each vial. One liter of this medium contained: 200 g NH_4HCO_3, 100 g KH_2PO_4, 10 g $MgSO_4.7H_2O$, 1.0 g NaCl, 1.0 g $Na_2MoO_4{\cdot}2H_2O$, 1.0 g $CaCl_2.{\cdot}2H_2O$, 1.5 g $MnSO_4{\cdot}7H_2O$, 0.278 g $FeCl_2$, 0.24 g $CoCl_2{\cdot}8H_2O$, 0.12 g $NiCl_2{\cdot}6H_2O$ and 0.06 g $ZnCl_2$. Vials were filled to the working volume with deionized water and pH was adjusted using HCl 10N or NaOH 2M. After sealing the vials with Wheaton rubber septum stoppers and aluminum rings, the headspace was purged with nitrogen gas for 15 seconds. Finally, for glucose and lactose experiments the vials were incubated under static conditions and hand-shaken before the headspace gas composition was measured. For CWP, the bottles were placed in a horizontal shaker at 150 rpm in an incubation room. All experiments were carried out at 37°C. Gas production and composition in the headspace were measured periodically as described in analytical methods.

2.2.3 Analytical methods

Gas production was measured using a liquid-displacement device filled with water (pH = 2). Hydrogen cumulative production was calculated for each vial considering the headspace composition and the volume of gas released at each time interval, using a mass balance equation (Van Ginkel *et al.* 2005; Argun *et al.* 2008). All gas volumes are reported at 1 atm and 25°C.

H_2, CO_2 and CH_4 were measured with a 1.0 ml Pressure-Lok® syringe (Valco Instruments, Houston, Texas, USA) by comparing a 300 µl sample with high purity standards (Alltech, Deerfield, Illinois, USA) using a gas chromatograph (GC,

6890N Network GC System, Agilent Technologies, Waldbronn, Germany) equipped with a thermal conductivity detector. The column used was a Hayesep D (Alltech, Deerfield, Illinois, USA) with the following dimensions: 10' x 1/8" x 0.085". Temperatures of the injection port, oven and the detector were 250, 60 and 250°C, respectively. Nitrogen was used as carrier gas with a flow-rate of 12 ml/min.

At the end of each experiment, 3 ml of liquid samples were taken and 60 µl of $HgCl_2$ (16 g/l) were added before centrifugation at 6610*g* for 15 minutes to minimize microorganisms activity (Park *et al.* 2005). The supernatant was diluted and filtered through a 0.22 µm membrane (Millipore, Bedford, Massachusetts, USA). Remaining substrate and fermentation end products, such as formic, acetic, propionic and butyric acids (VFA) were analyzed in the filtrate by capillary electrophoresis in the same run (Soga and Ross 1999). Analytes were quantified by comparison with high purity standards. For this purpose a capillary electrophoresis system (Agilent 1600A, Waldbronn, Germany) was used with a basic anion buffer (Agilent, pH = 12.1) and a fused silica capillary column (Agilent, id = 50 µm, L = 80.5 cm, effective length = 72 cm). Temperature and voltage were 20°C and –30 kV, respectively. The samples were injected with a pressure of 300 mbar for 6 s. Detection was carried out with indirect UV detection using a diode-array detector. The signal wavelength was set at 350 nm with a reference at 230 nm. A buffer flush for 4 min at 1 bar was performed prior to each run. Solvents such as acetone, ethanol, propanol and butanol were analyzed by injecting a 1 µl sample in a gas chromatograph 6890N equipped with an auto-sampler 7863 (Agilent, Wilmington, USA) and a capillary column HP-Innowax (30 m x 0.25 mm i.d. x 0.25 m film thickness; Agilent, Wilmington, USA). Helium was used as carrier gas at a flow rate of 1.5 ml/min. Temperatures for the injector and flame ionization detector (FID) were 220 and 250°C, respectively. The solvents analyses were performed with a split ratio of 1:0.1 and a temperature program of 35°C for 2

min, increased at 10°C/min to 80°C, and maintained at this temperature to a final time of 15 min. VSS were analyzed according to the Standard Methods (APHA *et al.* 1998).

2.2.4 Experimental design and data analysis

Once cumulative hydrogen production was calculated from experimental data, a modified Gompertz equation was used to fit the kinetics of biohydrogen production using KaleidaGraph 4.0 (Synergy software). This equation has been widely used to model gas production data (Lay 2001; Khanal *et al.* 2004; Lin and Lay 2005; Mu *et al.* 2007):

$$H(t) = H_{\max} \bullet \exp\left\{-\exp\left[\frac{2.71828 \bullet R_{\max}}{H_{\max}}(\lambda - t) + 1\right]\right\} \qquad (2.1)$$

where $H(t)$ (ml) is the total amount of hydrogen produced at culture time t (h); $H_{\max}$ (ml) is the maximal amount of hydrogen produced. $R_{\max}$ (ml/h) is the maximum hydrogen production rate; λ (h) is the lag time before exponential hydrogen production. HMY and VHPR were defined as response variables. HMY was calculated from H_{max} and defined as mol H_2/mol consumed substrate. VHPR was obtained from R_{max} standardized to the working volume (mmol H_2/l/h). As one aim of this work was to evaluate the effect of initial substrate concentration [S_0] and initial pH on both HMY and VHPR, experiments were conducted following a central composite experimental design (Table 2.1). As can be seen from Table 2.1, [S_0] varied from 5 to 25 g/l with a central value of 15 g/l and axial points at 0.86 and 29.14 g/l, while pH varied from 4.5 to 7.5 with a central point at 6.0 and axial points at 3.88 and 8.12. The central point was a triplicate run and the experimental design was run in duplicate for data analysis.

Table 2.1 Central composite experimental design.

Run	Real values		Coded values	
	X_1	X_2	x_1	x_2
1	5.0	4.5	-1	-1
2	5.0	7.5	-1	1
3	25.0	4.5	1	-1
4	25.0	7.5	1	1
5	15.0	3.88	0	-1.414
6	15.0	8.12	0	1.414
7	0.86	6.0	-1.414	0
8	29.14	6.0	1.414	0
9	15.0	6.0	0	0
10	15.0	6.0	0	0
11	15.0	6.0	0	0

To perform the fitting of the experimental design for both initial pH and [S_0] levels, coded variables were used according to Eq. (2.2).

$$x_i = \frac{X_i - X_i^*}{\Delta X_i} \qquad (2.2)$$

where x_i is the coded value of the ith test variable, X_i is the uncoded or normal value of the ith test variable, X_i^* is the uncoded value of the ith test variable at the center point, and ΔX_i is the step change value in the normal variables (Lay *et al.* 1999; Mu *et al.* 2006b). X_1 (g/l) and x_1 correspond to the real and coded values for [S_0] respectively while X_2 and x_2 correspond to the real and coded values for pH. The step change values for [S_0] and pH were set at 10.0 and 1.5, respectively. Experimental results obtained with this *a priori* design were analyzed using RSM due to its suitability in finding optimal values for the response variables as a function of experimental treatments.

The response variables (HMY and VHPR) were fitted using a polynomial quadratic equation to correlate each response variable to the independent variables ([S_0] and pH). The mathematical form of each quadratic equation is described in Eq. 2.3 :

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} x_i x_j \qquad (2.3)$$

where x_i are the independent variables, which could have an influence on the response variable y. β_0 is the constant of the model, β_i the *i*th linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the coefficient for the *ij*th interaction. RSM analyses were made using three-dimensional response surface plots constructed for each polynomial equation with Statgraphics Plus 5.0 software (Statistical Graphics Corp.).

2.3. Results and discussion

2.3.1 Kinetics of hydrogen production with lactose, cheese whey and glucose

As an example, kinetic experimental data obtained from each treatment during 46 hours of the experiment using CWP are shown in Figure 2.1a. After a lag period that ranged from 8 to 15 hours depending on the substrate and treatment, biohydrogen production started at different rates and to a different extent. For the three substrates, the hydrogen content in the headspace peaked at around 50 - 55 %, with 50 - 45 % CO_2. Methane was detected (<10 %) when CWP was used as substrate in all treatments. The profile of hydrogen content in the vial headspace, at central point conditions for glucose conversion to biohydrogen is shown in Figure 2.1b. For all substrates, Eq. 2.1 adequately described biohydrogen production showing regression coefficients (R^2) above 0.87 (Table 2.2).

The parameters H_{max} and R_{max} obtained from fitting Eq. 2.1 to the cumulative Bio-H_2 production data for each substrate are shown in Table 2.2. These parameters were used to calculate the response variables HMY and VHPR. Response variables were analyzed using RSM. Because R_{max} is not normalized to the reactor volume, it was not possible to make comparisons between the performances of different reactors. Therefore, VHPR was used as response variable instead of R_{max}. Furthermore, the use of standardized units for hydrogen production rates has been proposed by Levin *et al*. (2004) in order to facilitate the sizing of a bioreactor that

would be needed to supply hydrogen to a specific fuel cell for electricity generation.

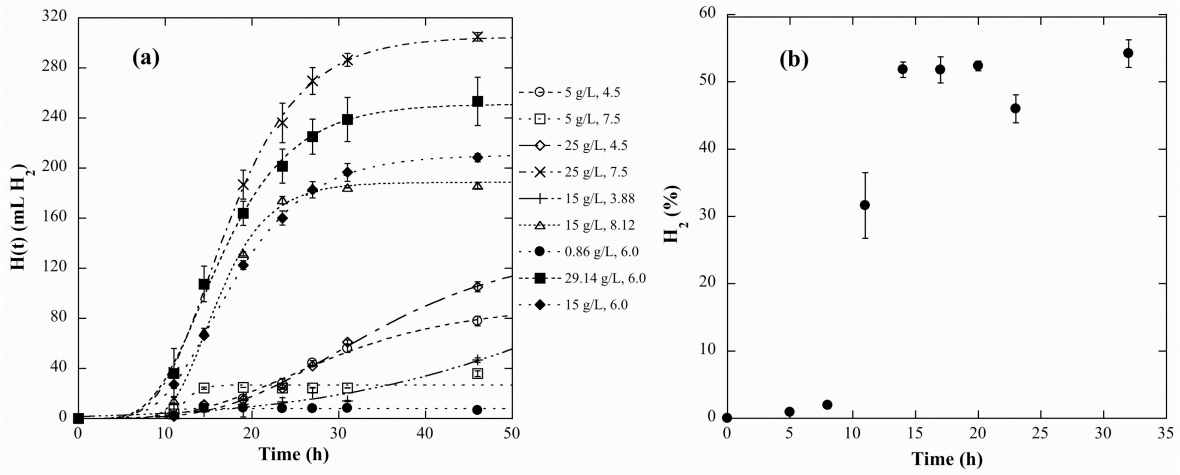

Figure 2.1. (a) Cumulative production of Bio-H_2 from CWP. Modified Gompertz equation fit for each treatment is shown in broken lines. (b) Hydrogen content in the biogas during the batch experiment with glucose at central point conditions (15 g glucose/l, pH 6.0). Standard deviations (SD) are presented as error bars.

2.3.2 Response surface analysis of HMY and VHPR

To examine the behavior of both HMY and VHPR, surface response plots were built. The following quadratic equations were used to draw these plots for HMY and VHPR:

Lactose

$$\textbf{HMY} = 2.609 - 0.568x_1 + 0.415x_2 + 0.036x_1^2 + 0.108x_1x_2 - 0.166x_2^2 \quad (2.4)$$

$$\textbf{VHPR} = 2.691 - 0.074x_1 + 1.606x_2 - 0.183x_1^2 - 0.180x_1x_2 + 0.429x_2^2 \quad (2.5)$$

CWP

$$\textbf{HMY} = 2.764 - 0.285x_1 + 0.064x_2 + 0.090x_1^2 + 0.508x_1\,x_2 - 0.822x_2^2 \quad (2.6)$$

$$\textbf{VHPR} = 5.318 + 1.183x_1 + 2.305x_2 - 0.323x_1^2 + 1.076x_1x_2 - 0.945x_2^2 \quad (2.7)$$

Table 2.2 Adjusted H_{max} and R_{max} for lactose, CWP and glucose.

Initial conditions: [S_0], pH	Lactose			CWP			Glucose		
	H_{max} (ml H_2)	R_{max} (ml H_2/h)	R^2	H_{max} (ml H_2)	R_{max} (ml H_2/h)	R^2	H_{max} (ml H_2)	R_{max} (ml H_2/h)	R^2
5 g/l, 4.5	62.4 ± 4.6	4.3 ± 1.7	0.99	91.5 ± 14	3.2 ± 0.8	0.99	54.8 ± 8.8	2.9 ± 0.1	0.99
5 g/l, 7.5	105.3 ± 1.2	13.6 ± 1.2	0.99	26.6 ± 0.2	8.3 ± 0.9	0.87	96.5 ± 4.3	19.6 ± 1.9	0.99
25 g/l, 4.5	32.6 ± 1.2	3.4 ± 1.6	0.97	140 ± 12.2	4.0 ± 0.1	0.99	48.8 ± 2.7	3.4 ± 0.6	0.98
25 g/l, 7.5	171.8 ± 1.4	10.9 ± 1.9	0.99	305.2 ± 3.1	19.6 ± 3.7	0.99	173.2 ± 4.5	21.2 ± 1.0	0.99
15 g/l, 3.88	42.8 ± 0.6	2.6 ± 0.3	0.99	29.8 ± 6.7	0.9 ± 0.1	0.91	34.9 ± 7.0	2.9 ± 0.6	0.98
15 g/l, 8.12	165.1 ± 7.9	12.8 ± 3.4	0.99	188.7 ± 0.5	17.9 ± 0.7	0.99	176.8 ± 15.2	21.6 ± 2.1	0.99
0.86 g/l, 6.0	20 ± 0.1	4.0 ± 2.1	0.90	7.0 ± 0.3	8.5 ± 0.1	0.93	9.0 ± 0.2	18.5 ± 1.7	0.98
29.14 g/l, 6.0	112.5 ± 11.6	5.5 ± 0.2	0.99	251.2 ± 19	16.3 ± 1.4	0.99	95.9 ± 1.4	9.7 ± 1.1	0.99
15 g/l, 6.0	108.7 ± 3.1	6.5 ± 0.1	0.99	210 ± 2.1	12.6 ± 1.5	0.99	101.1 ± 1.3	10.2 ± 0.5	0.99
15 g/l, 6.0	124.4 ± 0.4	6.3 ± 0.2	0.99	211 ± 2.7	13.2 ± 1.9	0.99	104.1 ± 3.9	11.9 ± 2.5	0.99
15 g/l, 6.0	116.1 ± 12.2	6.8 ± 1.1	0.99	197 ± 3.9	13.1 ± 0.1	0.99	115.7 ± 8.0	11.3 ± 0.6	0.99

Note: Data are given as mean values ± SD, n = 2.

Glucose

$$\textbf{HMY} = 1.319 - 0.086x_1 + 0.179x_2 - 0.181x_1^2 + 0.098x_1x_2 - 0.057x_2^2 \quad (2.8)$$

$$\textbf{VHPR} = 4.609 - 0.533x_1 + 3.141x_2 + 0.453x_1^2 + 0.118x_1x_2 + 0.075x_2^2 \quad (2.9)$$

In Eqs. 2.4 to 2.9, x_1 and x_2 are the coded variables for $[S_0]$ and pH respectively. Since the p values for each quadratic model equation (Eqs. 2.4 – 2.9) were below 0.005, thus these equations adequately described the behavior of experimental data. The regression coefficients (R^2) for HMY were 0.78, 0.63 and 0.63 for lactose, CWP, and glucose respectively, while for VHPR, R^2 were 0.83, 0.94 and 0.91 for lactose, CWP and glucose respectively.

Table 2.3 summarizes the highest HMY and VHPR predicted by the response surface analysis. Also, the parameters with the most significant effects on HMY and VHPR are indicated in Table 2.3.

2.3.3 Lactose and CWP

The analysis of variance for HMY using lactose as substrate showed that both initial lactose concentration and initial pH had a significant effect on HMY (Table 2.3). The highest experimental HMY was 3.6 mol H_2/mol lactose and was achieved under the same conditions as glucose. This yield represents 45% of the theoretical maximum of 8 mol H_2/mol lactose consumed (Collet *et al.* 2004). According to Fig. 2.2a, predicted by the quadratic model (Eq. 2.4), there is a tendency for HMY to increase as both initial lactose concentration decreases and initial pH increases. For VHPR, the effect of initial pH was greater than the initial lactose concentration. It was reflected in a low p value for initial pH and a higher figure for $[S_0]$ (p = 0.65). The highest experimental VHPR obtained was 5.6 mmol H_2/l/h under the same initial conditions as HMY (Table 2.3).

Table 2.3 Summary of significant effects from the ANOVA analysis and experimental conditions for best HMY and VHPR.

Substrate	Significant effects (p value < 0.10)		Highest HMY (mol H_2/mol substrate) and VHPR (mmol H_2/l/h) obtained, and conditions at which they were found	
	HMY	VHPR	HMY	VHPR
Lactose	pH (0.0005) $[S_0]$ (0.0001)	pH (0.0001)	3.6 ± 0.03 pH = 7.5 $[S_0]$ = 5 g/l	5.6 ± 0.48 pH = 7.5 $[S_0]$ = 5 g/l
CWP	pH^2 (0.0032) $[S_0]$ (0.0394)	pH (0.0001) $[S_0]$ (0.0001) pH•$[S_0]$ (0.0004) pH^2 (0.0002)	3.1 ± 0.04 (mol H_2/mol lactose) pH = 6.0 $[S_0]$ = 15 g/l	8.1 ± 1.5 pH = 7.5 $[S_0]$ = 25 g/l
Glucose	pH (0.0018) $[S_0]^2$ (0.0108)	pH (0.0001) $[S_0]$ (0.0557)	1.46 ± 0.07 pH = 7.5 $[S_0]$ = 5 g/l	8.9 ± 0.87 pH = 8.12 $[S_0]$ = 15 g/l

Figure 2.2c shows that the maximum HMY using CWP as substrate was found at pH 6.0. At that pH, HMY is barely sensitive to changes in $[S_0]$. That is to say, HMY has slightly higher values at low $[S_0]$ than at higher $[S_0]$. This is because the HMY response surface resembles a saddle (Oehlert 2000), with a zone at pH 6.0 in which HMY values decrease with increasing $[S_0]$ and then increase again but at a lower value than the initial one. The significant effects were $[S_0]$ and pH^2 (Table 2.3). Using CWP the experimental values for HMY at pH 6.0 decreased from 5.9 to 2.8 mol H_2/mol lactose as $[S_0]$ increased from 0.86 to 29.14 g CWP/l. Although 5.9 mol H_2/mol lactose is a very high yield, the low H_{max} (7 ml H_2) found with this treatment (Table 2.2, CWP: 0.86 g/l, pH 6.0) makes it impractical and therefore this condition is not reported as maximum in Table 2.3.

For VHPR, the effects of $[S_0]$, pH, the interaction ($[S_0]$·pH) and pH^2 were all significant (Table 2.3). A clear trend is observed in Fig. 2.2d with higher VHPR

values for both, higher pH and [S_0]. Therefore, it may be possible to find an optimal VHPR value by performing experiments at [S_0] higher than 30 g CWP/l and pH above 8.12. The highest VHPR found using CWP was 8.1 mmol H_2/l/h achieved at near neutral pH and under higher concentration than glucose and lactose (Table 2.3).

Ferchichi *et al*. (2005a) used diluted crude CW (*ca*. 41.1 g lactose/l) as carbon substrate and a pure *Clostridium* strain, and studied the influence of different initial pH (5 – 10) on the hydrogen production rate and yield in batch experiments. The authors found that HMY peaked at pH 6.0 with a value of 2.7 mol H_2/mol lactose and the VHPR was 9.4 mmol H_2/l/h. In another study with a *Clostridium* strain, Collet *et al*. (2004) used lactose (10 g/l) as carbon substrate in continuous culture obtaining HMY from 2.1 to 3 mol H_2/mol lactose and VHPR around 2.5 mmol H_2/l/h depending on the dilution rate at pH 7. Recently, Yang *et al*. (2007) performed both batch and continuous experiments using cheese whey permeate powder as substrate. In batch experiments with initial pH that ranged from 7.28 – 7.33 the authors obtained yields between 8 – 10 mM/g COD fed, achieved with anaerobic sludge as inoculum and uncontrolled pH. However, the hydrogen production rate was not reported for batch experiments. Best results were found by the authors in the continuous system (HRT = 24 h, organic loading rate = 12 g COD/l/d), at controlled pH (4 – 5) attaining yields between 1.8 – 2.3 mM/g COD and volumetric production rates up to 18.75 ml H_2/l/h.

In the present study, biohydrogen production from the lactose and protein present in cheese whey powder solution resulted in comparable HMY and VHPR values as reported in the previous works mentioned above (Collet *et al.* 2004; Ferchichi *et al.* 2005a). However, in this study the highest VHPR was obtained under more alkaline initial conditions.

2.3.4 Glucose

The analysis of variance showed a stronger effect of initial pH than initial glucose concentration ($[S_0]$, p = 0.1148) on HMY. However, there was a significant effect of the quadratic term $[S_0]^2$ (Table 2.3). From Fig. 2.2e it is clear that a simultaneous increase in $[S_0]$ and decrease in pH lowers HMY.

Some authors had reported HMY and VHPR from batch experiments using glucose as substrate and mixed microbial populations. Among these, Kawagoshi *et al*. (2005) obtained a HMY of 1.4 mol H_2/mol glucose working at an initial glucose concentration of 20 g/l; they found two pH values as suitable initial conditions for biohydrogen production: 6.5 and 7.0. Salerno *et al*. (2006) found the highest HMY (1.17 mol H_2/mol glucose) using low glucose concentration (3.76 g/l) at pH 6.2. Park *et al*. (2005) obtained 2 mol H_2/mol glucose also at pH 6.12. Furthermore, Zheng and Yu (2005) attained a HMY of 1.75 mol H_2/mol glucose at an initial glucose concentration of 10 g/l and pH 6.0. The highest experimental HMY found in this work was 1.46 mol H_2/mol glucose at initial pH above the values reported by Kawagoshi *et al*. (2005) and $[S_0]$ above the 3.76 g/l used by Salerno *et al*. (2006) (Table 2.3).

Regarding the VHPR, the quadratic model (Eq. 2.9) adequately described the variance of the experimental data (R^2 = 0.91). In this case, the effect of initial pH value was the most significant while $[S_0]$ had a lower effect (Table 2.3). As can be seen from Fig. 2.2f there is a clear trend in which an increase in pH, regardless of $[S_0]$, caused an increase in VHPR. The highest experimental rate was 8.9 mmol H_2/l/h (Table 2.3). Results published by other authors report similar figures for VHPR. Cheong and Hansen obtained 8.6 mmol H_2/l/h at a controlled pH of 5.7 and $[S_0]$ ~ 21.3 g/l (2006). Salerno *et al*. (2006) achieved 9 mmol H_2/l/h at pH 6.2 and $[S_0]$ = 3.76 g/l.

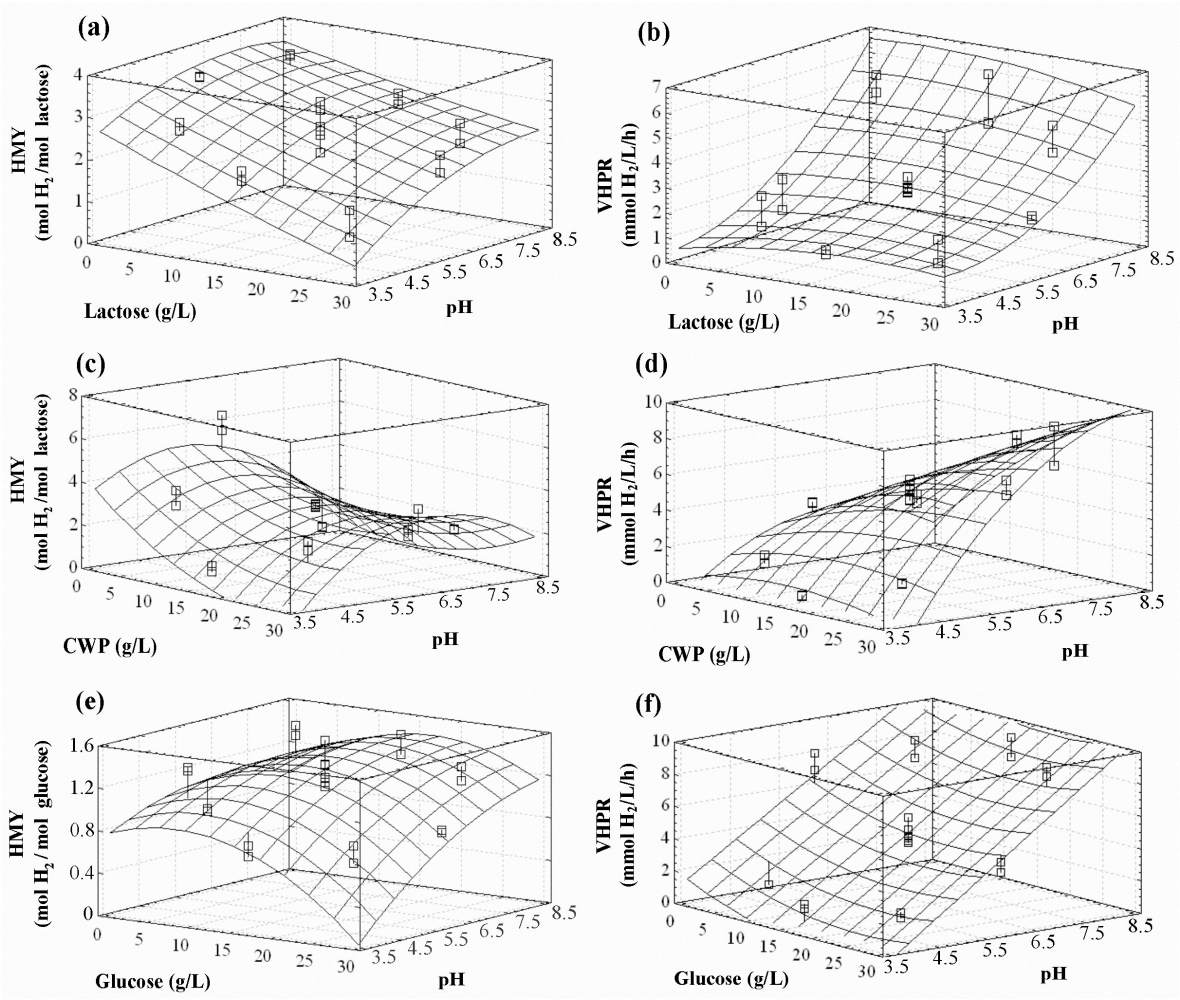

Figure 2.2 Hydrogen molar yield (HMY) and volumetric hydrogen production rate (VHPR) for lactose (a,b), CWP (c,d) and glucose (e, f). Experimental data are shown in squares with the standard error bars.

2.3.5 Analyses of fermentation end products and final pH in culture medium

Analyses of solvents such as acetone, ethanol, propanol and butanol were performed for the treatments with the highest VHPR using CWP as substrate but only ethanol was detected. Ethanol concentrations ranged from 10 to 50 mg/l (0.2 – 1 mM). As these concentrations were very low compared to those obtained of volatile fatty acids, only VFA were further analyzed as major fermentation end products.

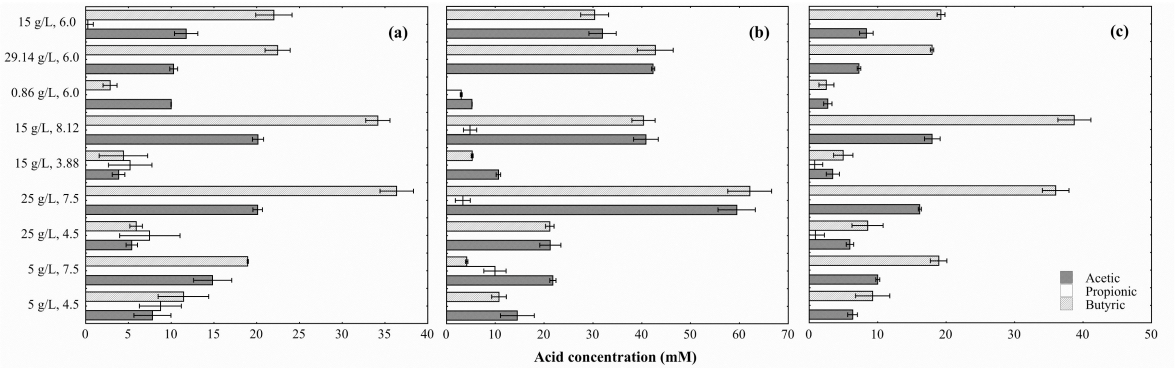

Figure 2.3 Total VFA concentrations (acetic, propionic and butyric acids) at the end of batch experiments for: (a) Lactose, (b) Cheese whey powder (CWP) and (c) Glucose.

The VFA found in the culture by the end of each experiment are shown in Figure 2.3. For the three substrates used, the acetic and butyric acids were the main metabolites (up to 65 mM) while propionic acid was found to a lesser extent (up to 10 mM). As a result of VFA production, pH decreased to acidic conditions for all substrates (Table 2.4). Final pH values were similar for glucose and lactose, but were higher for CWP. The reason for this is likely due to the anaerobic digestion of protein present in CWP which produces ammonia and, therefore, increases pH (de Vrije and Claassen 2003).

When lactose was used (Fig. 2.3a), the experimental conditions that yielded the highest H_{max} (Table 2.2) also resulted in high butyric acid concentration (*ca*. 35 mM). For these conditions acetic acid content was around 20 mM. At both, central point (15 g lactose/l, pH 6.0) and at (29 g lactose/l, pH 6.0) acid production was similar with acetic acid around 10 mM and butyric acid around 22 mM. Propionic acid was detected (below 10 mM) in treatments with initial pH ≤ 4.5.

Table 2.4 Final pH measured for lactose, CWP and glucose.

Initial conditions: [S_0], pH	Final pH		
	Lactose	CWP	Glucose
5 g/l, 4.5	3.82 ± 0.13	4.36 ± 0.04	3.90 ± 0.04
5 g/l, 7.5	4.7 ± 0.07	6.06 ± 0.05	4.69 ± 0.09
25 g/l, 4.5	3.69 ± 0.13	4.38 ± 0.01	3.69 ± 0.18
25 g/l, 7.5	3.89 ± 0.10	4.86 ± 0.12	3.86 ± 0.12
15 g/l, 3.88	3.79 ± 0.04	4.34 ± 0.09	3.78 ± 0.05
15 g/l, 8.12	4.06 ± 0.02	4.95 ± 0.04	4.05 ± 0.01
0.86 g/l, 6.0	5.56 ± 0.16	6.12 ± 0.03	5.49 ± 0.13
29.14 g/l, 6.0	3.89 ± 0.12	4.55 ± 0.10	3.84 ± 0.09
15 g/l, 6.0	3.86 ± 0.10	4.46 ± 0.01	3.84 ± 0.13
15 g/l, 6.0	3.82 ± 0.10	4.47 ± 0.04	3.80 ± 0.13
15 g/l, 6.0	3.76 ± 0.18	4.48 ± 0.01	3.87 ± 0.01

In most cases production of acetic and butyric acids, using CWP, was at about the same concentration (1:1, Fig. 2.3b). The treatment with the high VHPR (25 g CWP/l, pH 7.5) also yielded the highest concentration of both acetic and butyric acids at around 60 mM each. Treatments with [S_0] and pH above 15 g/l and 6.0, respectively, produced between 30 – 40 mM of acetic and butyric acids.

For glucose, the treatments with higher butyric acid concentration (>35 mM) and acetic acid content around 18 mM (Fig. 2.3c), correlated with the conditions in which both higher volumes of H_2 (H_{max}: Table 2.2) and higher VHPR were achieved. The treatments with either low pH (≤ 4.5) or [S_0] = 0.86 g glucose/l resulted in low concentrations (below 10 mM) of both butyric and acetic acids. Except for treatment with [S_0] = 0.86 g glucose/l and pH 6, in which acetic and butyric acids concentration was the same (*ca*. 3 mM); for the rest of experiments, the butyric content was higher than the acetic acid.

Quantification of fermentation end products such as VFA is important due to their role as regulators in metabolic shifting from acidogenesis (hydrogen production) to solventogenesis (production of acetone, ethanol, propanol or butanol) in clostridia which reduces hydrogen yield (Levin *et al.* 2004). Moreover, some VFA can be toxic or inhibitory to the H_2-producing microbial populations (Zheng and Yu 2005). As discussed by Van Ginkel and Logan (2005a), butyric acid could be more toxic than acetic acid in a hydrogen-saturated system, and although there is no agreement on the threshold value for shifting from acidogenesis to solventogenesis, it is reported that it could be from 2 – 30 mM of undissociated acids. Another important factor in metabolic shifting is pH. The optimum pH reported for solventogenesis is around 4.5 while for acidogenesis, it is 5.5 or higher (Jones and Woods 1986; Van Ginkel *et al.* 2001; Ferchichi *et al.* 2005a). In the present work, high partial pressures were likely to occur (not measured) in the treatments with the highest VHPR, due to high hydrogen accumulation observed in the headspace of the vials. This high hydrogen partial pressure (pH_2) is one of the reasons that lowers HMY because at $pH_2 > 10^{-4}$ atm, metabolic routes deviate from acetate production to other products such as butyrate (Angenent *et al.* 2004).

As previously mentioned, pH dropped for most of the treatments due to VFA production. For glucose and lactose, the final pH for treatments with high VHPR ranged from 3.8 to 4.7. When CWP was used, the pH of most treatments fell to a range of 4.3 – 6.1. Considering that the *p*Ka values for acetic and butyric acids are 4.76 and 4.81, respectively, undissociated butyric acid concentration for the treatments with the highest VHPR would be around 10 - 30 mM at the end of the experiments. This concentration of protonated butyric acid could have caused inhibition of the metabolism for hydrogen production.

It is known that inhibition of hydrogen production by butyric acid could be reduced by keeping the pH above 4.8, which helps to decrease the concentration of its undissociated acid. As a result, a process under controlled alkaline pH could overcome this inhibition. However, Figure 2.3 shows that at alkaline pHs (8.12 and

7.5) a larger amount of VFA were formed. These VFA could be fed into a second stage process for additional energy production, i.e. methane or more hydrogen, thus improving the energy/substrate yields (Redwood and Macaskie 2006; Davila-Vazquez *et al.* 2008b).

2.3.6 Overall performance comparison with previous studies

Although the results obtained in our work were similar to the reports cited above, one novel aspect of this work was the comparable values of HMY and VHPR at pH above the range (5 to 6) considered optimum for fermentative biohydrogen production (Van Ginkel *et al.* 2001). There are few reports in the literature in which biohydrogen production is achieved using mixed populations at pH above 7. Wang *et al*. (2006a) used a mixed population from an acclimated sewage sludge (CSTR, HRT = 6-12 h), previously acidified. Hydrogen was produced from sucrose up to an initial pH of 8.5, with an optimum initial pH of 7.5. In another work, biohydrogen was produced from starch in a pH range from 5.5 to 8.5, using acclimated sludge (previously heat-treated) (Wang *et al.* 2007). Therefore, it is possible to select hydrogen-producing organisms that can start to grow or germinate at an initial pH above 7. This microbial ability could be useful in processes in which alkaline pretreatments are used for the solubilisation of sugars from lignocellulosic biomass or also when used to enhance hydrogen production from organic matter (Cai *et al.* 2004). This would eliminate the need for reducing the pH to acidic levels before starting hydrogen production experiments after alkaline pretreatments. Due to the wide range between the high starting pH (above 7) and the final acidic pH, the fermentation time would be longer and consequently a larger amount of biohydrogen would evolve, minimizing the need for base addition. This ability may be related to higher values for HMY and VHPR. That is to say, the wider the pH range, the longer the lapse of time before the pH falls to harmful levels for the microbial cells (toxicity by VFA, high hydrogen partial pressure) and/or triggers a switch to hydrogen-consuming metabolic routes (solventogenesis). Regarding the effect of substrate concentration, for

glucose and lactose, the higher HMY were found at low substrate concentration, and it was the same case for VHPR with lactose (Table 2.3). This is in agreement with previous studies in which high initial concentrations caused high initial hydrogen production, increased hydrogen partial pressure and acid toxicity or pH inhibition (Van Ginkel *et al.* 2001). Therefore, it seems that low to moderate initial concentrations may be related to better hydrogen yield/production performance. However, one can consider that the inhibitory/toxic thresholds are specific to each system (type of substrate and inoculum) and thus RSM is an efficient tool to carry out further research.

2.4. Conclusions

RSM was a useful tool to model HMY and VHPR with quadratic equations using glucose, lactose and CWP as carbon substrates. The different behavior of the response variables for the tested substrates indicates that RSM is a robust tool to define optimal conditions for biohydrogen production when new substrates or inocula are tested.

Due to the higher trustworthiness of quadratic VHPR models for the three substrates ($R^2 > 0.83$), this variable may be selected as a design parameter. Then, in order to have high VHPR, the best initial conditions for glucose and lactose are: $[S_0]$ = 5 g/l and, pH 7.5. When CWP is to be used, higher substrate concentrations are recommended ($[S_0] \geq 15$ g/l at pH 7.5). HMY and VHPR obtained in this study were found at an initial pH above the reported optimum pH value for hydrogen production. These findings could also be useful when alkaline pretreatments are performed either for the solubilisation of sugars from lignocellulosic materials or for the conditioning of organic matter from wastes before hydrogen production.

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Chapter 3

Fermentative hydrogen production from cheese whey: Medium formulation effect

Summary

Hydrogen gas obtained from biomass *via* dark fermentation is considered as a sustainable and clean energy carrier. Batch fermentations were performed to assess the total hydrogen production (H_{max}), volumetric hydrogen production rate (VHPR), maximum lactose consumption (S_{max}), maximum lactose consumption rate ($R_{max,S}$), and hydrogen molar yield (HMY) using two mineral media formulation. Cheese whey powder (CWP) was selected as substrate and heat-treated anaerobic granular sludge as inoculum. The mineral media formulation was based either on carbonate (A) or phosphate (B). In both 80 ml or 2.4 l experiments, medium B yielded around twice the VHPR than the attained with medium A, but HMY only had a slight increment with the use of medium B. S_{max} , H_{max} and $R_{max,S}$ were also enhanced with medium B. Batch tests with initial 25 g/l of CWP (77% lactose) and initial pH of 7.5 showed a faster pH drop for medium A. The lower pH for medium A together with the butyrate concentration led to a decrease on lactose consumption compared to when medium B was used. Results suggest that butyrate levels and lower pH are the reasons for lower hydrogen production with medium A. *Clostridium perfringens* LNT6 and *Enterobacter* sp. were the main microorganisms detected in the experiments with medium B, while just a proteobacterium was detected in cultures with medium A. These findings are significant because the improvement of the VHPR of a fermentative process is critical for the scaling-up of hydrogen production processes to assess its practical application.

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3.1 Introduction

A large effort to find both renewable and sustainable energy sources is being undertaken around the world. Anaerobic biological processes commonly used to treat wastewaters are able to generate sustainable fuels such as methane or hydrogen, and rather than aerobic processes, are considered to be net energy producer systems (Calli *et al.* 2008b). Considerable attention has been paid to biohydrogen production from organic wastes such as wastewaters and organic residues or by-products (Van Ginkel *et al.* 2005; Kapdan and Kargi 2006). Regarding biohydrogen production, it is known that the by-products from the dairy industry represent a potential source of substrates for energy generation. In particular cheese whey (CW), a lactose-rich by-product, which accounts for the 85% of the total volume of processed milk (De León-Rodríguez *et al.* 2006). A dehydrated lactose-rich, cheap and easy-handling product obtained from CW, is known as cheese whey powder (CWP; Ozmihci and Kargi 2007).

According to Eq. 3.1, the maximum hydrogen molar yield (HMY) is 8 mol H_2 per mol of lactose consumed, when acetate is the main end product. HMY lowers to 4 mol H_2/mol lactose if butyric fermentation occurs (Eq. 3.2, Calli *et al.* 2008a). Moreover, the HMY could be lower than 4 mol/mol if other metabolites such as lactic acid, ethanol, butanol or acetone are also formed (Levin *et al.* 2004).

$$C_{12}H_{22}O_{11} + 5H_2O \rightarrow 4CH_3COOH + 4CO_2 + 8H_2 \quad (3.1)$$

$$C_{12}H_{22}O_{11} + H_2O \rightarrow 2CH_3CH_2CH_2COOH + 4CO_2 + 4H_2 \quad (3.2)$$

Biohydrogen generation by dark fermentation is highly dependent on the process conditions such as temperature, pH, mineral medium formulation, kind of inoculum, profile of organic acids produced, type of substrate and concentration, hydrogen partial pressure, and reactor configuration (Davila-Vazquez *et al.* 2008b). In general, pH is a key parameter in biological processes, therefore the bacterial media formulation always considers buffering compounds to reduce pH variations

during cultivations. There are few reports about biohydrogen production using CW, and in all cases the use of a carbonated compound in the buffer mineral media such as sodium or ammonium bicarbonate prevails (Ferchichi *et al.* 2005a; Yang *et al.* 2007; Davila-Vazquez *et al.* 2008a), presumably because carbonated-media were extensively and successfully used in anaerobic digestion processes (methanogenesis). Unlike methanogenic process where organic acids are consumed, in fermentative hydrogen production there is an intrinsic generation of organic acids (mainly acetic, propionic, and butyric) along with H_2, CO_2 and other metabolites, as aforementioned. These acids (H^+: Eq. 3.3), that could be toxic to cells, react with bicarbonates and generate additional dissolved CO_2, which also reduce the buffer capacity of the medium (Eq. 3.3).

$$HCO_3^- + H^+ \rightarrow H_2CO_3 \rightarrow H_2O + CO_2 \qquad (3.3)$$

As pH is an important parameter influencing the efficiency and productivity of fermentative biohydrogen process, the use of carbonate-buffered media is being reconsidered (Lin and Lay 2004; Wang *et al.* 2006a).

Thus the aim of this study was to evaluate biohydrogen production by mixed cultures using CWP as substrate and two different mineral media in batch experiments. The microbial communities were analyzed using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and a comparison in terms of HMY, VHPR, pH, and metabolites profiles was performed.

3.2 Materials and methods

3.2.1 Biohydrogen production experiments

Cheese whey powder (CWP, Land O´Lakes Inc., Minnesota, USA) was used as a source of lactose (77% w/w), and protein (11% w/w). All chemicals were purchased as reagent grade. Anaerobic granular sludge from a full-scale up-flow anaerobic sludge blanket reactor (UASB; San Luis Potosí, México) was used as inoculum for biohydrogen production. The UASB reactor treats wastewater from a candy factory in San Luis Potosí, México. The granular sludge was washed with tap water and then boiled for 40 minutes to inactivate methanogenic microflora. Batch experiments were conducted in duplicate using 120 ml serum vials with a working volume of 80 ml. CWP was used as substrate (25 g/l) and 4.5 g volatile suspended solids (VSS)/l of inoculum were used. An initial pH of 7.5 and vials were set as previously described (Davila-Vazquez *et al.* 2008a). Batch experiments were performed without pH control in order to follow the pH drop due to VFA production with each medium. Two different mineral media were used: carbonate-based (A, modified from Van Ginkel *et al.* 2001) and phosphate-based (B, modified from Wang *et al.* 2006a). The mineral media composition is shown in Table 3.1.

One scale-up batch experiment for each medium was carried out in a 3 l (2.4 l working volume) stirred glass reactor equipped with an ADI 1030 system controller and BioXpert 1.3 data-acquisition software (Applikon Biotechnology, Schiedam, The Netherlands). Mixing at 250 rpm was performed with two Rushton turbines and pH was monitored online using an autoclaveable pH electode (AppliSens, Applikon, Schiedam, The Netherlands). Temperature was kept at 37°C using an electric jacket. Initial CW concentration and pH were 25 g/l and 7.5, respectively. Gas production and composition in the headspace were measured periodically as described in analytical methods.

Table 3.1 Mineral medium composition: carbonate-based (A, modified from Van Ginkel *et al.* 2001) and phosphate-based (B, modified from Wang *et al.* 2006a).

Compound	Carbonate-based buffer (A: mg/l)	Phosphate-based buffer (B: mg/l)
NH_4HCO_3	2500	
KH_2PO_4	1250	
$MgSO_4.7H_2O$	125	
NaCl	12.5	
$Na_2MoO_4{\cdot}2H_2O$	12.5	12.5
$CaCl_2.{\cdot}2H_2O$	12.5	
$MnSO_4{\cdot}7H_2O$	18.75	15
$FeCl_2$	3.48	
$CoCl_2{\cdot}8H_2O$	3	3
$NiCl_2{\cdot}6H_2O$	1.5	
$ZnCl_2$	0.75	75
$NH_4H_2PO_4$		4500
Na_2HPO_4		11867
K_2HPO_4		125
$MgCl_2{\cdot}6H_2O$		100
$FeSO_4{\cdot}6H_2O$		25
$CuSO_4{\cdot}5H_2O$		5

3.2.2 Analytical methods

Gas production was measured using two different liquid-displacement devices filled with water (pH = 2). For the 120-ml vials, an inverted burette (250 ml) modified to have a gas-sampling port in the top was used, while in the case of the 3-l bioreactor a manometer calibrated to periodically count a fixed volume of gas was employed. Cumulative hydrogen production was calculated for each vial considering the headspace composition and the volume of gas released at each time interval, using a mass balance equation (Van Ginkel *et al.* 2005; Argun *et al.* 2008; Davila-Vazquez *et al.* 2008a). All gas volumes are reported as measured, 0.82 atm and 25°C.

H_2 and CO_2 were quantified using a gas chromatograph (GC, 6890N Network GC System, Agilent Technologies, Waldbronn, Germany) equipped with a thermal conductivity detector (Agilent Technologies, Waldbronn, Germany). The column used was a Hayesep D (Alltech, Deerfield, Illinois, USA). Temperatures of the injection port, oven and the detector were 250, 60 and 250°C, respectively. Nitrogen was used as carrier gas with a flow-rate of 12 ml/min.

Liquid samples were withdrawn at the end of each experiment (for the 120 ml vials) and periodically for the 3-l reactor as follows. 10 ml of liquid samples were taken and 60 µl of $HgCl_2$ (16 g/l) were added before centrifugation at 6610*g* for 15 min to minimize microorganisms activity (Park *et al.* 2005). Remaining substrate and fermentation end products, such as formic, acetic, propionic, and butyric acids (VFA) were analyzed in the filtrate by capillary electrophoresis. Solvents such as acetone, ethanol, propanol and butanol were analyzed using a gas chromatograph. Both VFA and solvents were quantified as previously described (Davila-Vazquez *et al.* 2008a). VSS were analyzed according to the *Standard Methods* (APHA *et al.* 1998).

3.2.3 Data analysis

Once cumulative hydrogen production was calculated from experimental data, a modified Gompertz equation was used to fit the kinetics of biohydrogen production and to obtain the parameters H_{max}, R_{max} and λ using KaleidaGraph ver. 4.0 (Synergy software). This equation has been widely used to model gas production data (Lay 2001; Khanal *et al.* 2004; Lin and Lay 2005; Mu *et al.* 2007):

$$H(t) = H_{max} \bullet \exp\left\{-\exp\left[\frac{2.71828 \bullet R_{max}}{H_{max}}(\lambda - t) + 1\right]\right\} \qquad (3.4)$$

where *H(t)* in ml is the total amount of hydrogen produced at culture time *t* (h); H_{max} (ml) is the maximal amount of hydrogen produced; R_{max} (ml/h) is the

maximum hydrogen production rate; λ (h) is the lag time before exponential hydrogen production. HMY and VHPR were defined as response variables; HMY was calculated from H_{max} and defined as mol H_2/mol consumed substrate whereas VHPR was obtained from R_{max} standardized to the working volume (ml H_2/l/h). For the experiments run in the 3-l reactor, a modeling of the substrate consumption was performed according to a modified Gompertz model (Eq. 3.5, Mu *et al.* 2007):

$$S_0 - S = S_{max} \bullet \exp\left\{-\exp\left[\frac{2.71828 \bullet R_{max,S}}{S_{max}}(\lambda - t) + 1\right]\right\} \qquad (3.5)$$

where S_0 is the initial lactose concentration (g/l); S is the lactose concentration (g/l) at fermentation time t (h); S_{max} is maximum lactose consumption (g/l); $R_{max,S}$ is the maximum lactose consumption rate (g/l/h); λ (h) is the lag time before exponential substrate consumption.

3.2.4 Microbial community analyses by 16S rRNA genes using DGGE

3.2.4.1 DNA extraction

Samples of 10 ml were withdrawn from the 120 ml-vials at the end of exponential hydrogen production and were stored at –20°C (15% v/v glycerol) until analysis. The protocols reported by Wisotzkey *et al.* 1990 and Sekiguchi *et al.* 1998 were optimized to be used on granular sludge samples as follows. In brief, prior to the DNA extraction, the samples were slowly thawed and 500 µl were taken and centrifuged at 7000*g* for 10 min. The pellet was washed two-times with phosphate buffer (10 mM, pH 7.5), resuspended in extraction buffer (10 mM Tris/HCl, pH 7.5, 50 mM EDTA, 0.5 M NaCl) and sonicated for 5 min in a ultrasonic processor (Sonics & Materials, Newtown, USA). Afterwards, the mixture was incubated with 10 µl of RNase (20 mg/ml, Invitrogen, Carlsbad, USA), 20 µl of lysozyme (20 mg/ml, USB, Cleveland, USA) and 15 µl of proteinase K (20 mg/ml, Invitrogen, Germany) during 2 h at 37°C and 350 rpm. 100 µl of SDS (10%) and 200 µl of

sodium acetate (5 M, pH 8) were added to the mixture and incubated for 10 min at 60°C. For the purification of the nucleic acids present in the mixture, one volume of chloroform-isoamylic alcohol (24:1) was added, mixed and centrifuged during 10 min at 7000*g*. This purification step was repeated two times with the liquid supernatant. To precipitate nucleic acids, one volume of isopropanol at -20°C was added to the supernatant and incubated for 3 h at -20°C before centrifugation at 4°C for 20 min at 7000*g*. The pellet was first washed with absolute ethanol and centrifuged at 7000*g* for 1 min, and then washed again with 70 % ethanol and centrifuged with the same conditions. Finally the DNA-containing pellet was air dried at room temperature and resuspended in 50 µl of sterile deionized water. A DNA integrity analysis was performed in 1% agarose gels, stained with ethidium bromide.

3.2.4.2 PCR amplification

Amplification of the hypervariable 3 region of the 16S rRNA gene from the purified nucleic acids preparations was carried out by PCR using *Pfu* polymerase (Biotools, Spain). The PCR primers used were the forward primer C356F (5´-**CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCC**CCTACGCGAGGCAGCAGCCTACGCGGGCAGCAG–3´), and the reverse primer 517R (5´-ATTACCGCGGCTGCTGG-3´). The GC clamp showed in bold letters was added at the 5´ end of the C356F primer. Reaction conditions were as follows: initial DNA denaturation at 95°C for 1 min using 1.5 U of *Pfu* polymerase, followed by 10 cycles of denaturation at 95°C for 30 s and annealing from 65 to 60°C for 30 s, lowering the temperature 0.5°C each cycle, and followed by an extension at 72°C for 1 min. In addition, 20 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 1 min; with a final extension at 72°C for 7 min were performed. The PCR product was

loaded onto a 1.5% agarose gel and stained with ethidium bromide to assess the size, purity and concentration of DNA.

3.2.4.3 DGGE analysis

DGGE was performed with Dcode Universal Mutation Detection System (Biorad, Hercules, California, USA). The PCR samples were loaded onto 10% polyacrylamide gels in 1 x TAE buffer (20 mM Tris-acetate, 10 mM sodium acetate, 0.5 mM EDTA, pH 7.4) with a denaturing gradient (urea-formamide) that ranged from 30 to 60%. Electrophoresis was carried out at 60°C at a constant voltage of 39 V during 14 h. After electrophoresis the gel was stained using SYBR© Safe for 30 min (Molecular Probes Inc., Eugene, Oregon, USA) before being visualized on a UV transilluminator (Biorad, Hercules, California, USA). The dominant bands were excised from the gel, eluted in 10 mM Tris-EDTA buffer (pH 7.5) overnight at 4°C. The eluted DNA was reamplified by PCR with the conditions mentioned before (See PCR amplification section). The PCR products from reamplification were sent to purification and sequencing to Molecular Cloning Laboratories (MCLAB, California, USA). Sequence data were analyzed with Bioedit-software and compared with sequences in GenBank (http://www.ncbi.nlm.nih.gov/blast/) and Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp).

3.3 Results and discussion

3.3.1 Biohydrogen production in the 120 ml vials

Batch fermentations carried out in 120 ml serum vials were conducted for biohydrogen production. After 44 h of fermentation, there was a clear difference in biohydrogen production experiments using the two media (Table 3.2). One considerable difference in the experiments was the lactose consumption with each medium; lactose was not detected at the end of the experiments with medium B, while in contrast only 63.7% of initial lactose was consumed using medium A. This significant difference is reflected in the higher maximum hydrogen production

achieved (H_{max}) using the phosphate-based (B) medium, which yielded around twice the volume produced with carbonate-based medium (A). Also the production rate (R_{max}) was enhanced with the use of medium B (Table 3.2).

Table 3.2 Kinetic parameters and performance of batch experiments with medium A (modified from Van Ginkel *et al.* 2001) and medium B (modified from Wang *et al.* 2006a).

	120 ml vials		3 l fermenter	
	Medium A	Medium B	Medium A	Medium B
H_{max} ; ml H_2	96 ± 6.8	224 ± 25	3944	6383
VHPR; ml H_2/l/h	124 ± 3.9	215 ± 19	236	400
S_{max} , g/l	-	-	12.1	17.3
$R_{max,S}$, g/l/h	-	-	1	2.7
HMY; mol H_2/mol lactose	1.1 ± 0.04	1.7 ± 0.18	1.5	1.8
Final pH	4.8 ± 0.01	5.8 ± 0.04	4.9	5.4
Acetic acid; mg/l	1523 ± 105	4159 ± 860	1097	2287
Propionic acid; mg/l	ND*	740 ± 43.8	2174	3416
Butyric acid; mg/l	1945 ± 78	2528 ± 154	1814	5048
Ethanol, mg/l	1036 ± 109	2409 ± 31	44	82
Lactose consumption (%)	63.7 ± 2.1	> 99	65	> 99

*Notes: ND = Not detected. Means are showed with ± standard deviation, n = 2

Due to the lost in buffer capacity for medium A, there was a difference of one unit in the final pH of the medium, being higher with the use of medium B. Consequently, due to the highest hydrogen production with medium B, total VFA concentration was 7427 mg/l, while the use of medium A resulted in a total VFA concentration of 3468 mg/l. Ethanol was the only solvent detected at around 2400 mg/l with the use of medium B, and near half the concentration with medium A. In terms of the profile of VFA, these results are similar to those obtained by Yang *et al*. (2007). Using a carbonate-based buffer, mixed microflora and CWP as substrate

the authors found mainly acetic and butyric acids with propionic acid present at low substrate-to-microorgamism ratio. Moreover, the presence of residual lactose was detected in their experiments with uncontrolled pH that fell from 7.33 to as low as 4.48. One difference is that the authors found a maximum VHPR of near 400 ml/l/h while in this work we report a maximum of 124 ml/l/h.

To our knowledge, there is only one report regarding lactose or CW fermentation for biohydrogen production using mixed cultures and phosphate-based medium (Calli *et al.* 2008a). The authors used pure lactose, as well as xylose, at thermophilic conditions at 55°C in both controlled and uncontrolled pH experiments in fed-batch operation for each substrate. For lactose, they found that best results were obtained with controlled pH at 5.3 with HMY reaching 3.45 mol H_2/mol lactose and VHPR was 57 ml/l/h. In the uncontrolled pH experiments, VHPR were below 9 ml/l/h and lactose consumption was 62% with an average HMY of 1.5 mol/mol lactose; the pH dropped from an initial value of 6.0 to 4.2. In all cases the initial lactose concentration was 2 g/l. The lactose consumption and HMY obtained by the authors are similar to the reported here for uncontrolled pH experiments with carbonate-based medium in which pH fell from 7.5 to 4.8. However, the VHPR we report for the mesophilic experiments are at least ten times higher than the best result reported by Calli *et al.* (2008a) in thermophilic controlled pH experiments. The reason for this is due to both higher initial substrate concentration (25 g/l) and initial pH of 7.5 used in this work, since previous findings showed that both factors had a significant effect on hydrogen production from either pure lactose or CWP (Davila-Vazquez *et al.* 2008a).

3.3.2 Biohydrogen production in the scale-up experiments

To further explore the behavior of pH, substrate consumption, metabolites production and hydrogen generation, one scale-up experiment was performed for each medium in a fermenter with a working volume of 2.4 l.

As noticed in Fig. 3.1 total hydrogen production was 6.4 l with medium B while 4 l H_2 were produced using medium A. The VHPR was also increased with the use of

medium B (Table 3.2). The increment in the working volume from vials to fermenter by a factor of 30, was reflected in the increase of H_{max} being the increment factor of 41 for medium A and 28.5 for medium B. However, the VHPR was nearly doubled from the vials to the fermenter for both media (Table 3.2).

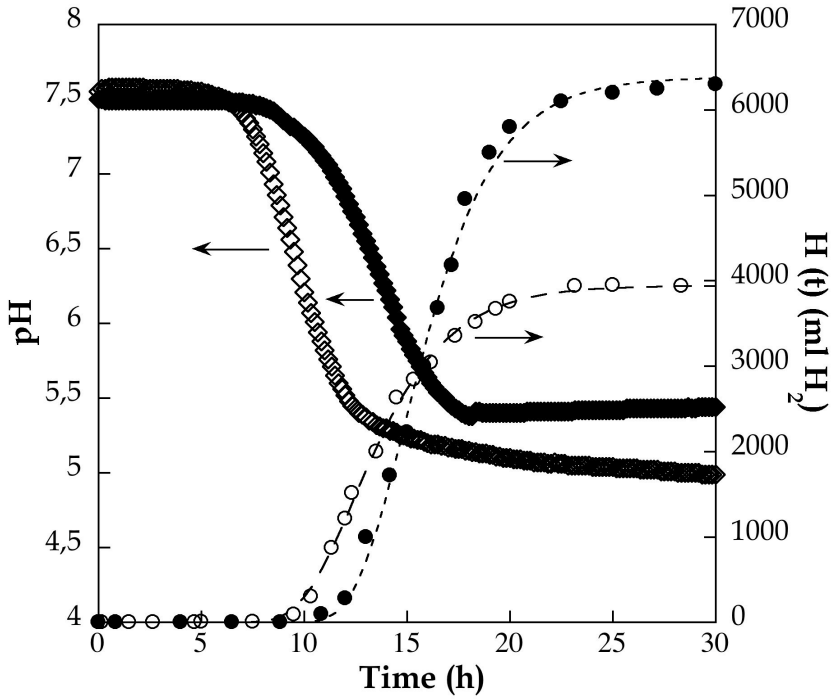


Figure 3.1. Biohydrogen production in the 3 l fermenter. Cumulative H_2 production using medium A (○) and medium B (●). The behavior of online pH for medium A (◇), and B (◆) is also shown.

Considering the lactose content (77%) in the CWP, it was expected to detect 19.25 g lactose/l as initial concentration in the batch experiments, however a maximum initial lactose concentration of around 16.5 g/l was measured (Fig. 3.2a). A possible reason for this could be adsorption of lactose to anaerobic granules and/or to protein present in CWP, and since the methodology includes the precipitation of the granules, only dissolved lactose could be detected. Therefore, all calculations were performed using dissolved lactose found in the medium.

Figure 3.2a shows that using medium A there is a point in which lactose is not further consumed by the microorganisms, reaching a residual concentration of around 6 g lactose/l. While with the use of medium B (Fig 3.2b) lactose was rapidly metabolized and was not detected in the culture medium after 18 h of fermentation.

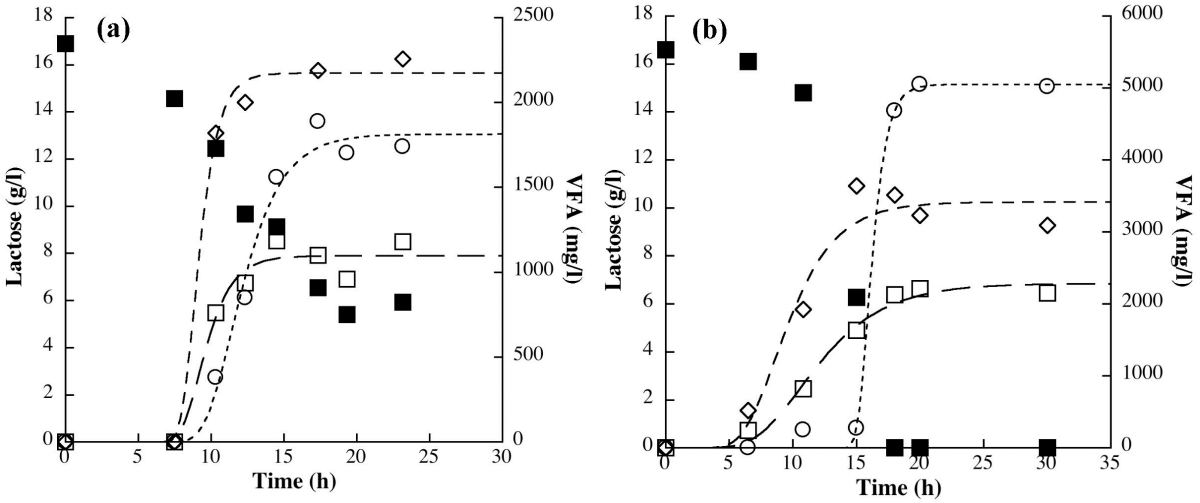


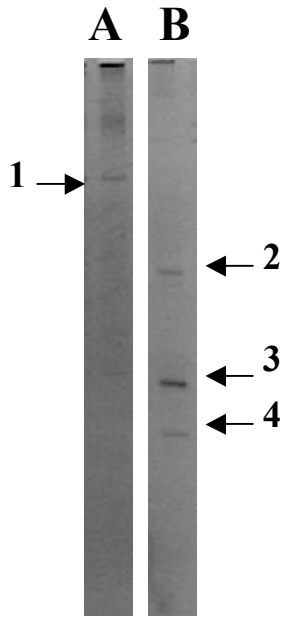
Figure 3.2. Lactose concentration (■), and volatile fatty acids production (□: acetic; ◇: propionic; ○: butyric acid) with medium A (**a**) and medium B (**b**) in fermentations for biohydrogen production with the 3 l fermenter.

This difference could be explained due to the different rates at which pH dropped for each medium (Fig. 3.1), and also due to the concentration of undissociated acids at certain fermentation times, because some VFA, such as acetic, butyric and propionic acids, are toxic to cells or inhibitory for the hydrogen production metabolism (Jones and Woods 1986; Van Ginkel and Logan 2005a). Both features (low pH and concentration of undissociated VFA) are connected because for a lower pH there is a higher amount of undisocciated acid form of the acid calculated using the Henderson-Hasselbalch equation (Van Ginkel and Logan 2005a). Therefore for medium A, the consumption of only 1.1 g lactose from fermentation time 17.3 h to 19.3 h, where consumption stopped, could be related to the concentration of undissociated acids such as butyric acid between those

fermentation times. At time 17.3 h (Fig 3.2a) the undissociated concentration of butyric acid (pH 5.16) was 583 mg/l (6.6 mM) and had a slight decrease at time 19.3 h. In the case of medium B, the concentration of butyric acid at time 15 h was only 21 mg/l (0.24 mM) at pH 5.88, this concentration is far below the reported threshold for butyrate toxicity to cells (2-30 mM). It suggests that the low butyric acid concentration in time 15 h allowed the bacterial cells to further metabolize the residual 6 g lactose/l in three hours, because from time 18 h lactose was not detected (Fig. 3.2b). This was reflected in a higher maximum lactose consumption (S_{max}) for medium B of 17.3 g/l calculated by the fitting of Eq. 3.5, although the real figure was 16.3 g/l as shown as initial lactose concentration in Fig. 3.2b. The low butyrate concentration with the use of medium B during the first 15 hours of fermentation could be the reason of a higher lactose consumption rate ($R_{max,S}$; Table 3.2) that was near three times the calculated for the experiment with medium A. Besides butyric acid concentration, the faster drop in pH for medium A could have a negative effect in cells and also inhibit hydrogen production metabolism. Figure 3.1, shows that the beginning of exponential hydrogen production with medium A occurs at time 12.3 h, and the pH had already dropped to 5.45, while for the same fermentation time the pH for medium B was 6.75. It is reported that the optimum pH for fermentative hydrogen production for different substrates is from 5.5 - 6 (Lay 2001; Van Ginkel *et al.* 2001), but hydrogen production has also been reported for higher pH (Wang *et al.* 2006a; Wang *et al.* 2007). However, pH below 5.0 has not been reported as optimum because it is around the pH where the switch from acidogenesis to alcohol production triggers (Oh *et al.* 2003). Thus, the lower pH at the beginning of the exponential phase using medium A could also explain the lower biohydrogen production compared to medium B because for the latter there was a bigger period of time before reaching a harsh low pH. Moreover, for medium B, pH fell to 5.4 which still is near the optimum reported range for hydrogen production, while for medium A the pH fell to below 5. It is hypothesized that these different rates of pH drop could also have an effect over the microbial communities that better adapt for each pH change.

3.3.3 Microbial community analyses

The effect of the two different culture media (A and B) on the microbial communities was studied with PCR-DGGE analyses with each medium in the 120 ml vials. Figure 3.3 shows that one clear band (1) was present in the experiment with medium A, while three bands were observed when medium B was used in batch fermentations.



Band	Microorganism
1	Uncultured gamma proteobacterium
2	Uncultured rumen bacterium clone L7A H10
3	*Clostridium perfringens* LNT6
4	*Enterobacter* sp. 76996

Figure 3.3 DGGE profile of partial 16S rRNA genes from batch experiments (120 ml vials) run with medium A (**A**) and medium B (**B**). The identified microorganisms corresponding to each lane/band in the DGGE profile are also shown.

The main microorganism detected in the experiments with medium A, was identified as an uncultured gamma proteobacterium (97% identity, accession number AY298738.1), while for medium B three microorganisms were observed: *Clostridium perfringens* LNT6 (96% identity, accession number EF589958.1), uncultured rumen bacterium clone L7A H10 (90% identity, accession number

EU381593.1) and *Enterobacter* sp. 76996 (98% identity, accession number AF227845.1) (Fig 3.3).

It is noteworthy to say that a identity of 90% is low to precise the genus, thus although the rumen bacterium clone was the highest match for the band number 2, further characterization of this sequence is needed (Drancourt *et al.* 2000). Species of *Clostridium* and *Enterobacter* are well known as efficient hydrogen-producing microorganisms (Collet *et al.* 2004; Chen *et al.* 2005; Collet *et al.* 2005; Ferchichi *et al.* 2005b; Levin *et al.* 2006; Mandal *et al.* 2006; Nath *et al.* 2006; Zhang *et al.* 2006; Shin *et al.* 2007). Therefore the selection of *Clostridium* or *Enterobacter* might be related to best results obtained with medium B. These findings are supported by a recent report in which bacterial populations from a mixed culture were -pH and substrate-dependent. The authors performed batch cultivations using cow dung sludge as inoculum and found *Klebsiella*, *Pseudomonas*, *Clostridium* and *Streptococcus* species at different initial pH (5.5 – 9) with xylose and cellulose as substrates (Lin and Hung 2008).

3.4 Conclusions

From the results showed in this work it is clear that the utilization of two different mineral media formulation, with CW as substrate, had a strong effect on Bio-H_2 production. Differences were observed regarding the microorganisms present in each culture condition: *Clostridium perfringens* and *Enterobacter* sp. were identified in experiments with medium B, while a proteobacterium was found with the use of medium A. Consequently, it was found that the use of medium B nearly doubled both the H_{max} and the VHPR, to those obtained with the use of medium A.

The kinetics of VFA production and pH drop in batch experiments suggested that deviations from metabolic hydrogen-production pathways occurred. Moreover, the kinetics had also an effect on the microbial community developed with two media and therefore these could be the reasons of higher hydrogen production with the use of medium B. These findings are significant because the enhancement of the VHPR of a fermentative process is critical for the scaling-up of fermentative

hydrogen production processes in order to assess its practical application in clean energy generation.

3.5 References

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Chapter 4

Continuous biohydrogen production using cheese whey: Improving the hydrogen production rate

Summary

Due to the renewed interest in finding sustainable fuels or energy carriers, biohydrogen (Bio-H_2) from biomass is a promising alternative. Fermentative Bio-H_2 production was studied in a continuous stirred tank reactor (CSTR) operated during 65.6 days with cheese whey (CW) as substrate. Three hydraulic retention times (HRT) were tested (10, 6 and 4h) and the highest volumetric hydrogen production rate (VHPR) was attained with HRT of 6 h. Therefore, four organic loading rates (OLR) at a fixed HRT of 6h were tested thereafter, being: 92.4, 115.5, 138.6 and 184.4 g lactose/l/d. The highest VHPR (46.61 mmol H_2/l/h) and HMY of 2.8 mol H_2/mol lactose were found at 138.6 g lactose/l/d; a sharp fall in VHPR occurred at an OLR of 184.4 g lactose/l/d. Butyric, propionic and acetic acids were the main soluble metabolites found, with butyric-to-acetic ratios ranging from 1.0 to 2.4. Bacterial species identified by PCR-DGGE at HRT of 10 h and 6 h were dominated by the *Clostridia* genus. The VHPR obtained in this study is the highest reported value for a CSTR system using CW as substrate and anaerobic sludge as inoculum. Thus, it was demonstrated that continuous fermentative Bio-H_2 production from CW can be significantly enhanced by an appropriate selection of parameters such as HRT and OLR. Enhancements in VHPR are significant because it is a critical parameter to determine the full-scale practical application of fermentation technologies that will be used for sustainable and clean energy generation.

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4.1 Introduction

The negative impacts on the global environment due to the intensive use of fossil fuels need to be reduced and reversed by replacing them gradually with sustainable and carbon-neutral energy carriers. There is an agreement that the use of biologically produced energy from biomass fulfills the requirements of being a green and sustainable process (Hawkes *et al.* 2007; Manish and Banerjee 2008). Therefore some options have been exploited such as the production of biodiesel, bioethanol and biohydrogen. The latter has very attracting features such as being the gas with the highest energy content per unit weight (143 GJ/ton), and a carbon-free fuel which only generates water when combusted or used in conventional fuel cells for electricity generation. Besides, biohydrogen is a low-solubility gas that can be easily separated from water, purified and used as energy carrier (Rittmann 2008). One approach used for biohydrogen production is by fermenting biomass (e.g. carbohydrates, wastewater, waste byproducts, etc.) and the biochemical routes are known as dark fermentation or fermentative hydrogen production. Among the microorganisms present in fermentative systems for hydrogen production are mainly *Clostridia*, *Bacillus* and *Enterobacter*, either in pure cultures or mixed populations (Davila-Vazquez *et al.* 2008b). As stressed by Kapdan and Kargi (2006), wastewaters from the food industry, and specially the dairy industry, have high potential to be used as raw materials for biohydrogen production. Around 10^8 tons/year of cheese whey are produced worldwide, with the risk of being a pollutant due to its high organic content (60 – 80 g COD/l) together with an inadequate disposal (Ozmihci and Kargi 2007; Gannoun *et al.* 2008). In spite of the great availability of lactose-rich wastes or byproducts from the dairy industry, few reports in biohydrogen production from these substrates exist (Calli *et al.* 2008a). Ferchichi *et al*. (2005a) used diluted cheese whey (*ca*. 41.1 g lactose/l) to study the influence of initial pH on biohydrogen production in batch experiments with a pure *Clostridium* strain. Another study with a pure culture reported the use of lactose (10 g/l) in a continuous regime at different pH and dilution rates (Collet *et al*. 2004). The use of mixed anaerobic cultures for continuous biohydrogen

production from CW was first reported by Yang *et al*. (2007). The authors tested different organic loading rates (OLR) at a fixed hydraulic retention time (HRT) and observed microorganisms related to the *Lactobacillus* genus and to a lesser extent *Clostridia* were detected by 16S rDNA analysis. Recently, a fed-batch process was performed by Calli *et al*. (2008a) to produce hydrogen from xylose and lactose at thermophilic conditions with inocula from compost slurry.

Although in several published reports authors are primarily concerned about improving hydrogen molar yields (mol H_2/mol substrate consumed), it is important to highlight that due to the *fermentation barrier* imposed by thermodynamics in native microbial cultures, more attention should be paid to raise volumetric hydrogen production rates (VHPR). Mainly because this is a critical parameter to determine full-scale practical application of fermentation processes for electricity generation, also because the higher the substrate concentration or OLR, the smaller the size and therefore the cost of the reactor needed (Levin *et al.* 2004; Kyazze *et al.* 2007). From what is so far published in the literature using CW or lactose in continuous cultures one can consider that hydrogen production rates could be improved by an appropriate selection of inocula and parameters such as OLR and HRT. Particularly, it can be hypothesized that shorter HRT and higher OLR than reported to date could raise VHPR. It should be noticed that for the scaling-up of a fermentation process the highest allowable OLR would be desirable to minimize the size of the reactor but also trade-offs between HMY and reactor size could be important (Kraemer and Bagley 2007).

Therefore, in this work the effect of different HRT and OLR with cheese whey powder solution as a synthetic dairy wastewater was studied in order to improve the reported VHPR and the hydrogen yields if possible. The microbial populations developed at each culture condition were analyzed using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and the main metabolites produced were analysed as well.

4.2 Materials and methods

4.2.1 Inoculum and substrate

Anaerobic granular sludge from a full-scale up-flow anaerobic sludge blanket (UASB) reactor was used as inoculum for biohydrogen production at a final concentration of 4.5 g of volatile suspended solids (VSS)/l. The UASB reactor treats wastewater from a confectionery factory at San Luis Potosí, México. Prior to use, the granular sludge was washed with three volumes of tap water and then boiled for 40 minutes to inactivate methanogens. The inoculum was stored at 4^{o} C. Cheese whey powder (CWP) was purchased from Land O´Lakes Inc, (Minnesota, USA). The soluble lactose content of CWP was 77% with 11% protein (w/w). Medium was supplemented with the following minerals (mg/l): Na_2HPO_4 11900; $NH_4H_2PO_4$ 4500; K_2HPO_4 125; $MgCl_2{\cdot}6H_2O$ 100; $ZnCl_2$ 75; $FeSO_4{\cdot}6H_2O$ 25; $MnSO_4{\cdot}7H_2O$ 15; $Na_2MoO_4{\cdot}2H_2O$ 12.5; $CuSO_4{\cdot}5H_2O$ 5; $CoCl_2{\cdot}8H_2O$ 3. This mineral medium was selected from previous experiments (medium B in chapter 3).

4.2.2 Experimental set-up

Continuous culture was performed in a 3-l bioreactor equipped with an ADI 1030 system controller and BioXpert 1.3 data-acquisition software (Applikon Biotechnology, Schiedam, The Netherlands). Mixing at 250 rpm was performed with two Rushton turbines; pH was monitored online (AppliSens, Schiedam, The Netherlands) and controlled at 5.9 during continuous operation dosing NaOH 10M with a peristaltic pump (20 rpm, Masterflex, Barnant, Illinois, USA). Temperature was kept at 37°C using an electric jacket. The bioreactor was fed and withdrawn using a peristaltic pump (1.6 – 100 rpm, Masterflex, Barnant, Illinois, USA). Operation started in batch mode and continuous regime began 12 h after. During the first three periods (A, B and C; Table 4.1) continuous operation was performed at a fixed CW feed concentration of 30 g/l but HRT was reduced from 10h to 4h stepwise, keeping a minimum operation time of 10-times the HRT for each period (A – G). From period D to G, a fixed HRT of 6h was selected and then, the OLR was increased by raising the CW concentration in the fed stepwise. The OLRs

tested ranged from 92.4 to 184.4 g lactose/l/d (Table 4.1). Foam production was controlled by addition of silicone antifoam (0.1%, AF Emulsion, Dow Corning, Midland, MI, USA). Gas production was measured using a liquid-displacement device filled with water (pH = 2; Fig. 4.1). All gas volumes are reported as measured (0.81 atm and 25°C).

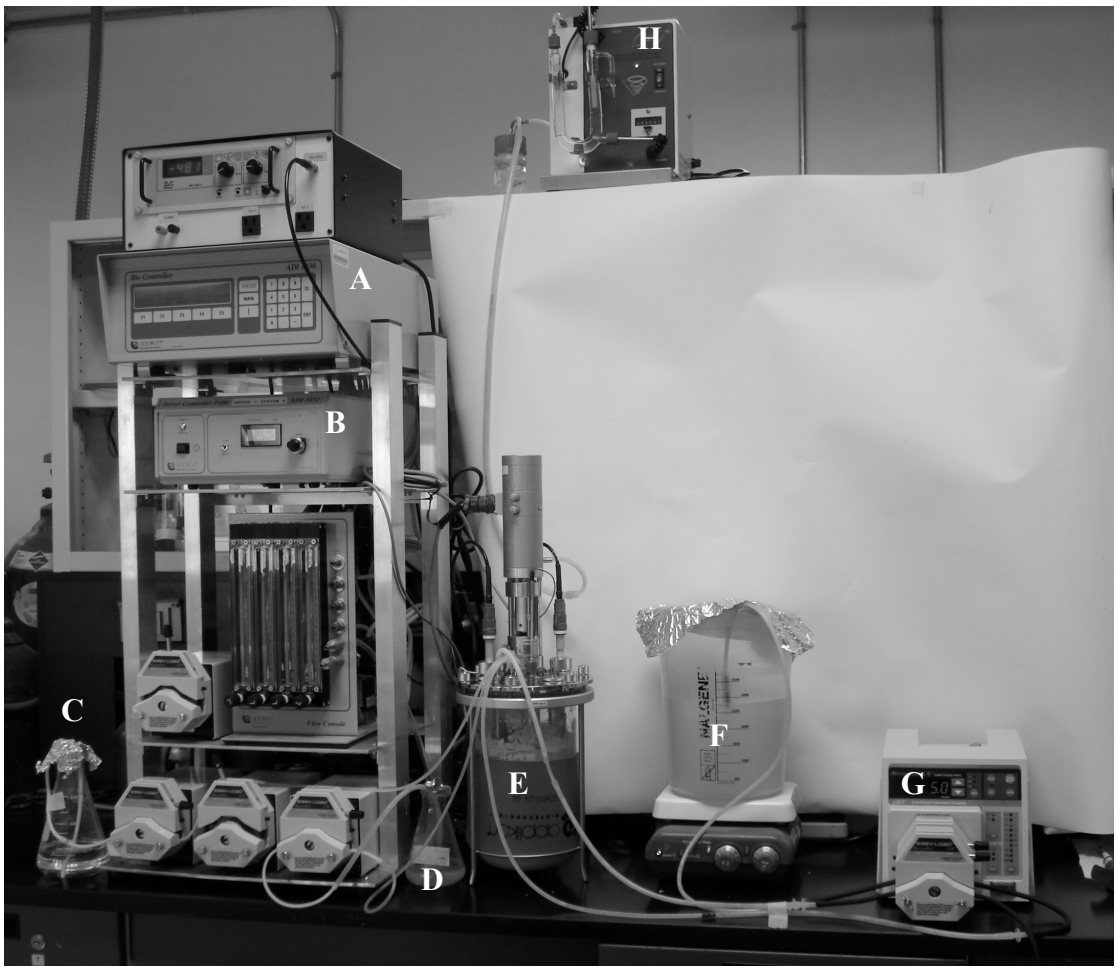

Figure 4.1. Continuous fermentation system. A: ADI 1030 system controller; B: stirrer controller; C: NaOH 10M ; D: antifoam; E: 3 l bioreactor; F: feed medium; G: peristaltic pump; H: liquid displacement device.

4.2.3 Analytical methods

H_2 and CO_2 were measured with a 1.0 ml Pressure-Lok® syringe (Valco Instruments, Houston, Texas, USA) by comparing a 300 µl sample from the reactor headspace with high purity standards (Alltech, Deerfield, Illinois, USA) using a gas chromatograph (GC, 6890N Network GC System, Agilent Technologies, Waldbronn, Germany) equipped with a thermal conductivity detector. The column used was a Hayesep D (Alltech, Deerfield, Illinois, USA) with the following dimensions: 10' x 1/8" x 0.085". Temperatures of the injection port, oven and the detector were 250, 60 and 250°C, respectively. Nitrogen was used as carrier gas with a flow-rate of 12 ml/min. Liquid samples were periodically withdrawn from the reactor and stored at –20°C before analysis. Remaining lactose, volatile fatty acids (VFA), and solvents such as acetone, ethanol, propanol and butanol were analyzed as previously described (Davila-Vazquez *et al.* 2008a). VSS were analyzed according to the Standard Methods (APHA *et al.* 1998).

4.2.4 Microbial community analyses by DNA extraction and DGGE

Ten mililiter samples were periodically withdrawn from the reactor and stored at –20°C with a final concentration of 15% glycerol until analysis. Twenty-three samples were selected, starting from day 0 to 53.4. The protocols followed for DNA extraction, PCR amplification of the 16S rRNA genes and DGGE analysis were already mentioned in sections 3.2.4.1, 3.2.4.2 and 3.2.4.3.

4.3 Results and discussion

4.3.1 Continuous hydrogen production

The bioreactor was operated during 65.6 days in 7 periods (A - G) under different HRT and OLR, and its performance is shown in Fig. 4.2. The features of each operation period are listed in Table 4.1. According to a previous study it was decided to set initial batch conditions at pH 7.5 and 30 g CW/l (Davila-Vazquez *et al.* 2008a). During the whole operation of the CSTR, hydrogen content in the gas

phase was between 49 to 58 % (v/v) with only CO_2 as the second gas detected. VHPR peaked at 1100 ml H_2/l/h before reaching the steady state at period A. Here CSTR started with a HRT of 10 h, feeding 30 g CW/l. During this initial period an average of 496 ml H_2/l/h (16.4 mmol H_2/l/h) evolved during 7.1 days of operation, with a HMY of 2.4 mol H_2/mol lactose. During this period, the mean total VFA produced was around 9700 mg/l, with a butyric-to-acetic acid (HBu/HAc) ratio of 2.4 (Table 4.1). The HBu/HAc ratio is proposed by some authors as a predictor of hydrogen yield, with reported values between 2.1 to 5.9 (Show *et al.* 2007). The reduction from a HRT of 10 h to 6h (keeping 30 g CW/l fed) increased the VHPR to 583 ml H_2/l/h (19.3 mmol H_2 /l/h) as a result of increasing OLR from period A to period B. This trend has been also reported by other authors (Van Ginkel and Logan 2005b; Show *et al.* 2007). Drops in hydrogen production rate at days 10.4, 13.3 and 21.2 which caused an increase in lactose present in the reactor were due to failures in the feeding pump. Although, it must be noticed that, after these perturbations, the system recovered and eventually returned to its previous performance. For this reason, it was decided to maintain period B during almost 23 days. For period B, total VFA production increased to 10642 mg/l with an HBu/HAc ratio of 2.05. With the exception of days 0.8, 1.9 and 20.8 propionic acid was not detected during the operation of periods A and B.

For period C the HRT was reduced to 4 h, holding again 30 g CW/l, which drastically led to a sharp fall in VHPR starting in day 31 presumably due to a wash-out of the hydrogen-producing bacteria (Fig 4.2).

As a result of the wash-out, lactose was detected in the medium up to 16.9 g/l at day 31.7. Moreover, the production of propionic acid triggered from day 31 reaching near 12000 mg/l at day 31.7. This is consistent with previous reports in which propionic-type fermentation is associated either with low production or consumption of H_2 (Hussy *et al.* 2003; Koskinen *et al.* 2007). Also both butyric and acetic acids dropped to 500 mg/l each, at day 32 lowering the HBu/HAc ratio to 1.0.

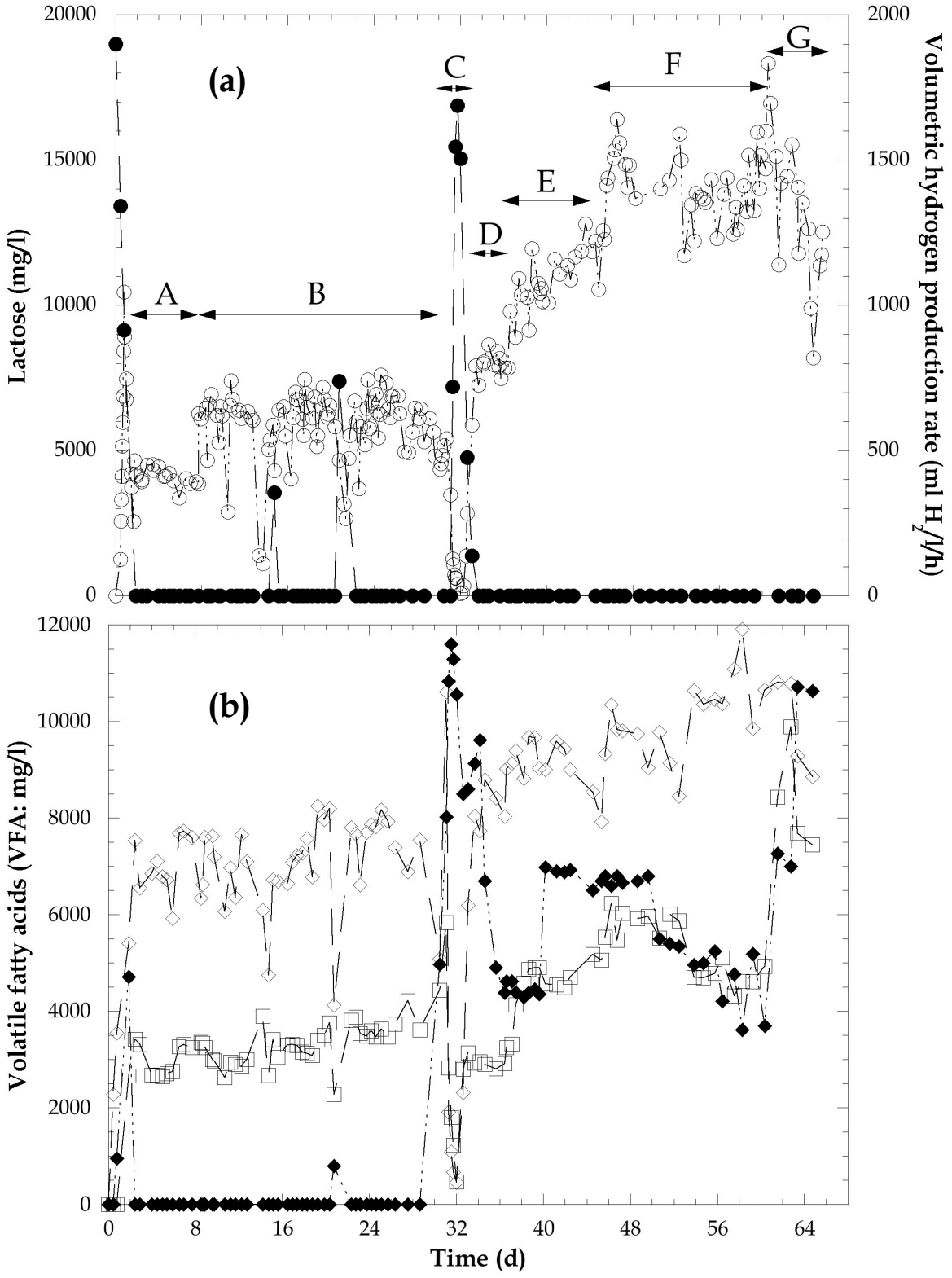

Figure 4.2 CSTR performance under A - G periods (For details see Table 4.1). (a): Residual lactose (●) and VHPR (○). (b): Profile of VFA. Acetic (□), propionic (◆) and butyric acid (◇).

Table 4.1 Performance of the CSTR for the production of biohydrogen under different operational conditions using cheese whey powder as substrate.

Parameters	Experimental periods						
	A	B	C	D	E	F	G
Duration (d)	7.1	22.8	1.7	3.9	8.9	14.9	6.3
Hydraulic retention time (h)	10	6	4	6	6	6	6
Organic loading rate (g lactose/l/d)	55.4	92.4	138.6	92.4	115.5	138.6	184.4
Volumetric hydrogen production rate* (mmol H_2/l/h)	$16.4^{a} \pm 5$	$19.3^{b} \pm 4$	$4.6^{c} \pm 5.7$	$23.32^{d} \pm 7$	$36.44^{e} \pm 3$	$46.61^{f} \pm 3.7$	$45.4^{g} \pm 9.1$
Hydrogen molar yield* (mol H_2/mol lactose)	$2.4^{h} \pm 0.7$	$1.7^{i} \pm 0.36$	$1.0^{j} \pm 1.9$	$2.1^{k} \pm 0.6$	$2.6^{l} \pm 0.2$	$2.8^{m} \pm 0.2$	$2.0^{n} \pm 0.4$
Total volatile fatty acids* (mg/l)	9723	10642	14600	17399	19133	20378	25617

*Mean values (± standard deviation), a: n=29 ; b: n=89 ; c: n=10 ; d: n=14 ; e: n=23 ; f: n=50 ; g: n=14 ; h: n=10 ; i: n=36; j: n=5 ; k: n=6; l: n=12; m: n=18; n: n=5.

Therefore, the change to a HRT of 4h was critical and had a detrimental impact on the VHPR, lactose consumption and VFA production. At this point the CSTR was re-inoculated with both initial heat-treated inoculum (50 ml, equivalent to 3 g VSS) and biomass saved from period B (20 ml, equivalent to 1.5 g VSS). Continuous operation at HRT of 6h (period D) was started after 8 h of batch regime. Even though periods B and D were operated with the same OLR and HRT, a higher VHPR (704 ml H_2/l/h) was attained in period D presumably due to differences in microbial populations before and after re-inoculation. During the short operation in period D (3.9 d) the HBu/HAc ratio returned to 2.4 and HMY reached 2.1. It should be noticed that from period C to D, propionic acid production decreased but not ceased. From this point and onwards the HRT was fixed at 6 h and the OLR was raised by increasing the CWP concentration in the fed. Thus a 25% increase in OLR from period D to E caused a 59% increment in VHPR while HMY was also raised from 2.1 to 2.6 and HBu/HAc ratio decreased from 2.4 to 2.0. One further increase of 20% in OLR resulted in a 35% higher VHPR from period E to F. Molar yield also had a slight upgrade during this change but HBu/HAc ratio decreased (Table 4.1, Fig. 4.2). The final increase to 184.4 g lactose/l/d during period G (33% more than period F) caused a fast drop in VHPR since day 60.5 (Fig 4.2a). This downward tendency in VHPR, together with the increase in propionic acid concentration from day 60 was interpreted as an indicative of a shock-load in the system due to the high OLR. Also the sudden increase in acetic acid from day 60 could be related to a diminished VHPR through H_2 consumption by acetogens (Kraemer and Bagley 2007). At this point, the operation of the CSTR was stopped after 6.3 days of operation of period G.

During the operation of the bioreactor, ethanol production attained values in a range from 100 to 600 mg/l, with the exception of period C in which the concentration dropped to 50 mg/l. Acetone, propanol and butanol were not detected. The same trend was observed for VSS with an average of 5000 mg/l, which decreased to 2000 mg/l during wash-out of the reactor (data not shown).

Table 4.2 Some reported VHPR and HMY under continuous or semi-continuous processes with lactose or cheese whey as substrates.

Inoculum	Carbon substrate	Volumetric H_2 production rate (mmol H_2/l/h)	H_2 yield (mol H_2/mol lactose)	Culture conditions [HRT (h), pH, Temperature (°C),OLR (g/l/d)]	Reference
Clostridium thermolacticum	Lactose (10 g/l)	2.58	3	17.2, 7, 58, 13.95	Collet *et al.* 2004
Anaerobic sludge	Cheese whey powder solution	1000 ml H_2/l/d	1.98 mM/g COD	24, 4-5, 35-38, 14	Yang *et al.* 2007
Compost (Fed-batch process)	Lactose (2 g/l)	1.61 2.55	3.7 3.2	22, 5, 55, 2.2 22, 5.3, 55, 2.2	Calli *et al.* 2008a
Anaerobic granular sludge	Cheese whey powder solution	23.32 36.44 46.61	2.1 2.6 2.8	6, 5.9, 37, 92.4 6, 5.9, 37, 115.5 6, 5.9, 37, 138.6	This study

Previous reports in fermentative Bio-H_2 poduction with an axenic *Clostridium* strain and mixed cultures using lactose or CW are presented in Table 4.2, in order to compare those results with the attained in this study.

In this work OLRs at least 6 times higher and HRTs shorter than other reported studies using lactose or CW were tested (Table 4.2). In particular, in the work by Yang *et al*. (2007), the authors used three HRTs (12, 18 and 24h) and attained better results at 24 h and 14 g COD/l/d with their highest VHPR of 1000 ml H_2/l/d. In the present work a VHPR of 46.61 mmol H_2/l/h (33768 ml H_2/l/d) at HRT of 6h is reported (period F, Table 4.1), which is near 34-times higher than the best obtained by Yang *et al*. (2007). It is also noteworthy to mention that in the best conditions, the authors achieved around 30% H_2 in the gas, along with the constant presence of methane and CO_2 (Yang *et al*. 2007). Moreover, other differences with the work by Yang *et al*. (2007) are remarked, they concluded that HRTs less than 24h did not favor the H_2 production from CW and reported that the best pH was in the range from 4.0 to 5.0. Despite of the different results there is an agreement that higher OLR may be effective for a greater VHPR as it is reported here.

The VHPRs achieved in this study were higher than those listed in Table 4.2 likely due to the different conditions tested, and mainly due to shorter HRT and higher OLR selected. As discussed by Van Ginkel and Logan (2005b) there is an inverse relation between OLR and HMY, thus the use of a higher OLR (to increase VHPR) leads to a diminished HMY as can be verified in Table 4.2. This compromise could be overcome by optimizing a fermentative process based on the VHPR and increasing the overall yield by using a second stage that will be fed with VFA to generate more energy in the form of H_2, methane or electricity (Redwood *et al.* 2008).

4.3.2 Microbial community analyses by PCR-DGGE

One reason for the differences found in H_2 fermentative system performances is the different microbial communities developed in the bioreactors under distinct culture conditions (Kim *et al.* 2006b; Koskinen *et al.* 2007). For this reason an

attempt to identify the microorganisms present during the operation of the CSTR was carried out by PCR-DGGE analyses. Figure 4.3 shows the DGGE pattern of the partial 16S rRNA genes amplified from the bioreactor samples under different HRT (10, 6 and 4h), from time zero (t_0) to operation day 53.4. As can be noticed in Fig. 4.3, species of the genus *Clostridium* were detected as the predominant bacteria in HRT 10 and 6h, but the intensity of these bands (A, B, F and G) diminished or dissapeared at the onset of HRT = 4h. *Clostridum* genus was detected again only after re-inoculation, which corresponds to lane 13.

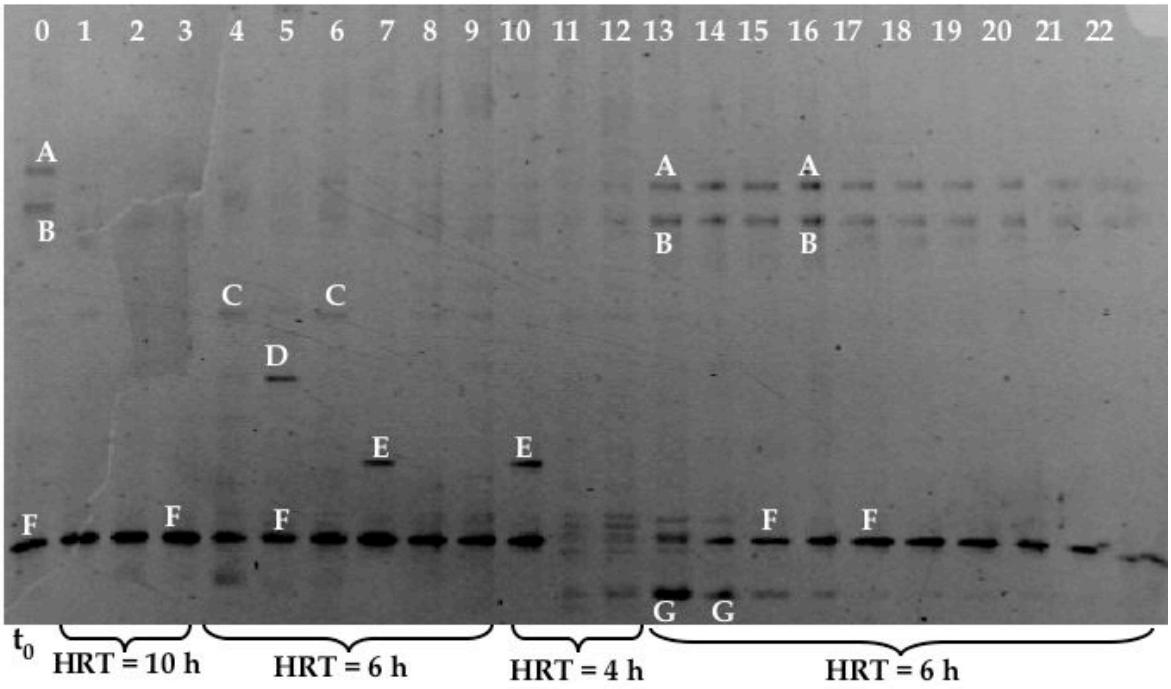

Figure 4.3 DGGE profile of the partial bacterial 16S rRNA genes amplified from the CSTR samples at t_0 and different HRTs. Lane number and sampling time (d), respectively, were as follows: 0=0, 1=2.9, 2=6.5, 3=6.9, 4=11.7, 5=14.7, 6=15.7, 7=18.2, 8=20.3, 9=28.7, 10=30.5, 11=31.7, 12=32, 13=33.7, 14=38.2, 15=40.2, 16=41.9, 17=44.5, 18=45.5, 19=47.3, 20=49.6, 21=51.6 and 22=53.4. The excised and sequenced bands (A – G) correspond to the following microorganisms: A: *Clostridium butyricum* CM-C86 (99% identity); B: *Clostridium butyricum* CM-C97 (96% identity); C: *Clostridium paraputrificum* (96% identity); D: *Enterococcus faecium* (99% identity); E: *Streptococcus* sp. (98% identity); F: *Clostridium* sp. (96% identity); G: *Clostridium butyricum* CGS6 (97% identity).

The dominance of clostridial species was already suspected based on the profile of soluble metabolites produced, due to butyrate and acetate were the main products (Chen *et al.* 2006).

During the wash-out of acidogenic bacteria that occurred at a HRT of 4h (Fig. 4.3: lanes 10, 11 and 12) it should be noticed that the band F, identified as *Clostridium* sp. disappeared at time 31.7 d at the same time that VHPR suddenly fell (Figs 4.2, 4.3). Recently, Chang *et al*. (2008) operated a CSTR inoculated with heat-treated sludge decreasing the HRT from 12, 8, 6, 4 to 3h. The authors could detect *Clostridium* strains by PCR-DGGE only from HRT 12 to 4h and when HRT decreased to 3h the active bacterial composition changed, resulting in the wash-out of clostridial strains. In another study, continuous production of Bio-H_2 at HRT from 12 – 2h resulted in a predominance of *Clostridium ramosum* even at the shortest HRT (Lin *et al.* 2006b). Moreover, an unidentified *Clostridium* sp. was found in a continuous reactor fed with starch operated at HRTs 2, 1 and 0.5h at pH 6.0. Other non-clostridial species such as *Streptococcus* sp., *Pseudomonas* sp., and *Dialister* sp. have been observed at HRTs of 12, 2, 1 and 0.5h (Cheng *et al.* 2008).

Thus, although other non-excised bands exist during the operation at HRT of 4h (times 31.7 and 32 d) it can be concluded that the main organism related to an efficient H_2-production in this system was *Clostridium* sp. identified from band F (Accession number AY188842).

Other *Clostridia* detected were *C. butyricum* CM-C86 (band A, accession number EU869239.1) and *C. butyricum* CM-C97 (band B, accession number EU869243.1) which were observed at time 0 and also due to the re-inoculation (day 33.7). Also *C. paraputrificum* (band C, accession number AY442815.1), *C. butyricum* CGS6 (band G, accession number AY540110). Occasionally *Enterococcus faecium* (band D, accession number AB243003.1) and *Streptococcus* sp. (band E, accession number EU932810.1) were detected during operation at HTR of 6h. As can be also noticed from Fig. 4.2 there are two bands (A and B) that were detected after re-inoculation corresponding to *Clostridium butyricum* CM-C86 and *Clostridium butyricum* CM-C97. These microorganisms were not detected in the period immediately before

wash-out (period B) and could be the reason for the slight improvement in VHPR and HMY for period D compared to B. This is consistent with reports in the literature where efficient hydrogen production using different *C. butyricum* strains and substrates were attained (Chen *et al.* 2005; Chen *et al.* 2008; Wang *et al.* 2008). Interestingly, *C. paraputrificum* was found as a low intensity band (C) apparently ubiquitous from time 0 to even at HRT of 4h. *Clostridium paraputrificum* has been reported as capable to produce H_2 from chitinous wastes (Evvyernie *et al.* 2001), moreover the overexpression of a [Fe]-hydrogenase gene using the same strain was reported by Morimoto *et al*. to enhance H_2 production (2005b).

Unlike the work by Yang *et al*. (2007) in which the authors found mainly *Lactobacillus* over *Clostridia*, here *Clostridia* species were detected during the operation of the CSTR at HRTs of 10 and 6h, shorter than the 24h used by those authors. It has been reported that *Lactobacillus* could be related to low H_2 production rates (Jo *et al.* 2007), then this feature could also explain the aforementioned differences in VHPR between this work and the reported by Yang *et al*. (2007). It must be remarked that although a very similar substrate (CWP) was used in both works, the selection of a suitable inoculum could also play an important role. In the work by Yang *et al.* (2007) the authors used a seed inoculum from an anaerobic digester with a HRT of 24 days, while for this work a granular sludge from a full-scale methanogenic reactor that has been operated for over 10 years with frequent periods of acidification was selected. Therefore it was hypothesized that the inoculum used here could be naturally enriched toward acidogenic bacteria.

4.4 Conclusions

The results from this study have shown that an enhancement of VHPR by increasing OLR using CW and mixed microflora, is feasible. It was demonstrated that shorter HRTs than previously reported with the use of CW, are recommended for better hydrogen production performance including higher VHPR and $\%H_2$ in the gas phase.

It was shown that HRT had a strong effect on the mirobial community detected by DGGE. Due that a wash-out of acidogenic bacteria was observed at a HRT of 4h, it was concluded that to achieve the best performance, a HRT of 6h was recommended. *Clostridium* sp. was the dominant microorganism of the microbial community in the bioreactor and was related to the best performance achieved in the CSTR. Moreover, it was shown that the OLR had a effect on the hydrogen production performance. Thus, in order to enhance VHPR, it is relevant to work with both appropriate conditions such as HRT and OLR and to select initial inocula that could be enriched toward efficient hydrogen-producing microorganisms.

Finally, enhancements in VHPR are significant because it is a critical parameter in the assessment of the full-scale practical application of fermentation technologies. Because the higher the VHPRs, the smaller the size and consequently the cost of the reactor needed for sustainable and clean energy generation from Bio-H_2 in the near future.

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Chapter 5

Perspectives, conclusions and final remarks

5.1 Perspectives for biohydrogen production

Even when there are many reports in the literature about biohydrogen production, only few economic analyses are available. In general, the molar yield of hydrogen and the cost of the feedstock are the two main barriers for the development of fermentation technologies. The main challenge to fermentative production of hydrogen is that only 15% of the energy from the organic source can typically be obtained in the form of hydrogen (Logan 2004). Consequently, it is not surprising that major efforts are directed to substantially increase the hydrogen yield.

The hydrogen production by Gram-positive bacteria such as *Clostridium* is shown in Fig. 5.1.

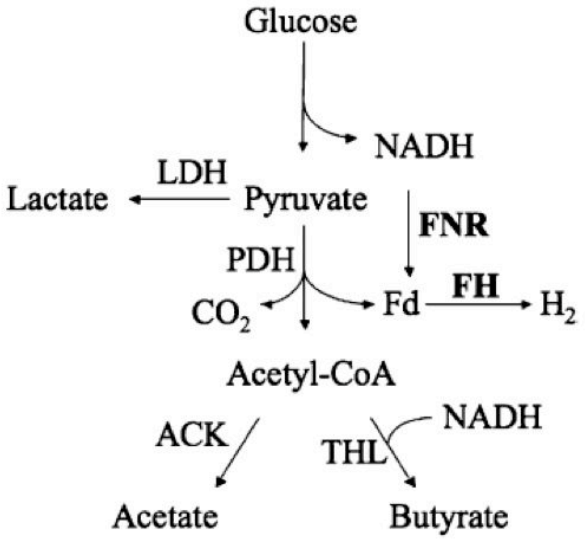

Figure 5.1 Metabolic routes of pyruvate in *Clostridium paraputrificum*. Key reactions in the generation of hydrogen are shown in bold. FNR: ferredoxin-NAD reductase; FH: [Fe]-hydrogenase.

The pathway for hydrogen production in clostridia uses two enzymes: ferredoxin-NAD reductase (FNR) and [Fe]-hydrogenase (FH). The overexpression of *hydA* gene encoding the FH has been used as a strategy to enhance hydrogen production. For instance, Morimoto *et al*. (2005a) reported that the hydrogen yield increased 1.7-times in recombinant *Clostridium paraputrificum* overexpressing *hydA*

gene with respect to a wild-type strain. Harada *et al*. (2006) proposed to disrupt *thl* gene encoding thiolase (THL), which is involved in the butyrate formation from *Clostridium butyricum*, like novel molecular strategies to improve the hydrogen production. Since *thl* defective mutant do not uptake NADH, it could be used for the hydrogen production by the FNR. Nevertheless, at this time, results on hydrogen production are not available. Overexpression of FNR could improve hydrogen production. However, strains overexpressing FNR have not been reported. Although it is now considered that the use of mixed microflora for hydrogen production has the advantage of lacking the need of sterile conditions in the fermentative bioreactor, the use of pure cultures (genetically modified) could emerge. As mentioned above, hydrogen molar yields may be increased through metabolic engineering efforts. At this moment the acceptability of genetically modified microorganisms is a challenge, since the possible risk of horizontal transference of genetic material. However, this can be ruled out by chromosomal integration and the elimination of plasmids containing antibiotic markers with available molecular tools (Datsenko and Wanner 2000). Moreover, the improvement of hydrogen production by gene manipulation is mainly focused on the disruption of endogenous genes and not introducing new activities in the microorganisms. New pathways must be discovered to directly take full advantage of the 12 mol of H_2 available in a mol of hexose.

The U.S. DOE´s 2015 program goal for fermentation technology is to realize a yield of 6 mol hydrogen per mol of glucose and achieve six months of continuous operation (Sverdrup *et al.* 2006). Nevertheless, the remaining energy in the unused substrate can be recovered by photobiological systems producing hydrogen, by methane production or by microbial fuel cells producing electricity (Logan 2004). According to a recent review, biological hydrogen production could equal the energy yield of bioethanol process only if the biohydrogen process achieves *ca*. 7.8 mol H_2/mol hexose (Redwood *et al.* 2008). To date this yield could not be attained by a single-stage, and a two-stage system would be required. Therefore, an immediate perspective from the results presented in this thesis would be to select a

second stage to produce more energy from the acidogenic effluent in order to improve the overall energy yield obtained with the first stage (Fig. 5.2). A proposed scheme of a two-stage (dual) system is described in Fig. 5.3 (Redwood *et al.* 2008). The authors assumed that a household might consume H_2 at a minimum rate of 573.6 mol/d, thus the feasibility of the decentralized application of a sequential dual system was evaluated by calculating the necessary reactor sizes and the feed requirements to meet this demand. The energy requirements of the process were not taken into account. If the potential H_2 yield (12 mol H_2/mol hexose) were to be distributed 4:8 between the 1st and 2nd stages, respectively, then the dark fermentation would be required to produce 191.2 mol H_2/d and the photobioreactor 382.4 mol H_2/day. Using published volumetric productivities, the authors calculated that a 80 l fermenter (containing an undefined mesophilic culture, *ca.* 0.1 mol H_2/l/h) and a 7684 l photobioreactor would be needed (Fig. 5.3).

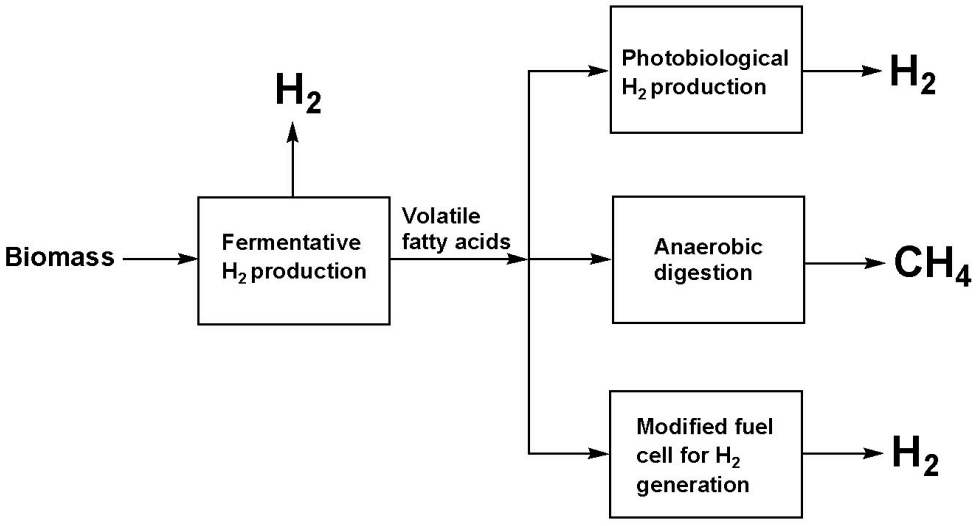

Figure 5.2. Scheme of some integrated strategies for hydrogen or energy (in the form of methane) production.

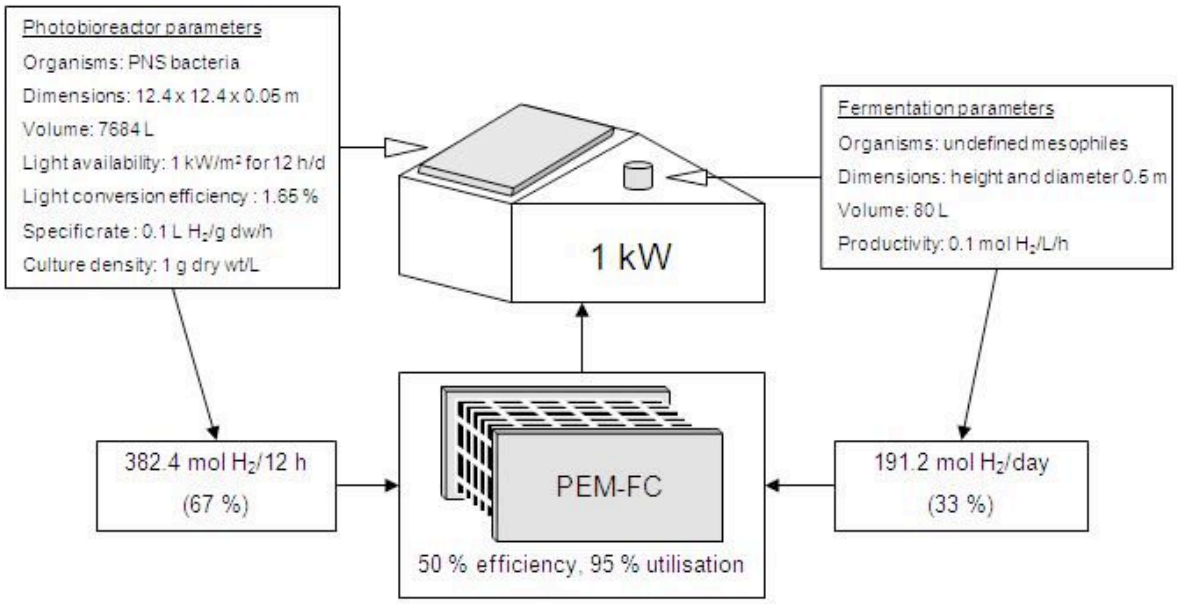

Figure 5.3 The diagram depicts one possible combination for a two stage system combining dark fermentation and purple non-sulfur (PNS) photosynthetic bacteria. PEM-FC stands for Proton Exchange Membrane Fuel Cell (From Redwood *et al.* 2008).

Thus, it is envisioned that success in the novel molecular strategies by re-directing metabolic pathways toward hydrogen production would have a strong impact in the reduction of bioreactors due to both increased efficiencies and higher hydrogen production rates.

de Vrije & Classen (2003) reported the cost of hydrogen production using a locally produced lignocellulosic feedstock. The plant was set at a production capacity of 425 Nm^3 H_2/h and consisted of a thermo-bioreactor (95 m^3) for hydrogen fermentation followed by a photo-bioreactor (300 m^3) for the conversion of acetic acid to hydrogen and CO_2. Economic analysis resulted in an estimated overall cost of € 2.74/kg H_2. This cost is based on acquisition of biomass at zero value, zero hydrolysis costs and excludes personnel costs and costs for civil works, all potential cost factors. Current estimation for hydrogen production cost is € 4/ kg

H_2 or € 30/GJ H_2. The estimation is done on the basis of process parameters which seem presently feasible.

Regarding feedstock costs, commercially produced food products, such as corn and sugar are not economical for hydrogen production (Benemann 1996). However, by-products from agricultural crops or industrial processes with no or low value represent a valuable resource for energy production. Nevertheless, besides hydrogen biological production, other biofuels (bioethanol, biodiesel, biobutanol, etc.) processes are being under development (Reisch 2006) and, eventually, the demand of agricultural by-products would increase its present low value. Wastewater has a great potential for economic production of hydrogen; only in the Unites States the organic content in wastewater produced annually by humans and animals is equivalent to 0.41 quadrillion British thermal units, or 0.46 EJ (Logan 2004). For a comparison, we now consume worldwide about 41.6 EJ/year and approximately 80% comes from burning fossil fuels (Rittmann 2008).

Currently, biologically produced hydrogen is more expensive than other fuel options and there is no doubt that many technical and engineering challenges have to be solved before a sustainable hydrogen economy can be implemented. Therefore it is considered that hydrogen biotechnology will play an important role in the energy market and will eventually emerge as a competitive technology together with other sustainable biofuel processes (Redwood *et al.* 2008).

5.2 Conclusions and final remarks

From the results presented in this work it is clear that in batch experiments the selection of a substrate, its initial substrate concentration, the initial pH and a proper mineral medium had a strong effect on fermentation parameters such as the maximum hydrogen production (H_{max}) and volumetric hydrogen production rate (VHPR). Differences were also observed regarding the microflora present in culture conditions with different media. These results have a potential practical application in the conversion of complex biomass to biohydrogen.

From the continuous experiments using cheese whey as substrate, the findings suggest that it is relevant to work with both appropriate conditions such as HRT and OLR along with the selection of initial inocula that could be enriched toward efficient hydrogen-producing microorganisms.

It is considered that improvements in the VHPR are of significance because it is a critical parameter in the assessment of the full-scale practical application of fermentation technologies. Because the higher the VHPRs, the smaller the size and consequently the cost of the bioreactor needed for sustainable and clean energy generation from Bio-H_2 in the near future. Although the continuous results from this work showed higher VHPR than previous reports with lactose or cheese whey (Table 4.2), the results are in general below than the reported with other substrates (glucose, sucrose, fructose) using either biofilm based/immobilized cells reactors (Wu *et al.* 2006; Lee *et al.* 2007) or granular sludge-based CSTR with biomass retention (Show *et al.* 2007) (Table 1.1). Thus, a perspective from this work is to use the best condition for higher VHPR as a first stage and couple it to a second stage such as another hydrogen-producing system using a photobioreactor or a methanogenic reactor, to use the remaining metabolites from the first stage to generate more energy and to improve the overall energy/substrate yield. As recommendations, a different reactor, such as a CSTR with biomass recycle or a biofilm-based reactor, could be tested to compare their productivity to those reported here for a system without biomass retention/recycling. It is hypothesized that VHPR could be at least doubled due to higher biomass retained in the system and the possibility to operate at both shorter HRT together with higher OLR, therefore avoiding the wash-out of the acidogenic bacteria.

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Curriculum vitae

The author of this dissertation, Gustavo Dávila Vázquez, was born in Colima, México on november 23rd, 1976. He received his Bachelor degree in Biochemical Engineering from the Instituto Tecnológico de Colima in 2000, and was awarded as the best alumnus from the Asociación Nacional de Facultades y Escuelas de Ingeniería (ANFEI).

His Master of Science degree in biotechnological processes was granted in 2003 from the Universidad de Guadalajara. The topic of his Master´s thesis was the biotransformation of pesticides using a fungal enzyme, being supervised by Dr. Rafael Vazquez-Duhalt at the Instituto de Biotecnología-UNAM.

From the summer of 2005, the author started his doctoral studies at the Instituto Potosino de Investigación Científica y Tecnológica in the Environmental Sciences Division under the main guidance of Dr. Elías Razo-Flores and the co-direction of Dr. Antonio De León Rodríguez from the Department of Molecular Biology.

In 2006, the doctoral protocol that generated this thesis was awarded with the *Dr. Sergio Sánchez Esquivel* prize from the Sociedad Mexicana de Biotecnología y Bioingeniería together with HyClone and Química Valaner companies.

From december 2008, the author will be assuming a research position at the Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco (CIATEJ). The address will be:

Email: gdv76@prodigy.net.mx

Unidad de Tecnología Ambiental, CIATEJ
Av. Normalistas No. 800
Col. Colinas de la Normal,
44270. Guadalajara, Jalisco.
México

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