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The Development and Microbiology of Bioprocesses for the Production of Hydrogen and Ethanol by Dark Fermentation

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The Development and Microbiology of Bioprocesses for the Production of Hydrogen and Ethanol by Dark Fermentation

Thesis for the degree of Doctor of Technology to be presented with due permission for public examination and criticism in Festia Building, Small Auditorium 1, at Tampere University of Technology, on the 26th of September 2008, at 12 noon.

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ABSTRACT

This work investigated the production of hydrogen and ethanol from carbohydrates by bacterial dark fermentation. Meso and thermophilic fermenters were enriched from the environment, and their H_2 and/or ethanol production in batch determined. Continuous biofilm, suspended-cell and granular-cell processes for H_2 or ethanol+H_2 production from glucose were developed and studied. Dynamics of microbial communities in processes were determined based on the 16S rRNA gene sequence analyses.

Mesophilic enrichment, obtained from anaerobic digester sludge, produced 1.24 mol-H_2 mol-glucose^{-1} in batch assays. Hydrogen production by the enrichment in a mesophilic fluidized-bed bioreactor (FBR) was found to be unstable – prompt onset of H_2 production along with butyrate-acetate was followed by rapid decrease and cease associated with propionate-acetate production. Intermittent batch (semi-continuous) operation allowed a momentary recovery of H_2 production in the FBR. The highest H_2 production rate (HPR) observed in FBR was 28.8 mmol h^{-1} L^{-1}, which corresponded to a relatively high hydrogen yield (HY) of 1.90 mol-H_2 mol-glucose^{-1}.

Mesophilic, completely-mixed column reactor (CMCR), with a similar inoculum and feed as used in the FBR, provided a prolonged H_2 production for 5 months. Highest HPR observed in the CMCR was 18.8 mmol h^{-1} L^{-1} (HY of 1.70 mol-H_2 mol-glucose^{-1}), while it in general remained between 1 and 6 mmol h^{-1} L^{-1}. Hydrogen production in the CMCR was decreased by shifts in microbial community metabolism from initial butyrate-acetate metabolism, first to ethanol-acetate, followed by acetate-dominated metabolism, and finally to propionate-acetate metabolism, which ceased H_2 production. The transitions of dominant metabolisms were successfully detected and visualized by self-organizing maps (SOMs). Developed Clustering hybrid regression (CHR) model, performed well in modeling the HPR based on the data on process parameters (pH, HRT) and metabolites (organic acids, ethanol, CO_2).

The instability of mesophilic processes (FBR and CMCR) was found to be due to rapid changes in microbial community structures after the start-up of continuous operation. The enrichment of organisms in bioreactors changed community metabolism away from H_2 (and butyrate-acetate) production. The FBR supported the growth of more diverse microbial community than that observed in the CMCR. *Clostridium butyricum* was the main H_2-producing organism in mesophilic bioreactors based on the metabolic pattern (e.g., high B/A –ratio) and on the 16S rRNA gene sequence analyses. The changes in quantities of *C. butyricum* (based on quantitative real time-PCR, and on proportion trends by DGGE) roughly corresponded to those in HPR.

Hydrogen production was more stable in the mesophilic CMCR than in the FBR. The instability of H_2 production in mesophilic reactors was likely related to the following reasons: Improper biocarrier in the FBR (low mass transfer of H_2, good adhesion of propionate-producers); unsuitable microbial community, e.g., presence of propionate-producers (BESA enrichment, no selection of spore-formers); too low bioreactor loading (caused sporulation of *C. butyricum* and favored the growth of propionate-producers).

Thermophilic isolate AK15, affiliated with *C. uzonii* (98.8%), produced relatively high amounts of H_2 from glucose (up to 1.9 mol-H_2 mol-glucose^{-1}) and xylose (up to 1.1 mol-H_2 mol-xylose^{-1}) in batch at 60°C. Batch ethanol production by another thermophilic strain, AK17, affiliated with *Thermoanaerobacterium aciditolerans* (99.2%), was amongst the highest reported for thermoanaerobes with ethanol yields of up to 1.6 mol-EtOH mol-glucose^{-1} and 1.1 mol-EtOH mol-xylose^{-1} in batch assays at 60°C. The HYs in batch by AK17 were

up to 1.2 mol-H_2 mol-glucose^{-1} and 1.0 mol-H_2 mol-xylose^{-1}. Further, AK17 tolerated up to 4%, v/v of exogenously added ethanol, and utilized main sugar residues found in lignocellulosic materials. Stable, long-term (3 months), co-production of ethanol and H_2 was achieved in an open system, CMCR by a co-culture of AK15 and AK17 at 60 °C. AK17 became dominant in the CMCR, producing promising ethanol yield of 1.35 mol-EtOH mol-glucose^{-1} and HPR of 6.1 mmol h^{-1} L^{-1} from glucose at the HRT of 3.1 h.

Extensive screening of Icelandic hot spring samples with glucose resulted in several H_2 and ethanol+H_2 -producing enrichment cultures, over a temperature range from 50 to 78 °C. One enrichment produced H_2 directly from cellulose at 70 °C. Enrichment 9HG, dominated by bacteria closely affiliated with *Thermoanaerobacter thermohydrosulfuricus* (100%), produced relatively high yields of ethanol (1.21 mol-EtOH mol-glucose^{-1}), and some H_2 (0.68 mol-H_2 mol-glucose^{-1}), from glucose in batch at 78 °C. Lactate production decreased the ethanol (0.69 mol-EtOH mol-glucose^{-1}) and H_2 (0.32 mol-H_2 mol-glucose^{-1}) yields in the continuous-flow bioreactor at 74 °C, and the yields were lower than those obtained in the batch fermentations. Co-production of ethanol+H_2 by 9HG was pH-dependent, and favored at the pH range of 6.5 to 7.1.

The hydrogen yield in batch (3.2 mol-H_2 mol-glucose^{-1}) by hot spring enrichment 33HL was among the highest reported for thermoanaerobes. The batch 33HL produced H_2 along with acetate. The dominant bacteria in the batch 33HL, *Thermobrachium celere* (100%) affiliated strains, did not thrive in continuous or semi-continuous open reactor systems fed with glucose. Continuous or semi-continuous reactor cultures with 33HL were dominated bacteria closely affiliated with *Thermoanaerobacterium aotearoense* (98.5 – 99.6%). These cultures produced H_2 along with acetate and butyrate.

High HY of 2.51 mol-H_2 mol-glucose^{-1} by 33HL was obtained in semi-continuous reactor at the HRT of 24 h at 58°C. High hydrogen production rate from glucose, 45.8 mmol h^{-1} L^{-1}, was obtained in continuous-flow reactor by 33HL at the HRT of 3h. Hydrogen production by 33HL was characterized by higher H_2 production efficiency (i.e., higher H_2 yield or specific H_2 production rate) than reported for mesophilic cultures. The 33HL readily formed granules in the continuous and semi-continuous reactor systems. Possessing good self-granulation, wide substrate utilization range and high hydrogen production efficiency, the 33HL is considered very suitable for thermophilic H_2 fermentation from carbohydrates.

This study demonstrated the H_2 or ethanol+H_2 production potential by thermophilic dark fermentation. Considering practical applications with the promising thermophilic cultures (AK17 and 33HL), continuous ethanol+H_2 or H_2 production from pentose sugars and real materials (i.e., organic wastes, lignocellulose hydrolysates) materials should be further studied. In this study, better stability and higher H_2 production was obtained by thermophilic dark fermentation processes compared to mesophilic processes. The better stability was related to more stable and less diverse microbial communities in the thermophilic systems compared to mesophilic systems. Further, this study demonstrated ready granulation and high H_2 production efficiency of thermophiles, which form basis for further development of thermophilic, high-rate H_2 production systems.

PREFACE AND ACKNOWLEDGEMENTS

This thesis is based on the work carried out mainly at the Department of Chemistry and Bioengineering (formerly Institute of Environmental Engineering and Biotechnology), Tampere University of Technology (TUT), Tampere, Finland, and partly at the Faculty of Natural Resource Sciences, University of Akureyri (UA), Akureyri, Iceland, and at the Research Center for Energy & Resources, Feng Chia University (FCU), Taichung, Taiwan.

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LIST OF ORIGINAL PAPERS

This thesis is based on the following original papers, referred to in this thesis by the roman numerals

I Koskinen PEP, Kaksonen AH, Puhakka JA. 2007. The relationship between instability of H_2 production and compositions of bacterial communities within a dark fermentation fluidized-bed bioreactor. Biotechnology and Bioengineering 97(4): 742-758. Copyright: Wiley-Liss Inc. a subsidiary of John Wiley & Sons, Inc.

II Nikhil, Koskinen PEP, Visa A, Kaksonen AH, Puhakka JA, Yli-Harja O. 2008. Clustering hybrid regression: a novel computational approach to study and model biohydrogen production through dark fermentation Bioprocess and Biosystems Engineering, in press, available on-line: DOI: 10.1007/s00449-008-0213-9. Copyright: Springer-Verlag.

III Koskinen PEP, Beck SR, Örlygsson J, Puhakka JA. 2008. Ethanol and hydrogen production by two thermophilic, anaerobic bacteria isolated from Icelandic geothermal areas. Biotechnology and Bioengineering, in press, available on-line: DOI: 10.1002/bit.21942. Copyright: Wiley-Liss Inc. a subsidiary of John Wiley & Sons, Inc.

IV Koskinen PEP, Lay C-H, Beck SR, Tolvanen KES, Kaksonen AH, Örlygsson J, Lin C-Y, Puhakka JA. 2008. Bioprospecting thermophilic microorganisms from Icelandic hot springs for hydrogen and ethanol production. Energy&Fuels 22:134–140. Copyright: American Chemical Society.

V Koskinen PEP, Lay C-H, Puhakka JA, Lin P-J, Wu S-Y, Örlygsson J, Lin C-Y. 2008. High efficiency hydrogen production from glucose by an anaerobic, thermophilic enrichment culture from Icelandic hot spring. Biotechnology and Bioengineering, in press, available on-line: DOI: 10.1002/bit.21948. Copyright: Wiley-Liss Inc. a subsidiary of John Wiley & Sons, Inc.

The author has also contributed the following publication, related to the topic of this thesis. In the text, the publication is referred to as Tolvanen et al. 2008.

Tolvanen KES, Koskinen PEP, Ylikoski A, Ollikka P, Hemmilä I, Puhakka JA, Karp M. 2008. Quantification of *Clostridium butyricum* with real-time PCR using lanthanide labeled probes and time-resolved fluorometry. International Journal of Hydrogen Energy 33:542–549.

THE AUTHOR'S CONTRIBUTION

Paper I:
Perttu Koskinen wrote the paper and is the corresponding author. He planned and performed the experimental work and interpreted the results.

Paper II:
Perttu Koskinen wrote the paper with Mr. Nikhil with equal contribution and is the second corresponding author. He planned and performed the experimental work (bioreactor experiments). Nikhil performed the modeling of bioreactor data and developed the Clustering Hybrid Regression model. Koskinen and Nikhil interpreted the results with equal contribution.

Paper III:
Perttu Koskinen wrote the paper and is the corresponding author. He planned the experiments and interpreted the results. He performed molecular analyses and ethanol inhibition test. Mr. S. Beck operated the bioreactor.

Paper IV:
Perttu Koskinen wrote the paper and is the corresponding author. He planned and performed majority of the experiments (bioreactor operation and temperature gradient experiment was performed together with Mr. C-H Lay), and interpreted the results. Ms. K. Tolvanen performed the PCR-DGGE analyses in the laboratory. Mr. S. Beck advised in the enrichment of H_2 and ethanol producers from hot springs.

Paper V:
Perttu Koskinen wrote the paper and is the corresponding author. He planned and performed the experimental work and interpreted the results. Mr. C-H Lay advised the bioreactor experiments and performed the GC and HPLC analyses of bioreactor samples.

The experimental work was carried out under the supervision of Prof. J.A. Puhakka (Papers I-V), Prof. A. Visa (Paper II), Prof. O. Yli-Harja (Paper II) assistant Prof. J. Örlygsson (Papers III, IV,V), Prof. S-Y Wu (Paper V), Prof. P-J Lin (Paper V) and Prof. C-Y Lin (Papers IV, V).

ABBREVIATIONS

AC	Activated carbon
ACF	Anaerobic contact filter
ADP	Adenoside diphosphate
AFC	Alkaline fuel cell
ASBR	Anaerobic sequencing batch reactor
ATP	Adenoside triphosphate
B/A	Butyrate to acetate
BEAMR	Bioelectrically assisted microbial reactor
BESA	2-bromoethanesulfonic acid
CHR	Clustering hybrid regression
CISBR	Carrier-induced sludge blanket reactor
CMCR	Completely-mixed column reactor
COD	Chemical oxygen demand
CSTR	Completely-stirred tank reactor
DGGE	Denaturing gradient gel electrophoresis
EAMFC	Electrochemically assisted microbial fuel cell
ED	Entner-Doudoroff
EM	Embden-Mayerhof
EPS	Extracellular polymeric substance
EtOH	Ethanol
FBR	Fluidized-bed bioreactor
FC	Fuel cell
FCV	Fuel cell vehicle
FHL	Formate-hydrogen lyase
FISH	Fluorescent *in situ* hybridization
H/D	Height to diameter
H_2	Molecular hydrogen
HPR	Hydrogen production rate (mmol h^{-1} L^{-1})
HRT	Hydraulic retention time
HY	Hydrogen yield (mol-H_2 mol-electron $donor^{-1}$)
ICSAB	Immobilized-cell-seeded anaerobic bioreactor
LR	Loading rate
MFC	Molten carbonate fuel cell
NAD^+	Nicotinamideadenine dinucleotide (oxidized form)
NADH	Nicotinamideadenine dinucleotide (reduced form)
NO_x	Nitrogen oxides
PAFC	Phosphoric acid fuel cell
PBR	Packed-bed bioreactor
pCO_2	Partial pressure of carbon dioxide
PCR	Polymerase chain reaction
PEMFC	Polymer or proton exchange membrane fuel cell
pH_2	Partial pressure of hydrogen
rRNA	Ribosomal ribonucleic acid
SHPR	Specific hydrogen production rate (mmol h^{-1} $g_{BIOMASS}^{-1}$)
SOFC	Solid oxide fuel cell
SOM	Self-organizing map
SO_x	Sulfur oxides
T	Temperature
UASB	Up-flow anaerobic sludge blanket reactor
VOC	Volatile organic carbon
VSS	Volatile suspended solids
WWTP	Waste water treatment plant

1 INTRODUCTION

By the year 2030, global energy demand is projected to grow by 50% from present (International Energy Agency 2007). Fossil fuels are used to meet our daily energy demands. This contributes to resource depletion, and environmental, and public heath problems (climate change, acid rain, ground level ozone, inhalable particles). Atmospheric CO_2 concentrations (379 ppm in 2005) have increased by almost 100 ppm compared to its pre-industrial level (Rogner et al. 2007). Global warming is evident based on the observations about the increased global average air and ocean temperatures, rising global average sea level, and widespread melting of snow and ice (IPCC 2007). Strikingly, eleven of the last twelve years (1995-2006) rank among the twelve warmest years recorded since 1850 (IPCC 2007). According to Intergovernmental Panel on Climate Change (IPCC) there is a very high confidence that human activities have contributed to the climate warming (IPCC 2007). Global average surface temperatures have risen 0.74 °C over the past 100 years, and depending on the emission scenarios, expected to increase from 1.8 to 4.0 °C, by the end of the 21st century (IPCC 2007). The temperature increase is widespread over the globe, and is greater at higher northern latitudes (IPCC 2007). The global warming is projected to result in serious impacts on ecosystems, food production, water resources, human health and the economics (IPCC 2007). Contrasting or additional theories to anthropogenic greenhouse gas emissions on global warming include increased solar activity (Rind 2002; Solanki et al. 2004) and cosmic rays (Svensmark 1998; Svensmark and Friis-Christensen 2007).

Presently, there is a global need and drive to introduce sustainable energy solutions. The Kyoto Protocol (United Nations 1997), aiming for global reductions in green house gas emissions (United Nations 2008), had been ratified, agreed or accessed by 176 countries by the December 2007 (United Nations 2007). Increased use of renewable energy will not only decrease the CO_2 emissions, but also improve energy security and create new jobs (European Commission 2006b). Renewables covered only 6.38% of primary energy consumption in the European Union countries in 2005 (22.94% for Finland) (European Commission 2006c), and the EU objective is to increase this share of renewables to 12% by 2010 (European Commission 1997). Further, the EU has set directive on increasing the share of renewables in vehicle fuel (petrol and diesel) up to 5.75 % until year 2010 (European Commission 2003a). U.S. has lately pushed forward increased usage of renewables – The US Energy Policy Act of 2005 (US Government 2005) states that the oil industry is required to blend 7.5 billion gal of renewable fuels into gasoline by 2012 (Gray et al. 2006). In the beginning of year 2006, China adopted "The renewable Energy Law" setting renewable energy a top priority in the Chinese energy strategy (Hu et al. 2005).

Today, the transport sector is highly dependent on fossil fuels (gasoline, diesel, kerosene etc.), and contribute to 21% of greenhouse gas emissions in the EU (European Commission 2006a). The renewable vehicle biofuels include e.g., bioethanol, biomethanol, biobutanol, biodiesel, biogas (biomethane) and biohydrogen (European Commission 2003a). Presently in the EU, the vehicle fuel is being supplemented with bioethanol (0.88 million TOE in 2006) or biodiesel (3.8 million TOE in 2006) to meet the requirements of renewables in fuel (Eurobserv'ER 2007a). Biogas production in the EU was 5.35 million TOE in 2006, and mainly used for heat and electricity production with a limited use as a vehicle fuel (EurObserv'ER 2007b). The theoretical biogas production potential in Europe is estimated to be up to 20% of natural gas consumption (Plombin 2003). The use of biohydrogen as a vehicle fuel is at the demonstration phase (Anonymous 2006).

Hydrogen is a secondary energy produced from primary energy sources, and therefore, considered as energy carrier, like electricity or gasoline, to transport energy to users

(Koroneos et al. 2004; Busby 2005). Unlike electricity, H_2 can be stored. Hydrogen is the most abundant element on the Earth (Schlapbach and Züttel 2001). Hydrogen has the highest energy content per mass unit of all compounds, about three times higher than that of liquid hydrocarbons (Schlapbach and Züttel 2001). The sources of hydrogen are versatile (biomass, organic wastes, water, fossil fuels) and globally distributed. In fuel cells (FCs), H_2 can be converted to electricity efficiently and without air emissions (Schlapbach and Züttel 2001; Dincer 2002). The potential uses of H_2 are many: as a fuel in traffic (vehicles, busses, airplanes etc.), as in stationary applications for generation of electric power and heat and in portable applications in electronic equipments.

Hydrogen can be produced from a variety of feedstock including fossil fuels, biomass and water. Even though H_2 conversion to energy in FCs is emissionless, the sustainability of H_2 energy depends on the production method (Turner 2004). Presently, H_2 is produced mainly from fossil fuels, which is not sustainable (Turner 2004). The H_2 production methods from renewables are numerous including (thermo)chemical, electrolytic, photolytic, radiolytical and biological methods (Riis 2006a). In this thesis, biohydrogen is defined as hydrogen produced by microorganisms (e.g., green algae, cyanobacteria or fermentative microorganisms). Green algae and cyanobacteria use the energy from sunlight to produce H_2 from water through an adapted photosynthesis, while fermentative bacteria are heterotrophs (Das and Veziroğlu 2001). Hydrogen dark fermentation processes have simpler process design (Nandi and Sengupta 1998) and higher production rates (Levin et al. 2004), but remain, so far, less studied than photobiological H_2 production.

Microbial fermentations offer an attractive alternative to produce sustainable energy. Fermentations can use various kinds of biomass or organic waste to produce energy carriers such as ethanol, butanol, methane or hydrogen (for reviews, see Zeikus, 1980, Claassen et al. 1999; Antoni et al. 2007). The present study focuses on the production of H_2 or ethanol plus H_2 by dark fermentation. The summary part of this thesis includes literature review on a) properties and applications of hydrogen and ethanol as energy carriers, b) hydrogen production methods, c) ethanol and hydrogen fermentations, d) bioreactor processes for continuous production of H_2 or EtOH+H_2. In the experimental part of this study a) mesophilic H_2-fermenters were enriched, and continuous H_2 production was studied in biofilm and suspended-cell processes, b) thermophilic H_2 or EtOH+H_2 –producers were enriched, and H_2 and EtOH+H_2 production characteristics were determined in batch assays, c) thermophilic H_2 or EtOH+H_2 production was studied in continuous, granular- and suspended-cell bioreactors, d) diversity and dynamics of microbial communities were determined in continuous-flow bioreactors.

2. HYDROGEN AND ETHANOL – PROPERTIES AND APPLICATIONS

2.1 Hydrogen and ethanol as energy carriers

Molecular hydrogen is a colorless, odorless and non-poisonous gas with very low specific gravity (Anonymous 2008a). H_2 is the lightest of all gases (Lide et al. 2007), 14.4 times lighter than air (Lovins 2005; Anonymous 2008a). Hydrogen condensates at very low temperature (-259.1 °C), and liquid H_2 has a specific gravity of only 70.8 g L^{-1} (Lide 2007) (Table 1). Hydrogen has the highest heating value of all fuels – 1 kg hydrogen contains as much energy as about 2.5 kg of natural gas or about 2.8 kg of gasoline (Das 1996). On the other hand, due to the low specific gravity, the volumetric energy density of H_2 is low – 3.7 L of liquid H_2 has the same energy as 1 L of gasoline (Anonymous 2008a). Therefore, storages, efficiently compacting H_2, are required (Schlapbach and Züttel 2001).

Hydrogen gas must be handled with extreme care, since hydrogen has a wide ignition range in the air, and low ignition energy (Busby 2005). Safety of H_2 fuel as compared to gasoline has been questioned. Even though H_2 has wide ignition range in air, H_2 leaks are diluted rapidly due to high diffusion coefficient (12 times higher than gasoline's) (Lovins 2005). The hydrogen flame in air is almost invisible making it difficult to spot (Busby 2005), but emits only 10% of the radiant heat compared to hydrocarbon fuel fire (Lovins 2005). Gasoline can be considered more flammable than hydrogen, as it has 4 times lower concentration limit required for burning, and lower self ignition temperature than hydrogen (Lovins 2005). In air atmosphere, H_2 does not explode easily and it rather burns. The theoretical explosion power is 22 times lower than that of gasoline (Lovins 2005). Hydrogen safety can be enhanced by storing H_2 in storage media (e.g., metal hydrides or glass microspheres) (Busby 2005) (See chapter 2.3).

Ethanol represents a renewable substitute for gasoline in today's vehicles. Pure ethanol is a colorless, flammable and volatile liquid with boiling and condensation temperatures of 78.3 and -114.1 °C, respectively (Kosaric and Vardar-Sukan 2001) (Table 1). Ethanol has lower volumetric energy content than gasoline, about 1.5 L of ethanol has the same energy content than 1 L of gasoline (Das 1996; US DOE 2006). On the other hand, ethanol is considered safer than gasoline, because of its low toxicity and better fire safety (Kosaric and Vardar-Sukan 2001).

Table 1. Properties of energy carriers; hydrogen, ethanol, methane and petroleum (Das 1996; Schlapbach and Züttel 2001; Busby 2005; Lide 2007; US DOE 2007b)

	Hydrogen	**Methane**	**Ethanol**	**Gasoline**
Density, gas (NTP) (kg $m^{3\ -1}$)	0.0899	0.651	N.A.	N.A.
Density, liquid (kg $m^{3\ -1}$)	70.8	422.6	789.3	720 -780
Melting point (°C)	-259.1	-182.3	-114.15	-40
Boiling point (°C)	-252.76	-161.15	78.29	N.A.
Lower heating value [MJ kg^{-1}, (kWh kg^{-1})]	119.9 (33.3)	50.0 (13.9)	N.A.	44.6 (12.4)
Energy per unit mass [MJ kg^{-1}, (kWh kg^{-1})]*	141.9 (39.4)	55.5 (15.4)	29.9 (8.3)	47.4 (13.2)
Energy per unit volume (GJ $m^{3\ -1}$)	10.10 (liquid) 0.013 (gas)	230 (liquid) 0.651 (gas)	23.6	34.85
Flame temperature (°C)	2045	1875	N.A.	2200
Self ignition temperature (°C)	585	540	423	228-501
Minimal ignition energy (mJ)	0.2	0.29	N.A.	0.24
Ignition limits in air (vol %)	4 – 75	5.3 – 15	4.3 -19	1.0 – 7.6
Flame propagation in air (m s^{-1})	2.65	0.4	N.A.	0.4
Diffusion coefficient in air (cm^2 s^{-1})	0.61	0.16	N.A.	0.05
Toxicity	No	No	No	Yes

* values represent the higher heating value (HHV), N.A. = not available

As compared to another gaseous fuel, methane, H_2 is considered to be more attractive energy carrier due to higher and scale-independent conversion efficiency to electricity in the fuel cells (FCs) (de Groot 2003); H_2 has conversion efficiency of 55-60 % (of the combustion value of H_2) compared to 33 % of that of methane, respectively (Van Groenestijn et al. 2002). Further, H_2 has a wider range of industrial applications compared to methane (Li and Fang 2007a).

2.2 Historical perspective of hydrogen and ethanol utilization

Important milestones in H_2 research and development have been listed in Table 2. Hydrogen was first isolated in the mid-1600s by Robert Boyle, who dropped iron nails into sulfuric acid, calling the H_2 gas evolved as "factitious air" (for reviews, see Hoffman 2002; Busby 2005). Roughly 100 years later, in 1766, Henry Cavendish identified hydrogen as a chemical element (calling it as inflammable air) and described its properties, e.g., density and molar weight. Cavendish also showed that burning H_2 in air produced water correcting the mistaken idea of water as a basic element. In 1783, Antoine-Laurent Lavoisier recognized the oxygen component of water, and gave hydrogen its modern name (= water producer) (for reviews, see Dunn 2002; Hoffman 2002; Busby 2005). In the end 1700s and early 1800s, hydrogen was used in hot air aeronautical balloons, and as a fuel in one of the first internal combustion engines. Hydrogen was also a rich component in "city-gas" used for heating and lighting purposes (for reviews, see Hoffman 2002; Busby 2005). In the 1920s and 1930s, hydrogen research was active and several H_2-utilizing transport applications were developed, from Zeppelin dirigibles to trains, buses and submarines (for a review, see Hoffmann 2002). The H_2 technology progress halted after the World War II due to low prices of oil and gasoline. The attention in H_2 energy raised again on 1970s during the energy crisis, but decreased after the oil prices dropped (Benemann 1996; Asada and Miyake 1999, for a review, see Hoffmann 2002). In the 1990s, the interest in H_2 raised again with the growing public awareness of negative global environmental impacts of fossil fuels (Benemann 1996).

Hydrogen production by microorganisms was revealed in the late 1800s (for reviews, see Benemann 1996; Hallenbeck and Benemann 2002). The basic research on H_2-producing bacteria was initiated in late 1920s (for a review, see Hallenbeck and Benemann 2002), and on microalgae in the early 1940s (Gaffron and Rubin 1942; for a review see Homann 2003). However, microbiological H_2 production was not considered as practical possibility until 1970s (Benemann 1996). In the 1970s and 1980s, the research in biohydrogen mostly concentrated on photobiological H_2 production (for reviews, see Benemann 1996, Asada and

Miyake 1999). The research on dark fermentative H_2 production gained more attention in the late 1990s with increasing amounts of studies until present.

Table 2. Some important mailstones in hydrogen research (for reviews, see Hallenbeck and Benemann 2002; Hoffmann 2002, Busby 2005; National Hydrogen Association 2007b)

Mid 1600s hydrogen was isolated by Robert Boyle
1776 H_2 identified as a chemical element by Henry Cavendish
1783 Hydrogen received it's modern name by Antoine-Laurent Lavoisier
1783 1st manned hydrogen balloon flight by JAC Charles
1794 Large-scale hydrogen generator operated in France
1800 H_2 production through water hydrolysis was invented by W. Nicholson and A. Carlisle
1839 1st workable fuel cell "a gas battery" developed by William Grove
1874 Concept of hydrogen economy was established by Jules Verne
1890s Commercial electrolysis cell developed
1896 Hydrogen production by microalgae detected by Jackson and Ellis
1898 H_2 liquefaction was established by James Dewar
1920s John B.S. Haldane described the structure, advantages and costs of hydrogen economy
1920s to 1930s H_2 exploited for aviation, e.g. German Zeppelin dirigibles carrying passengers on trans-Atlantic flights
1920s Pressurized electrolyzer developed by F. Lawaczeck and J.E. Noeggerath
1920s First commercial H_2 electrolyzers set-up
1929 Basic research on H_2-producing bacteria initiated by L.H. Strikland
1930s H_2 transportation using pipelines suggested F. Lawaczeck
1930s to 1940s H_2 powered trucks, buses, submarines designed by R. Erren
1932 H_2 powered railway system studied in Dresden, Germany
1932 Modern fuel cell developed by Francis T. Bacon
1937 Hindenburg airship disaster killing 37 passengers
1942 Basic research on H_2-producing microalgae initiated by Gaffrom and Rubin
1950s First proton exchange fuel cell (PEMFC) developed by General Motors for NASA space applications
1959 Practical fuel cell (5 kW), powering a welding machine, developed by F.T. Bacon
1959 1st fuel cell vehicle, a tractor, was demonstrated by Harry K. Ihrig (Allis-Chalmers Manufacturing company)
1960s NASA starts to apply H_2 fuel cells for electricity power in spacecrafts
1962 John Bockris releases plans for solar energy based H_2 power supplies to US cities
1970 Term "Hydrogen Economy" released by technicians in General Motors
1973 Oil crisis raises the interest in alternative fuels giving a boost to H_2 research
1974 International Association for Hydrogen Energy was established
1979 Solid, crystalline H_2 was created in laboratory
1988 1st H_2 powered airplane was flown in the U.S.
1989 Solid, semi-metallic H_2 was created in laboratory by Mao and Russell
1990 1st solar-powered H_2 production plant operated in Germany
1992 1st grid-independent solar house using H_2 storage operated in Germany
1993 1st fuel cell bus (by Ballard Power Systems) debuts in Vancouver, Canada
1993 Daimler-Benz displays its 1st NECAR I experimental fuel cell vehicle
1994 1st solar-powered H_2 production demonstration plant operated in Saudi-Arabia
1996 Liquid-H_2 -powered bus operated in Germany
1997 Hydrogen's blame for Hindenburg disaster challenged by A. Bain – the catastrophic fire was caused by static electricity and flammable airship skin material, not H_2
1998 1st U.S. residential fuel cell installed
1998 Iceland released ambitions for Icelandic hydrogen economy until 2030
1999 1st European H_2 fueling stations opened in Hamburg and Munich
2001 1st volume-produced PEM fuel cell wide industrial application released by Ballard Power Systems
2001-2006 European hydrogen bus demonstration project (CUTE) operating FC buses in 10 European cities. Different H_2 fueling, distribution and production systems were developed locally.
2007- Hydrogen FCV and fueling station demonstration program in California, U.S. – 24 H_2 stations and 179 FCVs in operation (Anonymous 2008b).

Throughout the history, around the world, human societies have discovered the fermentation of ethanol (for a review, see McGovern et al. 2004). Proofs of ethanol fermentation in China have been dated as far as 7000 B.C. (McGovern et al. 2004). In addition to being used as stimulants, ethanol drinks had medicinal value due to analgesic (pain killer) and disinfectant effects (for a review, see McGovern et al. 2004). Antoine Lavoisier discovered the biochemical reaction occurring during ethanol fermentation from sugars in 1789 (for a review, see Barnett 2003). In 1860, Louis Pasteur confirmed the role of yeast in ethanol fermentation (for a review, see Vaughan-Martini and Martini 1995; Barnett 2003). At present, ethanol is a chemical which is produced in largest volume by industrial fermentation (Kosaric and Verdar-Sukan 2001). Today, in addition to alcohols drinks, ethanol is also used in cleaning, in

cosmetics and pharmaceuticals, as a fuel supplement and antifreezing agent, in chemical industry as solvent and extractant, and as a feedstock for the production of several chemicals (e.g., acetaldehyde and acetic acid) (Lynd 1989; Kosaric and Verdar-Sukan 2001).

The production and utilization of ethanol as a vehicle fuel is mature technology with rather long history (Roehr 2001; Bothast and Schlicher 2005). In the beginning of 1900s, the early T model Ford allowed the use of either ethanol or gasoline as fuel (Bothast and Schlicher 2005). Until the late 1930s, the use of ethanol-gasoline blends was common and large ethanol production plants were set-up in the U.S. (Roehr 2001; Bothast and Schlicher 2005). However, the plants were closed after the World War II due to low oil prices (Roehr 2001). During the oil crisis in the 1970s, the interest in ethanol increased and large research programs were started (Rosillo-Calle and Cortez 1998; Roehr 2001). In Brazil, this led to an extensive development of vehicles running on 100% ethanol (Rosillo-Calle and Cortez 1998; Roehr 2001). In the mid 1980s, more than 90% of the cars sold in Brazil were running on 100% ethanol (Rosillo-Calle and Cortez 1998). Until the mid 2000 Brazil was the largest manufacturer of ethanol, but was overtaken by the U.S. with the present annual production of about 18 billion liters (about half of the global production) (Gnansounou et al. 2005; Gray et al. 2006; Angenent 2007).

2.3 Applications of hydrogen and ethanol

Hydrogen has multiple potential applications as energy carrier. Hydrogen can be used as a vehicle fuel, for stationary production of electricity and heat and as a fuel in portable electronics. Today, H_2 is mainly used as a feedstock in the industry with very limited use as a fuel or energy carrier, such as in spacecrafts (Ramachandran and Menon 1998; Das and Veziroğlu 2001; Busby 2005) (Table 3). Annually, about 450 billion m^3 (50 million tons) of H_2 is produced globally (Busby 2005; National Hydrogen Association 2007a). The main industrial applications of H_2 include manufacturing ammonia (about 60 %) and the oil refining (cracking and hydrogenation of hydrocarbons) (for reviews see Ramachandran and Menon 1998; Busby 2005; National Hydrogen Association 2007a).

Table 3. Main applications of hydrogen today (Ramachandran and Menon 1998; Busby 2005)

Application	Function
Petrochemical industry	Cracking and hydrogenation of hydrocarbons and removal of sulfur, nitrogen, oxygen and metals (hydroprocessing) for the production of gasoline, diesel, jet fuel, and other petroleum products
Fertilizer industry	Production of ammonia as a fertilizer feedstock
Chemical industry	Production of chemicals e.g., methanol, acetic acid, butanediol, benzene, butyraldehyde etc.
Food industry	Solidification of oils and fats
Metallurgical applications	As a reductant in the production of elemental metals
	As an oxygen scavenger
Mechanical industry	Arc welding with H_2 flame
Electricity supply	Cooling of generators, motors and transformers
Electronics industry	In the production of electrical components (to form reducing atmosphere)
Nuclear industry	As an oxygen scavenger to prevent corrosion
Glass manufacture	As an oxygen scavenger in float glass manufacture process
	Glass cutting with H_2 flame
Weather monitoring	H_2-filled weather balloons
Space exploration	Rocket fuel
	Providing electricity

H_2 in stationary power and heat applications

Stationary FCs provide reliable power with very consistent voltage output and high efficiency (40 to 85%) (US Fuel Cell Council 2004b). The waste heat of stationary FCs can be utilized for heating (cogeneration) or for further electricity production by using gas turbines. Residential scale FCs (3 to 10 kW) have been developed to provide primary or backup power for houses (US Fuel Cell Council 2004a). They can run independently or in parallel to an existing power grid (US Fuel Cell Council 2004a). Some of the FC types (see below) for stationary or residential power applications can use a variety of fuels as they can internally reform them to H_2 (US Fuel Cell Council 2004b). These FCs enable the utilization of conventional fuels (e.g., methane or propane) with existing distribution infrastructure (US Fuel Cell Council 2004a). For these reasons, stationary FC systems may provide many benefits to the industrial or residential power production.

H_2 as vehicle fuel

World's leading car manufacturers are conducting vast research efforts on the development and commercialization of fuel cell vehicles (FCVs). FCs offer cleaner and more efficient alternative to the internal combustion engine (US Fuel Cell Council 2004c). FCVs are estimated to be 1.5 to 2 times more efficient than vehicles with conventional combustion engine, and the exhaust gas consists of H_2O and no harmful air emissions (Schlapbach and Züttel 2001). As in stationary applications, some of the vehicle FCs can reform H_2 from a variety of fuels. FC technology has been demonstrated in a variety of vehicles including cars, buses, mopeds and even locomotives (US Fuel Cell Council 2004c). Proton exchange membrane (PEM) fuel cells are the most typical FCs for cars and buses, but also solid oxide FCs (SOFCs) are being evaluated (US Fuel Cell Council 2004c; Sequeira et al. 2007)

In addition to the FCVs, hydrogen can be used to fuel combustion engine vehicles. Hydrogen fueled combustion engine is less efficient (about half) than the hydrogen fuel cell (Schlapbach and Züttel 2001). However, H_2 fueled combustion engines are still more efficient than conventional diesel or gasoline engines (Das 1996). The emissions of H_2 fueled combustion engines compose mainly of water and some NO_x, but no CO, CO_2, SO_x, VOC and particulates as in the case of diesel and gasoline engines (Das 1996). The emission levels for road vehicles will have stricter terms in the future. European Union directive (Euro 5 standard) of cutting particulate and NO_x emissions in road vehicles will enter into force in September 2009 (European Commission 2008), and further emission cut regulations are projected in 2014 (Euro 6 Standard).

The use of H_2 as vehicle fuel has been greatly hindered by the lack of efficient onboard storage systems (Das 1996). To make an example, a regular car uses about 24 kg of gasoline per 400 km (7.5 L per 100 km). To cover the same range, a FCV needs about 4 kg of H_2 (Schlapbach and Züttel 2001). At room temperature and at atmospheric pressure, 4 kg of H_2 would take a volume of 45 m^3 corresponding to a balloon with a diameter of 5 m (Schlapbach and Züttel 2001). Alternatively, 4 kg of H_2 would correspond approximately 57 L of liquid H_2.

Ethanol as vehicle fuel

Ethanol is a very flexible vehicle fuel. Vehicles can run on 100% ethanol or it can be mixed with gasoline in any proportions. In the U.S., car manufacturers have developed vehicles called flexible fuel vehicles (FFV), which can use ethanol gasoline blends from 0 up to 85% of ethanol (US DOE 2006, 2007a). The FFVs include some modifications in materials and engine calibration system compared to regular cars (US DOE 2006). Presently in the U.S., the focus is on utilizing E85, a fuel mix containing 85 % of ethanol and 15% of gasoline (US

DOE 2006). Supplementation of 15% gasoline to ethanol improves the cold-start performance of vehicles (US DOE 2006). In 2007, there were more than 5 million FFVs capable of using E85 and more than 1000 fueling stations selling E85 (US DOE 2007a).

The advantages and disadvantages of ethanol fuel are listed in Table 4. In brief, ethanol engines have higher efficiency and result in less air emissions (except for aldehydes) than gasoline engines (Kosaric and Vardar-Sukan 2001; Niven 2005). The sustainability of fuel ethanol has been debated. Ethanol has low to negative (-80 to +40%) net energy value (NEV), i.e., energy needed in ethanol production over the life cycle versus energy released in ethanol combustion (for a review, Niven et al. 2005). The NEV is highly case dependent and is influenced by e.g., geography, climate and crop species, and by its cultivation, transportation and conversion processes. Regarding the life cycle analysis of gasoline-ethanol blends, the E10 fuel (10% of ethanol in fuel) offers only a minor or no advantages in greenhouse gas emissions and energy efficiency, and may increase the production of photochemical smog as compared to gasoline (for a review, see Niven 2005). E85 may decrease significantly the green house gas emissions, but substantial amounts of aldehydes are produced (27 times more than gasoline) (for a review, see Niven 2005).

Table 4. Advantages and disadvantages of ethanol as a vehicle fuel compared to gasoline (Kosaric and Vardar-Sukan 2001; Niven 2005; U.S. DOE 2006)

Advantages (+) Disadvantages (-)
Technical aspects
+ Higher efficiency in engines
+ Better fire safety (however, ethanol flame is less visible than gasoline)
+ 100% ethanol provides about 5% increase in engine power
- Lower energy density, higher fuel consumption per km
- 100% ethanol engines have poor cold start-up (additions of ether, benzene or gasoline needed)
- Larger evaporation losses than with gasoline
- Ethanol-gasoline blends have low water tolerance (small amounts of water can separate ethanol and gasoline)
- Ethanol can dilute the lubrication oils and be harmful to the engine
Environmental and public health aspects
+ Renewable, less CO_2 emissions (depending on the process and gasoline blend)
+ Less NO_x and CO emissions (depending on the gasoline blend)
+ Less particulate emissions
+ Lower benzene, VOC and 1,3 butadiene emissions
+ Less toxic than gasoline
- Ethanol-gasoline blends may cause more diverse contamination effects to soil and ground water than gasoline
- Higher aldehyde emissions (aldehydes highly irritant to mucous membranes, and a probable carcinogen)
- Large scale production of ethanol feedstock causes threats to decrease biodiversity
Socio-economical aspects
+ Domestic production, may increase energy security
+ Supports farmers and rural economies
- Lower net energy value than that of gasoline (fuel energy – energy needed for production and transportation)
- Uneconomic compared to gasoline, subsidies required
- The production of ethanol feedstock competes with food production, impacts food availability and prices
- Large land areas required for ethanol feedstock production

Hydrogen fuel cells

Hydrogen fuel cells (FCs) can be used to provide electricity for mobile (FC vehicles), stationary (electricity and heat power plants) and portable applications (digital devices, tools, power generators) (Dincer 2002; Busby 2005; Agnolucci 2007; Sequeira et al. 2007). FC technology is clean, quiet and flexible, and offers significant improvement in energy efficiency obtained with present technologies (Dincer 2002). Further, the durability and reliability of FCs have been demonstrated (Dincer 2002). In hydrogen FCs, H_2 combines with oxygen generating electricity, water and heat. Low temperature FCs provide emission free generation of electricity with high efficiency (in high temperature FCs small amount of NO_x is produced) (Schlapbach and Züttel 2001; Dincer 2002). Several kinds of fuel cells, distinguished by the type of electrolyte used, have been developed to meet the demands of different applications (Sequeira et al. 2007) (Table 5). Presently, the FC technology is in pre-

commercialization phase with several FC types commercially available, while the full commercialization still faces numerous technical and market uncertainties (for a review, see Hellman and van den Hoed 2007).

On-line, laboratory-scale electricity generation by proton exchange membrane fuel cell (PEMFC) connected to H_2 dark fermentation bioreactors has been recently demonstrated (Lin et al. 2007a; Jeon et al. 2008). Lin et al. (2007a) reported a stabile, long term (300d), electricity generation with PEMFC combined with a dark fermentation bioreactor. The biogas from dark fermentation reactors need to be carefully purified to remove H_2S, CO and CO_2, as these impurities may be detrimental to the FC materials (Levin et al. 2004).

Table 5. Hydrogen fuel cells (FCs) (for reviews, see Dincer 2002; McLean et al. 2002; US DOE 2002; Levin et al. 2004; Busby 2005; Sequeira et al. 2007)

FC type	Temperature range (°C)	Efficiency (%)	Capacities (kW)	Applications	Features
Polymer or proton exchange membrane fuel cell (PEMFC)	60 - 120	40 - 60	100 - 250	Vehicles, stationary power, military, portable	• Being applied in many passenger vehicles cars and buses • Potential uses to replace batteries in mobile applications + High power density + Operate at low temperatures, fast start-up + Flexible (rapid response to power demand) - Sensitive to impurities (CO_2, CO, H_2S)
Phosphoric acid fuel cell (PAFC)	150 - 220	37 - 55 (72 – 80)[a]	25 – 20000	Stationary power, large vehicles	• Arguably most developed FCs for commercial use • More than 400 trial installations worldwide + Reliable
Solid oxide fuel cell (SOFC)	800 – 1100	45 – 70 (70 – 85)[a]	200 - 5000	Stationary power, large vehicles	• 15 demonstration units installed • Typically applied as stacks of FCs + High tolerance to impurities + High power densities + Low value catalyst can be used - High temperatures required, long startup
Alkaline fuel cell (AFC)	23 - 250	50 -60	2 - 100	Vehicles, space missions, military	• Interest faded due to PEMFCs + Operate at low temperatures, fast start-up + Can achieve long lifetimes - Sensitive to impurities (CO_2, CO)
Molten carbonate fuel cell (MFC)	600 - 660	45 - 65 (70 – 85)[a]	250 - 3000	Stationary, space missions, military	• For large scale industrial or power applications + High efficiency + Relatively tolerant to impurities - High temperatures required, long start-up - Corrosive electrolyte limits durability

[a] with heat recovery

Hydrogen storage

The storability is one of the most important advantages of H_2 as energy carrier. However, the low volumetric energy content of H_2 gas compared to other fuels poses a problem for energy storage (Schlapbach and Züttel 2001; Busby 2005). The main H_2 storage methods today, compression (350 to 700 bar, in steel or lightweight composite tanks) and liquefaction, provide insufficient increase in energy density (for mobile applications) and also consume energy – about 10 % H_2's energy content is lost in compression and 30 to 40 % in liquefaction, respectively (Busby 2005; Riis et al. 2006b). Alternatively, better storage capacity may be achieved by binding H_2 in storage materials (through absorption, adsorption or chemical reactions), which also increase the safety of H_2 storage by preventing the evaporation and burning (Busby 2005; Biniwale et al. 2007) (Table 6). However, these methods are still struggling to overcome the weight penalty associated with the storage matrixes (Busby 2005). Other issues involved in the H_2 bonding matrixes include the cost, stability, recyclability and toxicity of the storage materials (Sakintuna et al. 2007) (Table 6). In comparison, the International Energy Agency (IEA) targets for H_2 storage systems in fuel cell vehicles (FCVs) include 5.0 $wt\%_{H2}$ of storage medium density, and with 80 °C H_2 liberation temperature (Riis et al. 2006b). The majority of H_2 today is produced on-site

without needs for storage or long-distance distribution (Busby 2005). For large scale H_2 storage, possibilities include salt caverns, depleted gas fields, and empty mines (Busby 2005).

Table 6. Hydrogen storage methods

Method	Storage capability (kg_{H2} m^{3-1})	Advantages (+) and disadvantages (-)	References
Compression (350 to 700 bar)	20.7 (350 bar) 30.0 (700 bar)	+Simple and well established process - Low volumetric energy density - Filling requires high pressures and some energy - Hydrogen losses by leakage - Low safety	for reviews, see Schlapbach and Züttel 2001; Busby 2005; Riis et al. 2006b
Liquefaction	33.3	+Well established process + Relatively high energy density in storage + Low storage pressure - Liquefaction requires large energy - Hydrogen losses by evaporation (short term storage) - Low safety	for reviews, see Sherif et al. 1997; Busby 2005; Riis et al. 2006b
Glass microspheres	5.4 wt%	+ Relatively safe (H_2 stored at low pressures) + Relatively high energy density in storage + Low cost containers can be used - Filling, and H_2 release require high temperature (300 °C) - Filling requires high pressures - Hydrogen losses by leakage	for reviews, see Dincer 2002; Riis et al. 2006b
Metal hydrides - Magnesium hydrides - Lithium hydrides	 7.7 wt% 96 (10 wt%)	+ Safe + High energy density in storage + Good reversibility in several hydrides + Low storage pressures - Heavy storage matrix - Hydrides with best storage capabilities require high temperatures for H_2 release - Heat exchanger required	Pinkerton et al. 2005; for reviews, see Busby 2005; Bououdina et al. 2006; Riis et al. 2006b; Sakintuna et al. 2007
Borohydrides ($NaBH_4$)	116 (10.8 wt%)	+ High energy density in storage + H_2 release at room temperature ($NaBH_4$) + Safe and controllable onboard H_2 generation + Low storage pressures - Storage medium must be regenerated off-board - Expensive, suitable for high-value applications	For reviews, see Riis et al. 2006b; Biniwale et al. 2007; Sequeira et al. 2007
Rechargeable organic liquids Cycloalkanes (e.g. methylcyclohexane)	62 (8.6 wt%)	+ Low storage pressures + High energy density in storage - High temperatures required for H_2 release (cycloalkanes) - Heat exchanger required (cycloalkanes) - Toxicity	for reviews, see Fakioglu et al. 2004; Riis et al. 2006b
Nanotubes - Carbon nanotubes (or nanofibres) - Boron nanotubes	 7.4wt% 2.6wt%	+ High theoretical H_2 storage capabilities + Light storage matrix + Plenty of raw material (carbon) available for their construction - Still in laboratory scale, no practical applications - High temperatures and/or pressures required - Difficulties in constructing the nanotubes	for reviews, see Lamari Darkrim et al. 2002; Hirscher et al. 2003; Fakioglu et al. 2004; Riis et al. 2006b

2.4 Hydrogen economy

Bockris, who has contributed to the development of the concept "hydrogen economy", defines it as "the utilization of hydrogen to transport energy from renewable sources over large distances; and to store it (for supply to cities) in large amounts" (Bockris 2002). Thus, hydrogen economy includes the production, storage, distribution, and use of hydrogen as an energy carrier (Turner 2004). The term of hydrogen economy was developed in the early 1970s by technicians of General Motors (Bockris 2002; Hoffmann 2002). However, the concept of hydrogen economy was developed far earlier (Dunn 2002; Turner 2004). In 1874, Jules Verne stated in the book of Mysterious Island that "water will be the coal of the future" (Verne 1874). The view by Jules Verne was based on the recognition of finite supply of coal and the possibilities of producing hydrogen by water electrolysis (for a review, see Turner 2004). Later, in the 1920s, Haldane developed the concept by including the H_2 energy storage potential, integrated it with H_2 production by wind-power, and estimated costs and social advantages of hydrogen transition (for reviews, see Hoffman 2002; Busby 2005).

The main drivers and barriers of hydrogen economy are listed in Table 7. European Commission has outlined that hydrogen economy would aid in sustaining high life standard,

and simultaneously providing a clean, safe, reliable and secure energy supply (European Commission 2003b). The main barriers of hydrogen economy are related to the immaturity of technology, the lack of commercially competitive technology and infrastructure, and economical risks.

The potential climate impacts of hydrogen economy are not well known. The potential environmental concerns of H_2 economy may be the increase in water vapor in the stratosphere due to unintended H_2 emissions (Tromp et al. 2003). The increase in water vapor can cause stratospheric cooling, increase ozone degradation, and change tropospheric chemistry and atmosphere-biosphere interactions (Tromp et al. 2003).

Table 7. Main drivers and barriers of hydrogen (and ethanol) as energy carrier (Wyman 1999; US Energy Agency 2002; Dunn 2002; Gosselink 2002; Australian Government 2003; European commission 2003b; Busby 2005; Marban and Solis 2007; van Ruijven et al. 2007).

Drivers of H_2 utilization as energy carrier
Environmental and public health impacts related to the use of fossil fuels • Global climate change • Air and water pollution • Acid rain
Energy security and decreases in geopolitical and price instability • Depletion of fossil fuel resources • Decreasing the dependency of fossil fuel • Geographical distribution of fossil fuel resources and its political impacts
Increasing energy demand • Growth of population • Economic growth, growth in energy intensity and standard of living
Technological advantages • Increasing energy efficiency • Increasing flexibility for balancing centralized and de-centralized energy systems
Unsafety of present energy production technologies (e.g. nuclear energy)
High market potential, growing markets
Energy production integrated with waste disposal
Directives and regulations • E.g., the proportions of renewables in vehicle fuel
Barriers of H_2 utilization as energy carrier
Immaturity of technology, lack of commercially competitive technologies • Hydrogen production, fuel cells, storage and distribution • CO_2 sequestration technologies • Fuel cell vehicles
Lack of infrastructure and high costs of creating it • Hydrogen production, fuel cells, storage and distribution
Difficulties in finding national, regional and global consensus for priorities in energy solutions
Availability of lower cost energy alternatives (e.g., coal, natural gas and nuclear power) • Prices for fossil fuels not high enough • Energy prices not yet affecting economic growth enough
Hydrogen safety issues
Market risks involved in H_2 transition
Doubts of sustainability • Non-renewable H_2 production pathways • Climate effects of H_2 unknown (Tromp et al. 2003)
Concerns and uncertainties of new technologies • Lack of knowledge

3 HYDROGEN PRODUCTION METHODS

Large-scale H_2 production was started in France as early as late 1700s (for a review, see Hoffmann 2002). First commercial hydrogen electrolyzers were established in the late 1920s, and in the 1960s the H_2 production shifted to fossil-fuel feedstock (Hoffmann 2002; Riis et al. 2006a). Today, the majority of hydrogen is produced from fossil fuels with the main methods being steam reformation of natural gas (48% of H_2 globally) or partial oxidation of coal (30 %) or oil (18%) (US Energy Agency 2002; National Hydrogen Association 2007a). Hydrogen production through water electrolysis (only 4% of global production) is considered feasible only when low-cost electricity is available or when high H_2 purities are required (Busby 2005; Riis 2006a; National Hydrogen Association 2007a).

The production of H_2 from fossil fuels results in greenhouse gas emissions (unless CO_2 sequestration is used), and therefore, H_2 economy based on renewable sources is more favorable (Turner 2004; Busby 2005; Riis et al. 2006a; National Hydrogen Association 2007a). The potential feedstock and process technologies for hydrogen production are numerous (Riis et al. 2006a) (Table 8). The H_2 production technologies include (thermo)chemical, electrolytic, photolytic, radiolytical and biological processes (Lay et al. 1999; Riis et al. 2006a).

In steam reforming, methane is mixed with water steam at high temperature and pressure producing a mix of H_2, and CO (syngas) (Riis et al. 2006a). The CO can be converted to H_2 (and CO_2) through water-gas shift reaction (Nath and Das 2003; Ni et al. 2006a,b; Riis et al. 2006a). The net conversion efficiency of the process is about 65% due to high energy inputs of the reforming process (Dincer 2002).

Nearly all kinds of biomass or fossil fuels can be thermally converted H_2 and other fuels via gasification or pyrolysis (Busby 2005; Ni et al. 2006b). Gasification aims at forming gaseous products, while pyrolysis aims at liquid products (Ni et al. 2006a). Pyrolysis is thermal decomposition of organic matter occurring in the absence of oxygen (for reviews see, Ni et al. 2006a; Sequeira et al. 2007). The main products of pyrolysis include H_2, CO_2, CO and hydrocarbon gases, liquids (oils, solvents and tar) and solid charcoal (for reviews, see Ni et al. 2006a,b). In gasification, the feedstock undergoes partial oxidation at high temperature (above 1000 K) producing gas (syngas: H_2, CO_2, CO, CH_4 and hydrocarbons), tar and charcoal (for reviews, see Ni et al. 2006a; Riis et al. 2006a; Florin and Harris 2007). The tar and charcoal fraction from pyrolysis and gasification can be further oxidized to H_2, CO_2 and CH_4 (Ni et al. 2006;a Florin and Harris 2007).

Electrolyzers have high energy efficiencies from 60 up to 85%, and the H_2 production process does not produce CO_2 emissions provided that the electricity for the operation is produced from renewable sources (Dincer 2002; Turner 2004; National Hydrogen Association 2007d). The need of electricity in water splitting decreases with increasing process temperature (Turner 2004; Riis et al. 2006a).

Table 8. Hydrogen production methods. The renewable feedstock are emphasized.

Process	Feedstock	Energy required	Emission(s)	Reference(S)
Thermal				
Steam reforming	Natural gas	Water steam at high temperature (700 to 850 °C) and pressure (3 to 25 bar)	CO_2, NO_x	for reviews, see Milne et al. 2001; Riis et al. 2006a; Ni et al. 2007
	Biogas or Bioethanol		NO_x	
	Methanol or **Biomethanol**	Water steam at 150 to 300 °C	CO_2, NO_x	Palo et al. 2007
Thermochemical gasification (partial oxidation)	Coal	Water steam and oxygen at high temperature(> 700 °C) (and pressure)	CO_2, NO_x	for reviews, see Milne et al. 2001; Nath and Das 2003; Ni et al. 2006a ; Riis et al. 2006a
	Heavy oil		CO_2, NO_x	
	Biomass or gas, organic wastes		NO_x	
Thermal pyrolysis	**Biomass, organic wastes**	Water steam at moderately high temperature (350 to 550 °C)	NO_x	for reviews, see Milne 2001; Ni et al. 2006a
Autothermal reforming (combination of steam reforming and gasification)	Natural gas	Water steam and oxygen at high temperature (800 – 1000 °C) and pressure (100 bar)	CO_2, NO_x (lower than in steam reforming)	for reviews, see Dincer 2002; Ni et al. 2006a ; Riis et al. 2006a
	Biogas or bioethanol		NO_x	
Direct solar gasification	**Biomass and wastes**	Heat (by sun light)	No	for reviews, see Milne et al. 2001; Nath and Das 2003
Supercritical gasification	**Biomass**	Water at super critical state (high pressure and temperature (400 to 700 °C)	No	for reviews, see Milne et al. 2001; Nath and Das 2003; Ni et al. 2006a
Water thermolysis	**Water**	Heat, high temperature required (~3000 °C)	No	for reviews, see Busby 2005, Riis et al. 2006a; Ni et al. 2006b
Electrochemical				
Electrolysis (Conventional or high temperature)	**Water**	**Electricity from renewables** (Solar photovoltaics, solar thermal, hydro or wind power, geo or ocean thermal energy or biomass)	No	for reviews, see Busby 2005; Riis et al. 2006a; National Hydrogen Association 2007d
	Water	Electricity from nuclear power	Nuclear waste from electricity production	for reviews, see Busby 2005 National Hydrogen Association 2007c
	Water	Electricity from fossil fuels	CO_2	for a review, see Busby 2005
Photoelectrochemical	**Water**	Direct sunlight	No	Dincer 2002
Thermochemical				
Thermochemical water splitting	**Water**	Heat (450 – 1000 °C) (from nuclear or solar energy)	Nuclear waste from heat production	for reviews, see Turner 2004; Riis et al. 2006a; National Hydrogen Association 2007c
Thermochemical H_2S splitting	H_2S			Ni et al. 2006b
Radiolytical	**Water**	High energy particles in nuclear reactor	Nuclear waste	Dincer 2002
Clean-up of industrial off-gases	Off-gas	Depends on application	Depends on application	Dincer 2002
As a by product .e.g. in the petroleum and chlorine manufacture	Petroleum, chlorine	Electricity, heat	Depends on application	Busby 2005
Biological				
Direct and indirect photolysis	**Water**	Light	No	for reviews, see Benemann 1996; Ghirardi et al. 2000; Das and Veziroĝlu 2001; Lopes Pinto et al. 2002; Melis 2002; Tamagnini et al. 2002
Photo and dark fermentation	**Biomass, organic wastes and waste waters**	Light (photofermentation), heating (< 80 °C)	Minor emissions of H_2S	for reviews, see Claassen et al. 1999; Das and Veziroĝlu 2001; Hallenbeck and Benemann 2002; Nath and Das 2003; Kapdan and Kargi 2006)
Electrochemically assisted microbial fuel cells	**Organic waste waters**	Electricity	Depends on electricity production method	Liu et al. 2005a,b, for reviews, see Angenent et al. 2004; Logan 2004; Hawkes et al. 2007
Enzymatic H_2 production	**Sugars (glucose, sucrose)**	Heat	No	Woodward and Orr. 1998; Woodward et al. 2000a,b.

3.1 Microbiological hydrogen production

A variety of microorganisms produce hydrogen in their metabolism. Some photosynthetic organisms, such as green algae and cyanobacteria produce H_2 directly from water. Photo and dark fermenting bacteria produce H_2 from organic substrates. Hydrogen production is due to reductant disposal catalyzed by two families of enzymes, hydrogenases and nitrogenases (Claassen et al. 1999, for reviews see Vignais et al. 2006; Vignais and Billoud 2007). The nitrogenase complex catalyzes the reduction of nitrogen gas to ammonia according to the following equation (for reviews, see Tamagnini et al. 2002; Vignais et al. 2006)

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi.$$

In the absence of N_2, the total electron flux is directed to the production of H_2. The reaction is irreversible, and can produce H_2 even at 100% hydrogen atmosphere, but is energy intensive requiring large quantities of ATP (Vignais et al. 2006). Hydrogenases catalyze the simplest of chemical reactions, the formation of molecular H_2 (for a review, see Vignais and Billoud 2007)

$$2H^+ + 2\ e^- \leftrightarrow H_2$$

The reaction is reversible and some of the enzymes are committed to consume H_2 (uptake hydrogenases, in the presence of electron acceptors), some to produce H_2 (in the presence of electron donor of low redox potential), or some can catalyze both reactions (bidirectional hydrogenases) depending on the environmental conditions (for reviews, see Vignais et al. 2006; Vignais and Billoud 2007). Most of the known hydrogenases are iron-sulfur proteins with two metal atoms at their active site, either Ni and Fe atoms ([NiFe]-hydrogenases) or two Fe atoms ([FeFe]-hydrogenases) (for a review, see Vignais and Billoud 2007).

The mechanisms, advantages and disadvantages of microbiological H_2 production methods are presented in Table 9.

Table 9. Microbial hydrogen production mechanisms − advantages and disadvantages

Mechanism	Organisms	Advantages	Disadvantages	References
Direct photolysis $2H_2O \xrightarrow{Light\ energy} 2H_2 + O_2$ **Indirect photolysis** $6H_2O + 6CO_2 \xrightarrow{Light\ energy} C_6H_{12}O_6 + 9O_2$ $C_6H_{12}O_6 + 6H_2O \rightarrow 12H_2 + 6CO_2$	Green algae, cyanobacteria	+ H_2 production from water + No need for organic electron donors (other than CO_2) + Higher solar light conversion efficiency than plants	- Discontinuous process - Low production rates - End product inhibition by O_2 - O_2 in the product gas may cause fire hazard	for reviews, see Benemann 1996,1997; Das and Veziroğlu 2001; Levin et al. 2004; Kapdan and Kargi 2006; Kovacs et al. 2006
Water-gas –shift reaction CO (g) + H_2O (l) → H_2 (g) + CO_2 (g)	Photohetero-trophic bacteria	+ Can remove CO (purification of CO from gas streams) + Relatively high production rates + No light required	- CO_2 present in the product gas - Low mass transfer	for reviews, see Benemann 1996,1997; Levin et al. 2004
Photofermentation $C_6H_{12}O_6 + 6H_2O \xrightarrow{Light\ energy} 12H_2 + 6CO_2$	Photohetero-trophic bacteria, purple, non-sulfur bacteria	+ H_2 production from various carbohydrates and organic wastes - High hydrogen yields, relatively high production rates + Can oxidize organic acids	- CO_2 present in the product gas - Effluent treatment required - Low light energy conversion efficiency - Impurity of product gas, traces of H_2S etc. - Requires light, discontinuous if not illuminated	for reviews, see Claassen et al. 1999; Das and Veziroğlu 2001; Hallenbeck and Benemann 2002; Levin et al. 2004; Kapdan and Kargi 2006
Dark fermentation e.g., $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2$	Heterotrophic bacteria	+ H_2 production from various carbohydrates and organic wastes + High H_2 production rates + No light required + Simpler process for engineering than the others + H_2 can be produced along with the high-value compounds (e.g. glucogenic acid and 1,3-propanediol)	- CO_2 present in the product gas - Incomplete oxidization of organic materials to H_2, low H_2 yields - Effluent treatment required - Impurity of product gas, traces of H_2S etc.	for reviews, see Benemann 1996; Nandi and Sengupta 1998; Das and Veziroğlu 001; Hallenbeck and Benemann 2002; Levin et al. 2004; Kleerebezem and van Loosdrecht 2007
Electrochemically assisted microbial fuel cells (EAMFC) $C_6H_{12}O_6 + 6H_2O \xrightarrow{Electric\ energy} 12H_2 + 6CO_2$	"Anodophilic" bacteria	+ Can oxidize organic acids + High H_2 yield	- CO_2 present in the product gas - Low efficiency - Low H_2 production rates	for reviews, see Angenent er al. 2004; Logan 2004; Hawkes et al. 2007; Cheng and Logan 2007

Direct and indirect photolysis

Some green algae and cyanobacteria are able to produce H_2 from water by direct or indirect photolysis. The H_2 production through photolysis is discontinuous, because it suffers from the feed back inhibition of the hydrogenase activity by oxygen (for reviews, see Benemann 1996; Melis 2002). Therefore, the H_2 and O_2-producing reactions need to be temporally or spatially separated (for a review, see Ghirardi et al. 2000). There are several ways of achieving this. Some non-heterocystous cyanobacteria achieve the temporal separation by using day-night cycles (for a review, see Benemann 1996). Alternatively, the sulfur deprivation of green algae *Chlamydomonas reinhardtii* results in the temporal H_2 production in light through the inhibition of oxygenic photosynthesis (for reviews, see Ghirardi et al. 2000; Melis 2002). Continuous H_2-production for over 5 months by *C. reinhardtii* has been reported in a two-stage photobioreactor combining aerobic phase in sulfur limitation and anaerobic, H_2 production phase (Fedorov et al. 2005). In heterocystous cyanobacteria (e.g. *Anabaena*), H_2 production occurs through indirect photolysis in spatially separated heterocysts under N_2-starvation by nitrogenase activity (for reviews, see Benemann 1996,1997; Asada and Miyake 1999).

Water gas-shift reactions

Some photoheterotrophic bacteria or archaea produce H_2 by using so called water-gas shift reaction pathway – utilizing CO as a sole energy source for ATP generation with the generation of CO_2 and H_2 (for reviews, see Levin et al. 2004; Henstra et al. 2007). The reaction is thermodynamically very favorable with a $\Delta G^{0'}$ of -20 kJ mol^{-1} (Levin et al. 2004). Reaction occurs both in light and dark, but in light H_2 is not accumulated. This is due to the oxidation of H_2 by uptake hydrogenases for supporting CO_2 fixation (Maness and Weaver 2002). The bacteria can potentially be used in the treatment of synthesis gas (CO and H_2) derived from the biomass gasification, or alternatively to remove CO from the gas stream prior to utilization in fuel cells (Maness and Weaver 2002; Merida et al. 2004; for a review, see Henstra et al. 2007). In addition to water-gas shift reaction, some of these bacteria, such as *Rhodopseudomonas palustris*, are also capable of photo or dark fermentative H_2 production (Oh et al. 2004c).

Photo and dark fermentations

In fermentation, H_2 is produced in the oxidation of organic compounds either with help of light energy (photofermentation) or without it (dark fermentation) (Claassen et al. 1999). Dark H_2 fermentation results in incomplete oxidization due to thermodynamic restrictions (see Chapter 6). Photofermentations enable the complete oxidization of carbohydrates to H_2 and CO_2, since these bacteria use the energy from sunlight to overcome the thermodynamic restrictions faced by dark fermenters. Generally, dark fermentations have higher H_2 production rates, but photofermentation processes have higher theoretical yields of H_2 (Das and Veziroğlu 2001). High H_2 yields have been achieved by integrating dark and photofermentation processes (see Chapter 8).

Photofermentative hydrogen production by some purple, non-sulfur bacteria occurs in nitrogen-deficient conditions by nitrogenases (Zürrer and Bachofen 1979; Fedorov et al. 1998; Levin et al. 2004). Generally, species belonging to the genera *Rhodobacter, Rhodomicrobium, Rhodopseudomonas*, or *Rhidospirillum* have been used in the photofermentation studies (Zürrer and Bachofen 1979; Segers and Verstraete 1983; Zhu et al. 1999; for a review, see Claassen et al. 1999). The fermentation rates are influenced by factors, such as pH, temperature, light intensity and wavelength and concentration and C/N values of the substrates (Hillmer and Gest 1977; Claassen et al. 1999). The substrate range for

photofermenters is wide (de Vrije and Claassen 2003), and photofermentative H_2 production have been demonstrated from several wastewaters (Zürrer and Bachofen 1979; Zhu et al. 1999). Dark fermentations will be discussed in detail in the following chapters, 5 to 8.

Microbial fuel cells

Microbial fuel cells (MFCs) use bacteria as catalysts to generate electric current while oxidizing organic and inorganic compounds (for reviews, see Rabaey and Verstraete 2005; Logan et al. 2006). Electricity production has been demonstrated from various substrates including carbohydrates (Rabaey et al. 2003,2005, Bond and Lovley 2003; Logan 2004), organic acids (Liu et al. 2005a,b; Oh and Logan 2005; Cheng and Logan 2007) and even from domestic or industrial wastewaters (Liu et al. 2004a; Min and Logan 2004; Oh and Logan 2005; Min et al. 2005; Rabaey et al. 2007) or organic matter in marine sediments (Bond et al. 2002). These anodophilic bacteria are able to mediate the electrons, released in the oxidation of electron donors, to extracellular anode electrode, where the electrons are passed through an external circuit to cathode, thus producing electric current (for reviews, see Logan 2004; Rabaey and Verstraete 2005; Logan and Regan 2006). A modification of a microbial fuel cell, called an electrochemically assisted microbial fuel cell (EAMFC) or a bioelectrically assisted microbial reactor (BEAMR), can produce hydrogen from carbohydrates (Cheng and Logan 2007; Liu et al. 2008; for a review, see Hawkes et al. 2007). The voltage applied on EAMFC (~300 - 410 mV with acetate) is substantially lower than that required for the electrolysis of water (1800 – 2000 mV) (Liu et al. 2005b,2008; Logan and Grot 2006). EAMFCs can be potentially used to treat the effluent of dark fermentation bioreactors (Logan 2004; Liu et al. 2005a,b; Oh and Logan 2005; Cheng and Logan 2007) – the voltage applied on the electrodes is able to overcome the energy barrier of hydrogen production from organic acids (Liu et al. 2008). With EAMFCs, high H_2 yields have been obtained from various organic acids, e.g. up to 3.95 mol-H_2 mol-acetate^{-1} (99% from theoretical maximum), or 8.01 mol-H_2 mol-butyrate^{-1} (80%), or 5.45 mol-H_2 mol-lactate^{-1} (91%) (Cheng and Logan 2007).

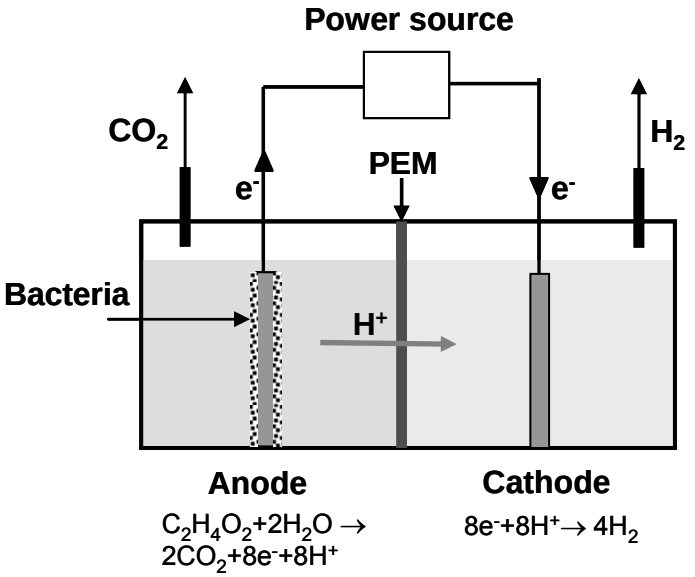


Figure 1. General schematic of electrochemically assisted microbial fuel cell (EAMFC) producing H_2 and from acetate (Adapted from Liu et al. 2005b). PEM = proton exchange membrane.

Comparison of H_2 production rates between different biological H_2 production systems

Table 10 compares the H_2 synthesis rates reported with different microbiological H_2 production systems. Highest H_2 production rates so far have been achieved with dark fermentation processes followed by photofermentation and water-gas shift reactions. The H_2 synthesis rates of direct and indirect photolysis have been far lower. Based on the calculations of Levin et al. (2004) using the highest H_2 production rate achieved with dark fermentation (627 mmol h^{-1} L^{-1}, Wu et al. 2006) , the volume of bioreactor required to provide H_2 for a 5 kW PEMFC would be about 190 L. In practice, the main concern is the scalability of the reactors, i.e., maintaining the high H_2 synthesis rates when scaling up the reactors (Levin et al. 2004). Further, the highest H_2 production rates have been obtained with model carbohydrate compounds (sucrose) while high rate H_2 production from real waste materials is yet to be proven.

Table.10. Comparison of H_2 synthesis rates of different microbiological H_2 production systems (Modified from Levin et al. 2004).

BioH_2 systems	**H_2 synthesis rate (mmol H_2 h^{-1} L^{-1})**	**Reference**
Direct photolysis	0.166	Kruse et al. 2005
Indirect photolysis	1.15	Weissman & Benemann 1977
Photo fermentation	158	Tsygankov et al. 1998a;b, in Levin 2004
Water-gas shift reaction	96.0	Levin et al. 2004
Dark fermentation	627	Wu et al. 2006
Microbial fuel cell	2.13	Cheng and Logan 2007

4 MICROBIAL FERMENTATIONS

Humankind has utilized microbial fermentations for thousands of years (Steinkraus 2004). When man evolved, he needed to consume the food before the microorganisms spoiled it. In some cases, microorganisms produced toxins, which caused illness and man learned to avoid it while in others pleasant flavors and textures were produced and man learned to appreciate such food (Steinkraus 2004). Such fermented food found today include e.g., wine, beer, cheese and yoghurt (Steinkraus 2004). Fermentations also preserve, increase the digestability the food, and enrich vitamins, proteins and amino acids (Steinkraus 2004; McGovern et al. 2004). At present, fermentations are utilized in numerous applications in the fields of food, pharmaceutical, chemical industry, energy production and waste management.

Fermentation is an internally balanced oxidation-reduction process. It takes place in environments, which lack terminal electron accepting compounds, such as oxygen, sulfate, nitrate and ferric iron (Madigan et al. 2000). In fermentation, ATP is produced by substrate level phosphorylation, where high energy intermediates with phosphate groups are formed that can couple energetically with the phosphorylation of ADP (Madigan et al. 2000). ATP yield in fermentation is low compared to the catabolism using terminal electron acceptors (respiration) – in the oxidation of glucose, 1-3 ATP per molecule is produced in fermentation as compared to 38 ATP per molecule generated in aerobic respiration. Due to low ATP yields, substrate level phosphorylation results in high end product formation compared to cell biomass synthesis rates (Madigan et al. 2000).

Fermentations, which do not require light energy have been named as dark fermentations. Fermentative H_2 production is an intermediate stage in the anaerobic degradation of organic material, which is a multistep process with series and parallel sets of reactions (Pavlosthathis and Giraldo-Gomez 1991). In environments lacking terminal electron acceptors, H_2 is produced to maintain the electron balance, i.e., to dispose the excess of electrons liberated in the oxidation of substrates (Madigan et al. 2000). In the nature, however, H_2 is not accumulated due to rapid consumption by H_2 utilizing bacteria, which maintain the low partial pressure of H_2 (pH_2), necessary for the anaerobic digestion to proceed (Madigan et al. 2000).

In the anaerobic degradation, organic polymers are first hydrolyzed by extracellular enzymes to monomers capable of passing through the cell membrane (Figure 2). The monomers are then fermented or anaerobically oxidized to intermediate products including organic acids (often called volatile fatty acids or VFAs) and alcohols and H_2 and CO_2 (acidification). Intermediate products are further degraded to acetate, H_2 and CO_2, which can be converted to methane by methanogenic bacteria (Pavlosthathis and Giraldo-Gomez 1991). Dark fermentation of H_2 from acetate (acetate oxidation) and other intermediated products (organic acids and organic alcohols) is possible only if the pH_2 is kept low in the system. H_2 consuming reactions, such as methanogenesis and homoacetogenesis, are obviously, undesirable in bioprocesses aiming at H_2 production.

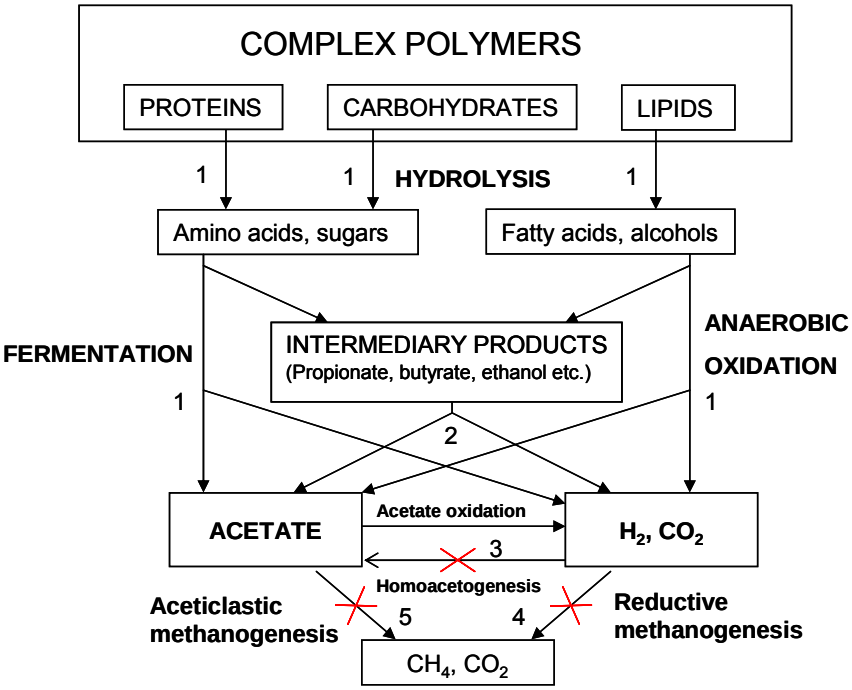


Figure 2. Anaerobic degradation of organic compounds and the microbial groups involved. 1, Fermentative bacteria; 2, hydrogen-producing acetogenic bacteria; 3, hydrogen-consuming acetogenic bacteria; 4, carbon dioxide-reducing methanogens; 5, Aceticlasctic methanogens (modified from Pavlosthathis and Giraldo-Gomez 1991). The crosses represent hydrogen consuming reactions, methanogenesis and homoacetogenesis, which are undesirable in H_2 producing reactors.

5 ETHANOL FERMENTATION

Ethanol production is the largest application of fermentation (Kosaric and Verdar-Sukan 2001). Majority of ethanol today is produced by *Saccharomyces* yeasts (Wiegel 1980; Ingram et al. 1999; Zaldivar et al. 2001; Gray et al. 2006; Lin and Tanaka 2006). An demonstration-scale alternative to ethanol fermentation includes gasification of lignocellulosic biomass to H_2 and CO (syngas), followed by e.g. thermochemical catalysis, such as Fischer-Tropsch process to form ethanol (for reviews, see Angenent 2007; Henstra et al. 2007; Phillips et al. 2007). Also microbiological pathways exist among meso- and thermophilic bacteria for syngas conversion to ethanol, e.g., by *Clostridium ljungdahlii* (Tanner et al. 1993; Abrini et al. 1994; Sakai et al. 2004; for a review, see Henstra et al. 2007).

Feedstock for ethanol fermentation

Corn (starch) and sugar cane (sucrose) are the most utilized feedstock for bioethanol production while several alternatives exist (Table 11) (Gray et al. 2006). Starch can be rather easily hydrolyzed with enzymatic treatment prior to fermentation (Gray et al. 2006). As supplies of presently used bioethanol feedstock are rather limited and compete with food production, lignocellulosic materials are seen as an attractive feedstock of the future (Gray et al. 2006). Lignocellulosic materials such as agricultural residues, forestry wastes, herbaceous and woody energy crops, could serve as low-cost and abundant feedstock for bioethanol (for reviews, see Lynd 1989; Olsson and Hahn-Hägerdal 1996; Wyman 1999; Zaldivar et al. 2001; Gnansounou et al. 2005). Increasing research efforts have recently been directed to lignocellulose-to-ethanol technologies (Angenent 2007), and several demonstration-scale processes exist (Abengoa 2008; Verenium 2008).

Table 11. Feedstock for ethanol production (Kosaric and Verdar-Sukan 2001; Senn and Pieper 2001; Zaldivar et al. 2001; Gnansounou et al. 2005; Lin and Tanaka 2006; Cardona and Sanchez 2007; Angenent 2007).

Feedstock	Geographical distribution
Sugar crops	
Sugar cane	Brazil, India, South Africa, Australia
Sugar beet	France, Europe
Potatoes	Germany and Eastern Europe
Fruits	Global
Starches	
Corn	U.S., South America, Europe
Wheat, Rye, Barley, Triticale (a hybrid or wheat and rye)	Western Europe, Eastern Europe, North America
Cassava (manioc, tapioca)	Brazil, China, Indonesia, Zaire
Sweet sorghum	China, Australia
Sweet potato	North and South America, South-East Asia
Jerusalem artichoke (Topinambur)	North America
Lignocellulosic biomass	
Industrial wastes	Global
- Waste sulfite liquors (pulp and paper industry)	
Forestry wastes	Global
Agricultural wastes	Global
Energy crops	Global
Food industry wastes	Global
Algal and/or cyanobacterial biomass	Global

Lignocellulose, the structural polymer of plants, is far more difficult and expensive to hydrolyze than starch (Ingram et al. 1999; Zaldivar 2001; Gray et al. 2006). In lignocellulose, cellulose (20–50 % of plant dry weight) fibers are embedded in covalently found matrix of hemicellulose (20–40 %), pectin (2–20%) and lignin (10–20%) thus forming very resistant structure for biodegradation (Ingram et al. 1999). Of the lignocellulosic constituents, cellulose, hemicellulose and pectin fractions are suitable for the production of bioethanol by fermentation (Ingram et al. 1999; Zaldivar et al. 2001). Lignin cannot be readily converted to ethanol, but can be used as fuel (i.e., burned) or utilized in the production of bioplastics

(Ingram 1999). The sugar residues of hemicellulose contain a varying mixture of hexoses (e.g., glucose, mannose and galactose), and pentoses (e.g., arabinose and xylose). The pentose sugars of hemicellulose, cellulose (a polymer of glucose), and pectin (a polymer of galacturonic acid) are not utilized by the wild-type strains of the main bioethanol-producing organisms, *Saccharomyces cerevisiae* and *Zymomonas mobilis* (Olsson and Hahn-Hägerdal 1996; Ingram et al. 1999; Zaldivar et al. 2001; Gray et al. 2006). The economically feasible production of ethanol from lignocellulosic materials requires efficient conversion of all the main carbohydrate constituents of this complex material to ethanol (Olsson and Hahn-Hägerdal 1996; Ingram et al. 1999; Zaldivar et al. 2001; Galbe and Zacchi 2002).

Thermophilic microorganisms have been suggested as potential producers of bioethanol from lignocellulosic biomass (for reviews, see Wiegel 1980; Lynd 1989; Lee 1997). Even though research efforts have improved the economical feasibility of lignocellulosic material conversion to ethanol, it still cannot compete with traditional feedstock (Wyman 1999). The steps of lignocellulose conversion to ethanol include (for reviews, see Hamelinck et al. 2005; Hahn-Hägerdal et al. 2006):

(1) Growing, harvesting, storing, and transporting of feedstock
(2) Pretreating lignocellulosic feedstock to open the cell-wall matrix and to remove lignin
(3) Exposing the feedstock to a mixture of purified enzymes to hydrolyze hemicellulose and cellulose to five- and six-carbon sugars
(4) Fermenting these sugars to ethanol
(5) Separating the produced ethanol

Improvements are needed in each of these steps to make the technology more cost-effective (Angenent 2007). From microbiological point-of-view, more effective and cost-efficient enzymes for the hydrolysis of feedstock, and more robust and efficient fermentative microorganisms are required (Gray et al. 2006; Angenent 2007). Solid-substrate fermentation of lignocellulosic materials by fungi, yeast and bacteria has been demonstrated, but the technology is not mature yet (Chinn et al. 2006, 2007, 2008; for reviews, see Lee 1997; Pandey et al. 2000; Pandey 2003). Many white-rot basidiomycetes (e.g., *Phanerochaete chrysosporium* and *Phlebia radiate*) and some actinomycetes have been employed for production of lignin-degrading enzymes and for delignifying lignocellulosic materials via solid-state fermentation (for a review, see Lee 1997).

Ethanol-producing microorganisms

Ethanol-producing microorganisms include yeasts, fungi and mesophilic and thermophilic bacteria (Table 12).The desired characteristics for ethanol fermentation microorganisms include the following (Kosaric and Vardar-Sukan 2001; Zaldivar et al. 2001 Dien et al. 2003).

• High ethanol yield per unit substrate oxidized and high ethanol productivity
• Broad substrate utilization range
• Substantial ethanol tolerance (> 4%)
• Minimal byproduct formation
• Robust growth (tolerance to changes in environmental parameters) and simple nutrient requirements
• Ability to grow on undiluted hydrolysates, and tolerance to inhibitors

Table 12. Ethanol-producing microorganisms (Adapted from Wiegel 1980; Olsson and Hahn-Hägerdal 1996)

Genus	Species	M/T[a]	EtOH yield hexose^{-1} [b]	EtOH yield xylose^{-1} [c]	References
Bacteria					
Bacteroides	*polyfragmatus*	M	1.07	0.73-1.01	Patel 1984; Patel et al. 1986
Geobacillus	*stearothermophilus*	T	1.0	N.A.	Atkinson et al. 1975
Clostridium	*thermocellum*	T	0.7 - 1.6	N.A	Sudha Rani et al. 1997; Ng et al. 1981; Bothun et al. 2004; for a review, see Zeikus 1980;
	saccharolyticum	M	1.2 - 1.6	0.9-1.0	Khan and Murray 1982; Asther and Khan. 1985
	uzonii	T	0.29 - 0.76	N.A.	Krivenko et al. 1990
Erwinia	*amylovora*	M	0.3 -1.5	N.A.	Sutton and Starr 1959
	chrysanthemi	M	<1.0 (1.46)[d]	0.72 (1.45)[d]	Tolan and Finn 1987;
Escherichia	*coli*	M	(2.20[e])[d]	(1.48 – 1.74[e])[d]	for reviews, see Ohta et al. 1990; 1991a; Yomano et al. 1998; Dien et al. 2003
Klebsiella	*oxytoca*	M	0.62 (1.96)[d]	0.52 (1.57)[d]	Ohta et al. 1991b
Sarcina	*ventriculi*	M	1.71	N.A.	for a review, see Cysewski and Wilke 1978
Raoultella	*planticola*	M	N.A.	0.22 (1.41)[d]	Feldmann et al. 1989
Ruminococcus	*albus*	M	0.5 – 0.8	N.A.	Iannotti et al. 1973
Spirochaeta	*aurentia*	M	0.8 - 1.5	N.A.	for a review, see Canale-Parola 1977
	litoralis	M	1.1 - 1.4	N.A.	for a review, see Canale-Parola 1977
	stenostrepta	M	0.8 - 1.5	N.A.	for a review, see Canale-Parola 1977
Thermoanaerobacter	*ethanolicus*	T	1.7 - 1.9	1.2 -1.4	Ng et al. 1981; Wiegel and Ljungdahl 1981; Lacis and Lawford 1988, 1991
	mathranii	T	N.A.	1.1	Larsen et al. 1997; Klinke et al. 2001
	thermohydrosulfuricus	T	1.1 - 1.5	1.1	Wiegel et al. 1979; Cook and Morgan 1994
Thermoanaerobacterium	*aciditolerans*	T	0.9	N.A.	Kublanov et al. 2007
	aotearoense	T	~ 1	~ 1	Liu et al. 1996
	polysaacharolyticum	T	1.08	N.A.	Cann et al. 2001
	thermosaccharolyticum	T	1.09	0.42 – 1.24	Lee and Ordal 1967; Mistry and Cooney 1989; Baskaran et al. 1995
	zeae	T	1.19	N.A.	Cann et al. 2001
Zymomonas	*mobilis*	M	1.9	(1.44-1.57)[d]	Lawford and Rousseau 1999; for reviews, see Swings and De Ley 1977; Zhang et al. 1995; Dien et al. 2003
Fungi					
Aurobasidium	*pullulans*	M	N.A.	0.69	Nigam et al. 1985
Fusarium	*avenaceum*	M	1.57	0.78	Suihko and Enari 1981
	gramineanum	M	1.65	0.72	Suihko and Enari 1981
	oxysporum	M	1.80	1.63	Suihko and Enari 1981
	sambucium	M	1.57	0.85	Suihko and Enari 1981
	solani	M	1.80	0.72	Suihko and Enari 1981
	sporotrichioides	M	1.72	0.26	Suihko and Enari 1981
Yeast					
Candida	*famata*	M	N.A.	0.65	Nigam et al. 1985
	shehatae	M	N.A.	1.08 - 1.47	Toivola et al. 1984; Slininger et al. 1985
	tenuis	M	N.A.	1.05	Toivola et al. 1984
Pachysolen	*tannophilus*	M	N.A.	0.92 -1.05	Slininger et al. 1985; Delgenes et al. 1986
Pichia	*segabiensis*	M	N.A.	0.82	Toivola et al. 1984
	stipitis	M	N.A.	0.98 – 1.41	Toivola et al. 1984 ; Slininger et al. 1985; Delgenes et al. 1986
Saccharomyces	*cerevisiae*	M	1.8 – 1.9	(0.26)[d]	Kötter and Ciriacy 1993; for a review, see Kosaric and Vardar-Sukan 2001
	uvarum	M	1.72	N.A.	for a review, see Kosaric and Verdar-Sukan 2001
Schizosaccharomyces	*pombe*	M	N.A.	(1.37)[d]	Chan et al. 1989

N.A.= not available; [a]mesophilic/thermophilic; [b]theoretical maximum 2 mol-EtOH mol-glucose-1; [c]theoretical maximum 1.67 mol-EtOH mol-xylose^{-1}; [d]recombinant strains in brackets; [e]higher than theoretical maximum, extra EtOH comes from the catabolism of complex nutrients (Ohta et al. 1991a)

The yeast species, which are of primary interest in industrial bioethanol production, include *Saccharomyces cerevisiae*, *S. uvarum* (*carlsbergensis*), *Schizosaccharomyces pombe*, and *Kluyveromyces* species (Kosaric and Vardar-Sukan 2001). The main advantage of yeasts compared to bacteria include higher ethanol tolerance, up to 18 %, (Kosaric and Vardar-Sukan 2001; Lin and Tanaka 2006).

A great variety of bacteria is capable of producing ethanol (Table 12), however, majority of them produce several side products, which decrease ethanol yields (Wiegel 1980; Kosaric and Vardar-Sukan 2001). Of the bacteria, only mesophilic *Zymomonas mobilis* and thermophilic *Thermoanaerobacter ethanolicus* can be regarded as strict ethanol producers (Wiegel and Ljungdahl 1981; Kosaric and Vardar-Sukan 2001; Lin and Tanaka 2006). *Z. mobilis*, traditionally used in the production of Mexican pulque-drink, has significantly higher ethanol productivity (3-5 fold) than yeast, and relatively high ethanol tolerance (up to 12%) (for reviews, see Sprenger 1996; Dien et al. 2003). However, the ethanol industry relies on *S. cerevisiae* mostly due to its hardiness (robustness) (Dien et al. 2003). However, alike *S. cerevisiae*, natural strains of *Z. mobilis* are not able to utilize pentose sugars of lignocellulosic materials (Olson and Hahn-Hägerdal 1996; Ingram et al. 1999; Zaldivar et al. 2001; Galbe and Zacchi 2002; Gray et al. 2006). The main advantage of many thermophilic bacteria is their metabolic diversity, i.e., the capability of degrading great variety of carbohydrates of lignocellulosic feedstock (Sommer et al. 2004). Other advantages and disadvantages of thermophilic bacteria are shown in Table 13.

Table 13. Advantages and disadvantages of using thermophilic bacteria and thermophilic temperature range for bioethanol fermentation (Wiegel 1980; Lynd 1989)

Advantages
+ Wide substrate utilization range
+ Direct fermentation of some biopolymers, e.g., starch (and cellulose), possible
+ Enhanced separation of ethanol, better possibilities for continuous distillation
+ Beneficial physical properties of growth medium (reduced viscosity and surface tension, increased diffusion rates and substrate solubility)
+ High reaction rates
+ Low risk of contamination by undesired microorganism, including pathogens
+ No aeration required
+ Easier to heat than cool bioreactors
+ Low cell yields, high product yield per substrate
Disadvantages
- Low ethanol tolerance
- Advanced technology required
- Production of side products (organic acids)
- Lower substrate tolerance
- Complex growth factor requirements
- Costs from bioreactor heating

Metabolically engineered organisms for ethanol production

Ethanol yields (Table 12) and/or substrate utilization range of microorganisms, mainly *S. cerevisiae*, *Z. mobilis*, *E. coli*, *Klebisella oxytoca* and *Erwinia chrysanthemi*, have been improved by genetic engineering (for reviews, see Olsson and Hahn-Hägerdal 1996; Zaldivar et al. 2001; Dien et al. 2003). Xylose and arabinose oxidising *S. cerevisiae* (genes imported from yeast *Pichia stipitis* and bacterium *Lactobacillus plantarum* or *Bacillus subtilis*) (Kötter and Ciriacy 1993; Moniruzzaman et al. 1997; Becker and Boles 2003; Karhumaa et al. 2006; Wouter Wisselink et al. 2007) and *Z. mobilis* (genes from *E. coli*) (Zhang et al. 1995,1998; Deanda et al. 1996) have been constructed. Very promising ethanol yields from pentoses have been obtained by recombinant *Z. mobilis* (75-95% of theoretical maximum), but the production is limited by low tolerance to acetic acid (Lawford and Rousseau 1999, 2002; for reviews, see Zaldivar et al. 2001; Dien et al. 2003). Nearly theoretical ethanol yields from glucose by recombinant strains of *E. coli* (Alterthum and Ingram 1989; Ohta et al. 1990;

1991a), *Klebsiella oxytoca* (Ohta et al. 1991b) and *E. chrysanthemi* (Tolan and Finn 1987) have been obtained by the insertion of genes from *Z. mobilis*. Engineered *K. oxytoga* is a potential organism for direct conversion of cellulolytic materials to ethanol (for a review, see Dien et al. 2003). More large scale demonstrations of ethanol production with genetically engineered organisms (GMOs) are required to prove their applicability in commercial processes (Dien et al. 2003).

6 HYDROGEN DARK FERMENTATION

6.1 Fermentation of glucose

The main reaction stoichiometries involved in the dark fermentative H_2 and ethanol production from glucose are listed in Table 14. Complete oxidation of glucose to CO_2 and H_2 is not thermodynamically favorable reaction in standard conditions. The conversion of glucose to acetate and butyrate are thermodynamically favorable reactions and sufficient to support microbial growth (Thauer et al. 1977; Han and Shin 2004a), whereas, further conversion of acetate and butyrate to H_2 and CO_2 are unfavorable reactions in standard conditions (Thauer et al. 1977). The conversions of acetate and butyrate to H_2 are thermodynamically favorable only when the pH_2 of the system is maintained very low. E.g., Lee and Zinder (1988) reported a threshold of pH_2 of 50 Pa for thermodynamic favorability for acetate oxidation at 60 °C by a thermophilic co-culture. This threshold has not been achieved with the present gas extraction systems used in H_2 dark fermentation processes. Therefore, the theoretical maximum H_2 yield is considered to be 4 mol-H_2 mol-glucose^{-1} associated with acetate as sole end product of dark fermentation (Claassen et al. 1999; Levin et al. 2004).

Table 14. Reaction stoichiometries of dark fermentation of glucose

Reaction	Stoichiometry	$\Delta G^{0'}$ (kJ reaction^{-1})	References
Complete oxidation of glucose	$C_6H_{12}O_6 + 12H_2O \rightarrow 12H_2 + 6HCO_3^- + 6H^+$	+ 3.2	Thauer et al. 1977
Acetate production	$C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 4H_2 + 2HCO_3^- + 4H^+$	- 206.3	Thauer et al. 1977
Butyrate production	$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2CH_2COO- + 2H_2 + 2HCO_3^- + 3H^+$	- 254.8	Thauer et al. 1977
Ethanol production	$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3CH_2OH + 2HCO_3^- + 2H^+$	- 235.0*	Ren and Gong 2006
Acetate and ethanol production	$C_6H_{12}O_6 + 3H_2O \rightarrow CH_3CH_2OH + CH_3COO^- + 2H_2 + 2HCO_3^- + 3H^+$	- 215.716	Hwang et al. 2004; Ren and Gong 2006
Lactate production	$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOO^- + 2H^+$	- 198.1*	Kim et al. 2006c
Butanol production	$C_6H_{12}O_6 + H_2O \rightarrow CH_3CH_2CH_2OH + 2HCO_3^- + 2H^+$	- 280.5	Chin et al. 2003
Propionate production	$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COO^- + 2H_2O + 2H^+$	- 359.0*	Hussy et al. 2003
Valerate production	$C_6H_{12}O_6 + H_2 \rightarrow CH_3CH_2CH_2CH_2COO^- + HCO_3^- + H_2O + 2H^+$	- 330.9*	Ren and Gong 2006
Acetogenesis	$4H_2 + 2HCO_3^- + H^+ \rightarrow CH_3COO^- + 4H_2O$	- 104.6	Thauer et al. 1977
Acetogenesis	$C_6H_{12}O_6 \rightarrow 3CH_3COO^- + 3H^+$	- 310.6*	Kim et al. 2006a
Acetate fermentation to H_2	$CH_3COO^- + 4H_2O \rightarrow 4H_2 + 2HCO_3^- + H^+$	+ 104.6	Thauer et al. 1977; Stams 1994
Butyrate fermentation to H_2	$CH_3CH_2CH_2COO^- + 10\ H_2O \rightarrow 10H_2 + 4HCO_3^- + 3H^+$	+ 257.3	Thauer et al. 1977; Stams 1994

*calculated based on the Gibbs free energies of formation provided in Thauer et al. 1977

6.2 Biochemical pathways of hydrogen and ethanol fermentation

Fermentative bacteria, lacking the ability of utilizing terminal electron acceptors, face a problem of disposing electrons provided in the oxidation of electron donors, i.e., the organisms need to regenerate the cytoplasmic electron carrier NAD (oxidize NADH) to maintain the glycolysis (Madigan et al. 2000). The means of regenerating NAD include the formation of H_2 and/or reduced products such as lactate, ethanol, butyrate, succinate and propionate (Stams 1994). In addition to serve in the regeneration of NAD, the formation of butyrate, succinate and propionate is coupled with the formation of ATP through substrate level phosphorylation (Madigan et al. 2000). The formation of acetate is coupled with the formation of ATP, but not with the regeneration of NAD.

The two main fermentation pathways of carbohydrate oxidation by H_2- and ethanol-producing microorganisms include Embden-Mayerhof (EM) (glycolysis) and Entner-Doudoroff (ED) pathways (Wiegel et al. 1980). Clostridia and enteric bacteria, the main groups of dark fermentative H_2-producers, use the EM pathway in the oxidation of carbohydrates generally resulting in a mixed-acid fermentation with several end products (Wiegel et al. 1980; Nandi

and Sengupta 1998; Hallenbeck 2005). The EM pathway yields two net ATPs, and one NADH per mol glucose oxidized (Nandi and Sengupta 1998; Madigan et al. 2003; Hallenbeck 2005). The ED pathway is used by many pseudomonads and other Gram-negative bacteria (Madigan et al. 2000).

A strict ethanol-producing bacterium, *Zymomonas mobilis*, uses the ED pathway gaining only one ATP per mol of glucose, a half of that obtained by *S. cerevisiae* through the EM pathway (for reviews, see Wiegel 1980; Sprenger 1996; Dien et al. 2003). This feature together with high number (50% of total proteins) and activity of enzymes involved in the ED pathway results in highly efficient funneling of carbon to fermentation products instead of biomass (2% yield only) (for reviews, see Sprenger 1996; Dien et al. 2003; Lin and Tanaka 2006). Therefore, *Z. mobilis* has very high ethanol yields (> 95 % of theoretical maximum), and 3 to 5 times higher ethanol productivity than *S. cerevisiae* (Sprenger 1996).

Among enteric bacteria and clostridia, two different enzymatic systems are being used for the metabolism of pyruvate formed in the glycolysis; Enteric bacteria use pyruvate formate lyase (PFL), while clostridia use pyruvate ferredoxin (flavodoxin) oxidoreductase systems (for reviews, see Nandi and Sengupta 1998; Hallenbeck and Benemann 2002; Hallenbeck 2005) (Figure 3). The breakdown of pyruvate results in the production of acetyl-CoA and either formate (enteric bacteria) or reduced ferredoxin (Fd[red]) (clostridia) (Figure 3). The breakdown of acetyl-CoA serves a two way means for maintaining cell growth while generating a variety of fermentation end products – Acetyl-CoA is used to obtain ATP (acetate production) via substrate level phosphorylation, and to regenerate NAD needed to maintain glycolysis (e.g., ethanol, butanol production), or for both needs (butyrate, propionate, succinate production) (Madigan et al. 2000; Temudo et al. 2007). An alternative way to regenerate NAD occurs via formation of lactate directly from pyruvate (Hallenbeck et al. 2005; Temudo et al. 2007). Enteric bacteria produce hydrogen through formate by using a formate-hydrogen lyase complex (FHL) (Figure 3A), while clostridia produce H_2 through Fd(red) (Figure 3B)(Nandi and Sengupta 1998; Hallenbeck 2005). The H_2 yields in enteric bacteria are limited by incomplete degradation of formate by FHL and the formation of lactate (and ethanol) for NAD regeneration. Therefore, in general, H_2 yields by enteric bacteria from glucose remain at about half of the theoretical maximum of 2 mol-H_2 mol-glucose^{-1} (Hallenbeck 2005).

In clostridia, the generation of ATP from acetyl-CoA degradation proceeds, in general, through the formation of acetate and butyrate which are linked with H_2 production (Figure 3B, Table 14) (Nandi and Sengupta 1998). In addition, the NAD regenerating reactions occur (i.e., ethanol, butanol, acetone, lactate), that are not linked with H_2 production (Figure 3B). The theoretical H_2 production maximum for clostridia takes place, when acetate is the sole end product (4 mol-H_2 mol-glucose^{-1}), while butyrate production results in a maximum of 2 mol-H_2 mol-glucose^{-1} (Nandi and Sengupta 1998; Hallenbeck 2005) (Table 14). From the breakdown of pyruvate, only 2 mol-H_2 mol-glucose^{-1} can be obtained. Additional H_2 can be obtained by the activity of NADH-ferredoxin oxidoreductase, which recycles NAD and produces Fd(red) (Hallenbeck 2005). However, the reduction of hydrogenase by NADH is thermodynamically favorable only at low pH of hydrogen, and therefore, the NADH is generally used to drive more energetically favorable reactions of butyrate or ethanol instead of acetate (Stams 1994; Hallenbeck 2005). At elevated temperatures, the thermodynamic conditions are more favorable for the production of additional H_2 through NADH reduction hydrogenase (Stams 1994; Hallenbeck 2005).

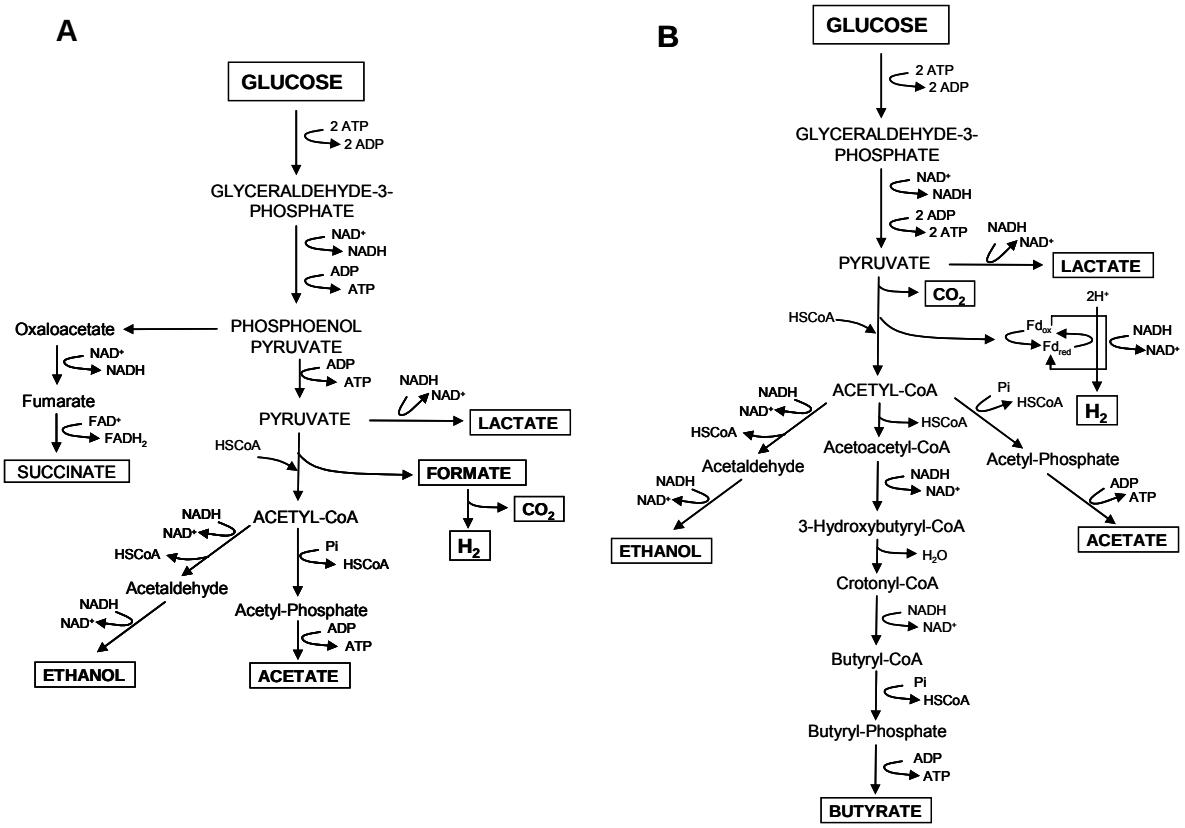


Figure 3. Glucose oxidation pathway of *Escherichia coli* (**A**) (Modified from Aristidou et al. 1999; Turcot et al. 2008) and *Clostridium butyricum* (**B**) (Modified from Saint-Amans et al. 2001).

Especially among hyperthermophiles, some modifications exist in the classical EM or ED pathways allowing increased H_2 production. *Thermotoga maritima* ferments carbohydrates via a mixture of conventional EM (85%) and ED pathways (15%) allowing a nearly theoretical conversion of glucose to H_2 (4 mol-H_2 mol-glucose^{-1}) (Schröder et al. 1994; Selig et al. 1997; for a review, see Schäfer and Schönheit 1995). Hyperthermophilic archaeon *Pyrococcus furiosus* uses a modified EM pathway in which Fd(red) is the main electron carrier instead of NAD, which could result in theoretical H_2 formation. However in practice, the metabolism is sensitive to pH_2 and alanine is produced instead of acetate decreasing the H_2 yield (Kengen and Stams 1994; for reviews, see Schäfer and Schönheit 1995; Verhees et al. 2003; Hallenbeck 2005).

6.3 Hydrogen dark fermentation microorganisms

A variety of microorganisms, including bacteria, archaea and yeast, in a wide temperature range, are capable of H_2 production by dark fermentation. The organisms used in H_2 dark fermentation studies include obligate anaerobes, facultative anaerobes and aerobes (in anaerobic conditions) (Table 15) (Nandi and Sengupta 1998; de Vrije and Claassen 2003). Clostridia and enteric bacteria are the most studied bacterial genera in dark fermentative H_2 production. Clostridia are obligate anaerobic, Gram-positive, rod-shaped and spore-forming bacteria (Chen et al. 2002). Enteric bacteria are facultatively anaerobic, oxygen tolerant, Gram-negative and non-sporulating rods (Madigan et al. 2000). The use of facultative anaerobes together with obligate anaerobes in H_2 fermentation process is beneficial since facultative anaerobes reduce the oxygen to water and create an anaerobic environment for the O_2-sensitive obligate anaerobes, and thus avoid the addition of reducing agents in the growth medium (Yokoi et at. 1998a).

Table 15. Microorganisms used in hydrogen dark fermentation studies and their H_2 yields

Genus	Species	M/T[a]	HY (mol-H_2 mol-hexose^{-1})[b]	References
Obligate anaerobes				
Acetomicrobium	*flavidum*	T	4	Soutschek et al. 1994
Acetothermus	*paucivorans*	T	3.5	Dietrich et al. 1988
Caldicellulosiruptor	*saccharolyticus*	T	3.3 – 3.6	van Niel et al. 2002, 2003; Kadar et al. 2003 ; de Vrije et al. 2007
Clostridium	*acetobutylicum*	M	1.8 - 2.0	Podesta et al. 1996; Noike et al. 2002, Chin et al. 2003; Zhang et al. 2006a; Lin et al. 2007b; Wang et al. 2008
	bifermentans	M	N.D.	Wang et al. 2003a; Lin et al. 2007b
	beijerinckii	M	1.96 – 2.81	Taguchi et al. 1993; Lin et al. 2007b
	butyricum	M	1.1 – 2.3	e.g., Karube et al. 1982; Crabbendam et al. 1985; Heyndrickx et al. 1986; 1987, 1990; Kataoka et al. 1997; Noike et al. 2002; Chen et al. 2005; Ogino et al. 2005; Lin et al. 2007b
	diolis	M	N.D.	Matsumoto and Nishimura 2007
	pasterianum	M	2.08 - 2.4	Brosseau and Zajic 1982; Heyndrickx et al. 1990
	paraputrificum	M	1.0 - 2.2 (2.4)[f]	Evvyernie et al. 2000, 2001; Morimoto et al. 2005
	thermocellum	T	1.05 - 1.6	Sparling et al. 1997; Islam et al. 2006; Levin et al. 2006
	thermolacticum	T	1.1 – 1.5	Collet et al. 2003, 2004
	tyrobutyricum	M	1.47	Zhu and Yang 2004; Lin et al. 2007b
	uzonii	T	0.55 – 0.67	Krivenko et al. 1990
Fervidobacterium	*pennavorans*[d]	T	N.D.	van Ooteghem et al. 2004
Petrotoga	*miotherma*[d]	T	N.D.	van Ooteghem et al. 2004
Pyrococcus[c]	*furiosus*	T	3.0 – 3.5	Schäfer and Schönheit 1992; Kengen and Stams 1994; Ma et al. 1994
Ruminococcus	*albus*	M	0.59 - 2.52	Iannotti et al. 1973; Miller and Wolin 1973; Ntaikou et al. 2008
Spirocheta	*thermophila*	T	2.95	Janssen and Morgan 1992
Thermoanaerobacterium	*thermosaccharolyticum*	T	1.4 - 2.53	Sjolander 1937; Ueno et al. 2001b; O-Thong et al. 2008
Thermococcus[c]	*litoralis*	T	N.D.	Belafi-Bako et al. 2006
	kodakaraensis	T	N.D.	Kanai et al. 2005
Thermohydrogenium	*kirishi*	T	0.53	Teplyakov et al. 2002
Thermotoga	*elfii*	T	3.3	de Vrije et al. 2002; van Niel et al. 2002 ; Kadar et al. 2003; van Ooteghem et al. 2004
	maritima	T	1.56 - 4.0	Schröder et al. 1994; Nguyen et al 2007
	neopolitana[d]	T	0.85 - 1.84[e]	van Ooteghem et al. 2001; 2004; Nguyen et al 2007
Thermosipho	*africanus*[d]	T	N.D.	van Ooteghem et al. 2004
Facultative anaerobes				
Candida[h]	*maltosa*	M	~0.90[e]	Lu et al. 2007
Citrobacter	*amalonaticus*	M	1.12 - 1.24	Oh et al. 2007, 2008a
	intermedius	M	1.0 - 1.5	Brosseau and Zajic 1982
Escherichia	*coli*	M	0.75 – 2.55 (1.82 - 3.12)[f]	Ordal and Halvorson 1939; Blackwood et al. 1956; Chittibabu et al. 2006; Yoshida et al. 2006 ; Turcot et al. 2008
Enterobacter	*aerogenes*	M	0.4 - 1.7	Tanisho and Ishiwata 1994, 1995; Yokoi et al. 1997; Rachman et al. 1997, 1998; Palazzi et al. 2000; Fabiano and Perego 2002; Nakashimada et al. 2002; Ito et al. 2004; Ogino et al. 2005
	asburiae	M	0.54	Shin et al. 2007
	cloacae	M	2.1 – 3.4	Kumar et al. 2000, 2001; Kumar and Das 2000, 2001a,b
Ethanoligenens	*harbinense*	M	1.93	Wang et al. 2008 ; Xing et al. 2008
Klebsiella	*pneumoniae*	M	N.D.	Solomon et al. 1995
Aerobes				
Alcaligenes	*eutrophus*	M	N.D.	Kuhn et al. 1984
Bacillus	*coagulans*	M	2.28	Kotay and Das 2007
	licheniformis	M	0.34 – 1.04	Kalia et al. 1994; Kumar et al. 1995

[a]mesophilic/thermophilic; [b] HY = hydrogen yield; [c]archaea; [d]produce H_2 in microaerobic conditions (6-8 % O_2 [v/v]); [e]calculated based on the information given; [f] yield in brackets obtained by genetic engineering; [g] H_2 yield calculated per mol N-acetylglucosamine (GlcNAc), a monomer of chitin; [h] yeast species

Hydrogen dark fermenting microorganisms can be easily enriched from various natural and engineered environments. Different waste treatment processes have been the most used sources of microorganisms for dark fermentative H_2 production systems (Table 16). Heat or extreme pH can be used to enrich spore-forming H_2 -fermenters and to inactivate H_2-consuming methanogens. Different methods, such as heating in an oven (e.g. 2 h at 100 °C) (Lay et al. 2003) or on a pan (e.g. 104 °C for 2 h) (van Ginkel et al. 2001), boiling (e.g. 15 min) (Lay 2000), acid (pH 3 for 24h) (Chen et al. 2002) or base treatment (pH 10 for 24h) (Chen et al. 2002) have been successfully used to select for spore-forming H_2-producers from various environments. In addition, forced aeration of seed material can be used to enrich H_2 fermenters (Ueno et al. 1995; 1996).

Table 16. Sources of microorganisms for fermentative H_2 production experiments

Source of microorganisms	**Reference**
Activated sludge from WWTP	Wang et al. 2003a; Kawagoshi et al. 2005
Anaerobic digester sludge from WWTP	e.g., Han and Shin 2004a; Lin and Chang 2004; Kawagoshi et al. 2005
Settling tank, WWTP	e.g., Chen and Lin 2001; Fang et al. 2002a;b; Yu et al. 2002b
Cattle dung	Kumar et al. 1995; Vijayaraghavan et al. 2006a,b; Yokoyama et al. 2007a,b
Sludge from reactor treating fructose-manufacturing wastewater	Liang et al. 2002
Soybean-meal fermentor	e.g. Mizuno et al. 2000b; Noike and Mizuno 2000
Palm oil mill effluent sludge, sludge compost, CREST compost	Morimoto et al. 2004
Mixture of thermophilic household compost, methanogenic landfill simulation reactor, landfill solid waste and garden soil.	Nielsen et al. 2001
Aerated sludge compost	Ueno et al. 1995; 1996
Composted soil	Van Ginkel et al. 2001
Compost pile	Khanal et al. 2004; Kawagoshi et al. 2005
Soils	Van Ginkel et al. 2001;Logan et al. 2002; Kawagoshi et al. 2005
Sludge from pig manure digester	Lay et al. 2003

WWTP = wastewater treatment plant

Genetic engineering of dark fermentative H_2-producers

Genetic engineering of dark fermenting bacteria aims at redirecting electron fluxes towards H_2 production (Benemann 1996; Hallenbeck and Benemann 2002; for a review, see Vardar-Schara et al. 2008). The means include the elimination of uptake hydrogenases or non-H_2 producing pathways, overexpression or modification of H_2-producing hydrogenases, or insertion of H_2-producing pathways (Hallenbeck and Benemann 2002; Nath and Das 2004; Yoshida et al. 2006) (Figure 3A). Hydrogen production by *E. coli* has been successfully increased by overexpression of formate-hydrogen-lyase (FHL) and by direction of metabolism towards pyryvate formate lyase (PHL) (disrupting succinate and lactate production pathways) (Yoshida et al. 2005, 2006, 2007). Without genetic engineering, by the addition of suicide substrates, Kumar et al. (2001) improved the H_2 yield of *E. cloacae* up to 3.4 mol-H_2 mol-glucose^{-1} by creating mutants lacking alcohol (ethanol and butanediol), butyrate and lactate production pathways.

So far, only a few reports exist on genetic engineering of *Clostridia* for H_2 fermentation. Morimoto et al. (2005) overexpressed hydrogenase gene of *C. paraputrificum* resulting in 1.7 fold increase in H_2 yield compared to the wild-type strain. Genetic engineering of *Clostridia* so far, has mainly focused on the maximization of solvent (*C. acetobutylicum*, Boynton et al. 1996; Green et al. 1996; Green and Bennet 1998) or acid (*C. tyrobutyricum*, Zhu et al. 2005) production rather than H_2. Low amounts of genetic data available and difficulties in gene transformation (as Gram-positive organisms) have likely limited the genetic modification of *Clostridia*.

Idea of incorporating microalgal oxidizing pentose phosphate pathways (PPP) into dark fermenting bacteria have been proposed (Hallenbeck and Benemann 2002). Stoichiometric H_2 yields (12 mol-H_2 mol-glucose^{-1}) have been obtained with the enzymes involved in PPP pathway in *in vitro* assays (Woodward et al. 2000b). Further, the microaerobic conditions have been suggested to enhance the H_2 production by facultative anaerobic bacteria (Nath and Das 2004; Chen et al. 2006b). With *Klebsiella pneumoniae* in microaerobic conditions, the TCA cycle would provide more ATP to run the H_2-producing nitrogenase with higher H_2 production potential (theoretical maximum 6.68 mol-H_2 mol-glucose^{-1}) than in anaerobic condition (2.86 mol-H_2 mol-glucose^{-1}) (Chen et al. 2006b). However, only a certain, limited level of oxidative phosphorylation of NADH and $FADH_2$ is allowed, making the control of metabolic fluxes a highly challenging task for metabolic engineers (Chen et al. 2006b).

6.4 Feedstock for H_2 dark fermentation

In terms of H_2 production rates and yields, carbohydrates are the most suitable feedstock for H_2 fermentation followed by proteins and peptides, while H_2 production from fats is considered very limited (Tables 17 and 18) (Okamoto et al. 2000; de Vrije and Claassen 2003; Lay et al. 2003). Majority of H_2 dark fermentation studies have been conducted with model compounds, mainly with glucose or sucrose. Glucose is the monomeric unit of the most abundant biopolymers, cellulose and starch while sucrose is a major component in some crops (see Chapter 5) and food industry wastes (sugar industry, brewing etc.) (Chang and Lin 2004). Hydrogen production from xylose, a sugar constituent of hemicellulose, has been demonstrated (Taguchi et al. 1995a; Lin and Cheng 2006; Lin et al. 2006b; Calli et al. 2008; Wu et al. 2008b). In a similar manner, H_2 production from lactose, a main carbohydrate constituent of diary wastes, has been demonstrated (Collet et al. 2003,2004; Calli et al. 2008). Further, H_2 production from the sugar polymers, starch and cellulose, has been reported (e.g. Taguchi et al. 1995b; Ueno et al. 1995; Lay 2001; Yokoi et al. 2002; Zhang et al. 2003). In addition, H_2 fermentation from chitin and N-acetyl-D-glucosamine, the monomer of chitin, has been demonstrated by *Clostridium paraputrificum* M-21 (Table 15) (Evvyernie et al. 2000).

Hydrogen production has been demonstrated from several wastes and potential energy crop materials (Tables 17 and 18). Pilot-scale H_2 production has been demonstrated from molasses (Ren et al. 2006), spent grains (Chou et al. 2008), citric acid production waste water (Yang et a. 2006), office paper slurry (Ueno et al. 2007) and food waste (Kim et al. 2005a). Depending on the feedstock and microorganisms used, the material may require pretreatment with processes, such as mechanical cutting or crushing (Kalia and Joshi 1995; Okamoto et al. 2000; Kim et al. 2004b), acid, enzymatic hydrolysis (Okamoto et al. 2000; Palazzi et al. 2000; Kadar et al. 2003, 2004; Wang et al. 2003a; Fan et al. 2006b,c) or sterilization (Tanisho and Ishiwata 1994,1995; Wang et al. 2003a). The treated feedstock may need to be diluted (Tanisho and Ishiwata 1994,1995) and supplied with nutrients and buffers prior to feeding to the reactors (Yu et al. 2002b).

Table 17. Batch H_2 production from actual wastes and wastewaters

Microorganism	Conditions		Substrate (concentration [g/L])	H_2 production		Reference
	T (ºC)	Initial pH		HY (mol H_2 mol-hexose^{-1})	HPR (mmol h^{-1} L^{-1})	
Mixed culture	35	N.A.	Bean curd waste	**2.54**	N.A.	Noike and Mizuno 2000
Mixed culture	35	6.0	Bean curd waste	**2.54**	N.A.	Mizuno et al. 2000b
Mixed culture	37	4.5	Rice slurry	**2.5**	N.A.	Fang et al. 2006
Caldicellulosiruptor saccharolyticus	N.A.	N.A.	Sweet sorghum juice	**2.32**	21	Claassen et al. 2004
Mixed culture	55	5.5	Food waste	**1.8***	3.8 $mmol_{H2}$ g_{VS}^{-1*}	Shin et al. 2004
Mixed culture	35	N.A.	Wheat Bran	**1.73**	N.A.	Noike and Mizuno 2000
Mixed culture	37	N.A.	Food waste	**1.5***	21.6 $mmol_{H2}$ g_{TVS}^{-1} d^{-1*}	Lay et al. 2005
Mixed culture	35	N.A.	Rice bran	**1.29**	N.A.	Noike and Mizuno 2000
Mixed culture	35	6.0	Food waste + sewage sludge (80%:20%)	**1.01**	0.9 $mmol_{H2}$ g_{VSS} h^{-1}	Shin et al. 2003
Mixed culture	26	N.A.	Potato starch	**0.59**	N.A.	Logan et al. 2002
Mixed culture	37	N.A.	Organic fraction of municipal solid waste	**7.48** $mmol_{H2}$ g_{TVS}^{-1*}	N.A.	Lay et al. 1999
Mixed culture	36	7.0	Cornstalk (15)	**6.22** $mmol_{H2}$ g_{TVS}^{-1*}	2.1 $mmol_{H2}$ g_{TVS}^{-1} L^{-1}	Zhang et al. 2007a
Mixed culture	37	N.A.	Rice	**3.46** $mmol_{H2}$ g_{VS}^{-1*}	N.A.	Okamoto et al. 2000
Mixed culture	36	7.0	Beer lees (20)	**2.86** $mmol_{H2}$ g_{TVS}^{-1*}	N.A.	Fan et al. 2006b
Mixed culture	36	7.0	Wheat straw (25)	**2.83** $mmmol_{H2}$ g_{TVS}^{-1*}	0.42 $mmol_{H2}$ g_{TVS}^{-1} h^{-1*}	Fan et al. 2006c
Mixed culture	40	6.0	Grains (solid conc. 3 wt%)	**0.54** mmol $_{H2}$ g_{VS}^{-1*}	6.7 $mmol_{H2}$ g_{TVS}^{-1} d^{-*1}	Chou et al. 2008
Mixed culture	37	N.A.	Lean meat	**0.22** $mmol_{H2}$ g_{VS}^{-1*}	N.A.	Okamoto et al. 2000
Mixed culture	37	N.A.	Chicken Skin	**0.17** $mmol_{H2}$ g_{VS}^{-1*}	N.A.	Okamoto et al. 2000
Mixed culture	37	N.A.	Fat	**0.17** $mmol_{H2}$ g_{VS}^{-1*}	N.A.	Okamoto et al. 2000
Mixed culture	37	N.A.	Egg	**0.15** $mmol_{H2}$ g_{VS}^{-1*}	N.A.	Okamoto et al. 2000
Mixed culture	37	7.5	Pineapple waste	**5.92** mol_{H2} g_{COD}^{-1}	15.9 mmol L l^{-1} h^{-1*}	Wang et al. 2006
Mixed culture	RT	N.A.	Candy production wastewater	**7.06** $mmol_{H2}$ g_{COD}^{-1*}	N.A.	Van Ginkel et al. 2005
Mixed culture	RT	N.A.	Potato – processing wastewater	**5.82** $mmol_{H2}$ g_{COD}^{-1}	N.A.	Van Ginkel et al. 2005
Mixed culture	35	6.0	Food waste + sewage sludge (87%:13%)	**5.10** $mmol_{H2}$ g_{COD}^{-1*}	N.A.	Kim et al. 2004b
Mixed culture	RT	N.A.	Apple - processing wastewater	**4.15** $mmol_{H2}$ g_{COD}^{-1*}	N.A.	Van Ginkel et al. 2005
Mixed culture	RT	N.A.	Municipal wastewater	**1.66** $mmol_{H2}$ g_{COD}^{-1*}	N.A.	Van Ginkel et al. 2005
Mixed culture	37	N.A.	Brewery mixture	**1.12** $mmol_{H2}$ g_{COD}^{-1*}	8.5 mmol g_{VSS}^{-1} d^{-1}	Fan and Chen 2004
Mixed culture	35	N.A.	Municipal waste water sludge + sludge filtrate	**0.88** mmol $_{H2}$ g $_{COD}^{-1*}$	N.A.	Wang et al. 2003b
Mixed culture	55	6.0	Starch	N.A.	15.2 $mmol_{H2}$ g_{VSS}^{-1}·d^{-1*}	Zhang et al. 2003

N.A. = not available; RT= room temperature

*calculated based on the information provided

Table 18. Continuous or semi-continuous H_2 production from actual waste waters and solid wastes

Reactor type	Microorganism	Conditions			Substrate (concentration [g L^{-1}])	H_2 production		VSS (gL^{-1})	Reference
		T (ºC)	pH	HRT (h)		HY (mol H_2 mol-hexose^{-1})	HPR (mmol h^{-1} L^{-1})		
Waste waters									
PBR	*E. coli*	37	6.0	2.7	Molasses	3.12	**97.4**	N.A.	Chittibabu et al. 2006
CSTR + granules	*Enterobacter aerogenes*	38	6.0	N.A.	Molasses (20)	2.5[a]	**36**	6.2	Tanisho and Ishiwata 1994
ASBR	Mixed culture	N.A.	6	12	Paper and food slurry	N.A.	**8.3**	N.A.	Ueno et al. 2006
CSTR	Mixed culture	60	6.8	12	Sugar factory wastewater (31.9 g_{COD}/L)	2.59[a]	**8.3**	N.A.	Ueno et al. 1996
CSTR	Mixed culture	32	5.2	15	Sugar beet extract	1.7	**~7.5**[*]	N.A.	Hussy et al. 2005
UASB	Mixed culture	55	5.5	2	Rice winery wastewater (34 g_{COD}/L)	2.14[a]	**6.6**	11.7	Yu et al. 2002b
CSTR	Mixed culture	37	5.5-6.0	18	Brewery waste	1.78 $mmol_{H2}$ g_{COD}^{-1}[*]	**5.3**	N.A.	Fan et al. 2006a
ASBR	Mixed culture	37	5.5-6.0	8	Dehydrated brewery waste	N.A.	**~5.2**[*]	N.A.	Fan and Chen 2004
CSTR	Mixed culture	30	5.2	15	Wheat starch (7.5)	1.9	**5.0**	N.A.	Hussy et al. 2003
ACF	Mixed culture	N.A.	N.A	72	Mixed fruit peel waste (84 gL^{-1} as VS)	19.1 $mmol_{H2}$ g_{VS}^{-1}[*]	**3.4**[*]	N.A.	Vijayaraghavan et al. 2007
ASBR	Mixed culture	55	5.5	96	Palm oil mill effluent	2.24	**2.6**[*]	N.A.	O-Thong et al. 2007
ACF	Mixed culture	N.A.	5	72	Palm oil mill effluent	N.A.	**1.8**[*]	N.A.	Vijayaraghavan and Ahmad 2006
CSTR	Mixed culture	55	5.5	120	Food waste	2.2	**1.7**	N.A.	Shin and Youn 2005
ASBR	Mixed culture	35	6	6	Sweet sorghum extract	0.7	**1.5**[*]	10.2	Antonopoulou et al. 2008
UASB	Mixed culture	35-38	6.8-7.2	12	Citric acid wasterwater	0.84	**1.2**	N.A.	Yang et al. 2006
CSTR	Mixed culture	35	~4.5	4.2	Molasses	N.A.	**1.0**	~ 7	Ren et al. 2006
ACF	Mixed culture	N.A.	N.A.	288	Jackfruit peel waste	16.6 $mmol_{H2}g_{VS}^{-1}$[*]	**0.9**[*]	N.A.	Vijayaraghavan et al. 2006a
CSTR	Mixed culture	35-38	4.5	24	Cheese processing wastewater	1.8 $mmol_{H2}$ g_{COD}^{-1}[*]	**0.9**[*]	N.A.	Yang et al. 2007
CSTR	Mixed culture	55	N.A.	29	Olive pulp	0.32 mmol g_{TS}^{-1}	**0.58**	N.A.	Gavala et al. 2005
ASBR	Mixed culture	35	6.0	72	Grains	N.A.	**0.35**	N.A.	Chou et al. 2008
CSTR	Mixed culture	35	4.0-4.3	6	Molasses	2.01[a]	**N.A.**	10.6	Ren and Gong 2006
Solid wastes									
LBR	Mixed culture	37	N.A.	144	Food waste	12.9 $mmol_{H2}/g_{VS}^{-1}$	**6.3**	N.A.	Han and Shin 2004b
ASBR	Mixed culture	37	N.A.	30	Food waste	N.A	**5.5**	N.A.	Shin et al. 2005
ASBR	Mixed culture	35	N.A.	36	Food waste + steamed rice	N.A.	**3.7**	N.A.	Kim et al. 2005a
ASBR	Mixed culture	35	5.5	48	Kitchen waste	1.2 $mmol_{H2}$ g_{COD}^{-1}	**2.5**	N.A.	Li et al. 2006
ASBR	Mixed culture	28	~4.6	24	Dairy wastewater	N.A.	**0.066**[*]	N.A.	Venkata Mohan et al. 2007
LBR	Mixed culture	35	N.A.	5-10	Food waste	19.3 $\%_{H2}$ $COD_{removed}^{-1}$	N.A.	N.A.	Han and Shin 2004a
ASBR	Mixed culture	55	5.5	84	Food and paper waste	15.0 $mmolH_2$ gVS^{-1}[*]	N.A.	N.A.	Valdez-Vazquez et al. 2005

[*]calculated based on the information provided

ACF = Anaerobic contact filter; ASBR = anaerobic sequencing batch reactor; CSTR = Completely-stirred tank reactor; HY= hydrogen yield; HPR = hydrogen production rate; LBR = Leaching bed reaction; N.A. = not available; UASB = Up-flow sludge blanket reactor

6.5 Factors affecting dark fermentative H_2 production

The metabolism of H_2 dark fermenting bacteria is highly dependent on the physicochemical conditions in the process. To maximize the H_2 yields, bacterial metabolism should be directed towards acetate production and away of producing reduced end products (e.g. alcohols and more reduced organic acids), which include H_2 that has not been liberated as gas (Hawkes et al. 2002; Levin et al. 2004). Physicochemical factors affecting H_2 and ethanol dark fermentation are listed in Table 19.

Table 19. Main factors affecting hydrogen and ethanol production

Factor	Effect(s)	References
Temperature	- Affects fermentation metabolism, activity and microbial composition - See Table 20	Yokoi et al. 1995; Evvyernie et al. 2000; Fang and Yu 2001; Fabiano and Perego 2002; Yu et al. 2002a,b; Chin et al. 2003; Hussy et al. 2003; Zhang et al. 2003; Lin and Chang 2004; Morimoto et al. 2004; Gavala et al. 2006; Lee et al. 2006a; Mu et al. 2006; Valdez-Vasquez et al. 2006; Georgieva et al. 2007; Yokoyama et al. 2007b; for reviews, see Li and Fang 2007a; Li et al. 2007
pH	- Affects fermentation metabolism, activity and microbial composition - Low pH decrease H_2 and increase solvent (e.g. ethanol) production (inhibition of hydrogenase activity). - Extreme pH (low or high) can be used to select spore-forming organisms - Affects cell membrane charge and the transport of compounds through the membrane - Affects enzyme activity - Affects toxicity of harmful substances	Heyndrickx et al. 1986;1987; Kalia et al. 1994; Yokoi et al. 1995; Majzat et al. 1997; Lin and Chang 1999; Evvyernie et al. 2000,2001; Kumar and Das 2000; Lay 2000; Fang and Yu 2001; Van Ginkel et al. 2001; Chen et al. 2002; Fabiano and Perego 2002; Fang and Liu 2002; Horiuchi et al. 2002; Liu and Fang 2002; Yu et al. 2002b; Chin et al. 2003; Hussy et al. 2003; Zhang et al. 2003; Collet et al. 2004; Fan et al. 2004, 2006b,c; Hwang et al. 2004; Khanal et al. 2004; Kim et al. 2004a; Wu and Lin 2004; Chen et al. 2005; Kawagoshi et al. 2005; Shin and Youn 2005; Cheong and Hansen 2006; Fang et al. 2006; Lin and Cheng 2006; Lin et al. 2006b; Ren and Gong 2006; Vijayaraghavan and Ahmad 2006; Koskinen et al. 2007b; for reviews, see Hawkes et al. 2007; Li and Fang 2007a; Li et al. 2007; Ren et al. 2007
Substrate concentration and loading rate (Food to microorganism [F/M] ratio)	- Affects fermentation metabolism, activity and microbial composition - High substrate loading may decrease H_2 production and increase solvent (.e.g. ethanol) production (substrate inhibition, improper F/M-ratio)	Harris et al. 1986; Lacis and Lawford 1988,1991; Dabrock et al. 1992; Lay et al. 1999; Fang and Yu 2001; Kumar and Das 2001; Lay 2001; Van Ginkel et al. 2001; Yu et al. 2002a; Chin et al. 2003; Kadar et al. 2003 ; Lee et al. 2003; van Niel et al. 2003; Wu et al. 2003; Fan et al. 2004, 2006b,c; Oh et al. 2004b; Sommer et al. 2004; Wu and Lin 2004; Chen et al. 2005; Kim et al. 2005b,2006c, Shin and Youn 2005; van Ginkel and Logan 2005b; Chen et al. 2006a; Fang et al. 2006; Kyazze et al. 2006; Ren and Gong 2006; Lin and Cheng 2006; Georgieva and Ahring 2007; Ren et al. 2007; Zhang et al. 2007b; for a review, see Kraemer and Bagley 2007
Hydraulic retention time (dilution rate)	- Affect the fermentation mechanism, activity and microbial composition - Generate "hydraulic selective pressure", which effect the microbial composition, and granulation - Low HRT favors H_2 production - Too low HRT may result in washout of H_2 (or ethanol) producers - Too low HRT decrease H_2 production due to substrate inhibition and improper F/M ratio - Low HRT can be used to wash out methanogens or homoacetogens	Heyndrickx et al. 1986; Dabrock et al. 1992; Nakamura et al. 1993; Ueno et al. 1996; Majzat et al. 1997; Francese et al. 1998; Rachman et al. 1998; Lin and Chang 1999; Lay 2000; Chen et al. 2001; Chang et al. 2002; Fang and Yu 2002; Liu and Fang 2002; Yu et al. 2002b; Hussy et al. 2003; Lin and Jo 2003; Lee et al. 2003; 2004a,b, 2006a,b; Wu et al. 2003, 2005a, 2006a,b; Chang and Lin 2004; Collet et al. 2004; Han and Shin 2004a; Lin and Chang 2004; Oh et al. 2004b; Fan et al. 2006a; Gavala et al. 2006; Lin et al. 2006a,c; Zhang et al. 2006b, 2007b,c,2008a,b; Cheong et al. 2007; Wang and Chang 2008; Vijayaraghavan and Ahmad 2006; Yu and Mu 2006; Antonopolou et al. 2008; for reviews, see Hawkes et al. 2002; 2007; Li and Fang 2007a
Hydrostatic pressure (effecting partial pressures of gases)	- Affects fermentation metabolism - Increase in hydrostatic pressure may improve ethanol yield, e.g. *C. thermocellum* or decrease it	Bothun et al. 2004
Partial pressure of hydrogen (pH_2)	- Affects fermentation metabolism, activity and microbial composition - Increased pH_2 decreases H_2 production, but may improve ethanol production - Increase in pH_2 decreases the regeneration of NADH leading to the formation of reduced products	Wiegel et al. 1979; Ben Bassat et al. 1981; Wiegel and Ljundahl 1981; Lamed et al. 1988; Lovitt et al. 1988; Kataoka et al. 1997; Mizuno et al. 2000a; Nielsen et al. 2001; Liang et al. 2002 ; Hussy et al. 2003, 2005; van Niel et al. 2003; Kim et al. 2006a; Kraemer and Bagley 2006; Kyazze et al. 2006; Liu et al. 2006; for reviews, see Hawkes et al. 2002,2007; van Groenestijn et al. 2002; Levin et al. 2004; Kraemer and Bagley 2007
Partial pressure of CO_2	- May affects fermentation metabolism and activity - May affect the activity of acetogens and methanogens	Tanisho et al. 1998; Park et al. 2005; Kim et al. 2006a; for a review, see Hawkes 2007
Ethanol concentration (ethanol tolerance)	- High concentrations inhibit growth due increasing membrane instability - Ethanol tolerance depends high on bacterial species - Bacteria generally more vulnerable to self-produced acids than externally added	Lovitt et al. 1988; Baskaran et al. 1995; Sudha Rani et al. 1996; Sudha Rani and Seenayya 1999; Lynd et al. 2001; Georgieva et al. 2007, for reviews, see Lynd 1989, Zaldivar et al. 2001; Burdette et al. 2002;

Stirring (effects partial pressure of gases)	- Affects fermentation metabolism - Vigorous stirring can increase H_2 production, and decrease ethanol production	Lamed et al. 1988
Fermentation products (organic acids)	-May affect the fermentation metabolism, activity and microbial composition - The passage of undissociated acids through cell membrane followed by their dissociation uncouples proton motive force - High concentrations of undissociated acids may decrease H_2 production and increase solvent production - High acid concentrations may lead to cell lysis - Sensitivity depends on organism and on acids, longer acids generally more toxic - Sensitivity to organic acids depends on pH (pH affects the dissociation of acids) - Bacteria generally more vulnerable to self-produced acids than externally added	Chin et al. 2003; Van Niel et al. 2003; van Ginkel et al. 2005; Zheng and Yu 2005; Kyazze et al. 2006; for a review, see Jones and Woods 1986
Inhibitory compounds in complex substrates or waste streams	- Sensitivity to inhibitory compounds is strain-dependent. -Compounds (organic acids, alcohols, alhedydes etc.) released in the hydrolysis of lignocellulosic substrates may be toxic to microorganism. Toxicity increase with increasing hydrophobicity of compounds - Na-ion. Inhibitory at high concentrations. - Heavy metals, e.g., Cu, Ni, Zn, Cr, Cd, Pb. Micronutrients, but inhibitory at high concentrations.	Zaldivar and Ingram 1999; Zaldivar et al 1999, .2000; Klinke et al. 2001; Kadar et al. 2003; Hao et al. 2006; Li and Fang 2007b; Lin and Shei 2008; for a review, see Zaldivar et al. 2001
Composition of growth media - Buffers - Nutrients - Growth factors	- Buffers, phosphate and carbonate. Needed to resist pH change. Carbonate buffers release CO_2. - Macronutrients (e.g., N, P, S, Mg, Ca, Na.). Essential for cell growth. Needed in synthesis of macro and micromolecules in cells. • Calcium is important in the granulation of cells. Increase mechanical strength of the granules. - Micronutrients (trace elements). Often play structural role in enzymes. • Iron concentration. Important e.g., in the formation and activity of hydrogenases and redoxins • Zinc concentration, essential for ethanol production in some organisms - Growth factors (e.g., Peptone, tryptone, yeast extract). Contain vitamins, amino acids, purines and pyrimidines. Vitamins fuctions often as parts of coenzymes. Generally required by thermophiles.	Mistry and Cooney 1989; Heyndrickx et al. 1990; Lee et al. 2001; Ueno et al. 2001a; van Niel et al. 2002; Kadar et al. 2003; Lee et al. 2004a; Lin and Lay 2004a,b,2005; Liu and Shen 2004; Chen et al. 2005; Lay et al. 2005;; Zhang et al. 2005; Wang et al. 2006, Zhang and Shen 2006; Yang and Shen 2006; Avci and Dönmez 2006; Chang and Lin 2006; Kim et al. 2006b; Lin and Chen 2006;.for reviews, see Madigan et al. 2000; Hawkes et al. 2007; Li and Fang 2007a

Growth inhibition of H_2-consuming microorganisms

The growth inhibition or absence of H_2-consuming microorganisms, such as methanogens, acetogens and sulfate reducers, is a prerequisite for high-rate H_2 production. The growth of methanogens in a H_2-producing reactor can be avoided by adjusting the operational parameters, such as HRT, pH and temperature, or by inhibitory compounds. There are three major inhibitors of methanogens: oxygen, 2-bromoethanesulfonic acid (BESA) and acetylene (Sparling et al. 1997). Oxygen cannot be continually used as inhibitor in H_2 fermenting systems, since it inhibits the activity of H_2 fermenters. BESA is a structural analog, and therefore a competitive inhibitor, of the coenzyme M in methanogens (Taylor and Wolfe, 1974) and is rather specific, and sensitive, for methanogens only (Sparling et al. 1987, 1997). However, some BESA-tolerant methanogens have been reported (Smith 1983; Santoro and Konisky 1987), and BESA may also decrease the H_2 production by dark fermentation (Wang et al. 2003a).

Low pH (<5.5) is generally considered to inhibit the growth of methanogens (Kraemer and Bagley 2007), while too low pH inhibits also the activity of hydrogenases resulting in production of more reduced products (Lay 2000). Weakly acidic pH alone may no be enough to inhibit H_2-utilizing methanogens. Methanogenic activity has been detected even at pH 4.5, which already initiated solventogenesis (production of acetone and butanol) in H_2 –producing bacteria (Kim et al. 2004a). The growth rates of methanogens and homoacetogens are generally lower than those of H_2-producers (Ueno et al. 1996; Chen et al. 2002). Methanogens and homoacetogens can, therefore, be washed out by using short HRTs. The HRTs should not, however, exceed the critical value, where H_2 producers are washed out from the reactor (Chen et al. 2002). During the bioreactor operation, heat shocks (e.g., 75 °C, 1h) can be used to re-select spore-forming bacteria (Chang et al. 2002; Sung et al. 2002; Wu et al. 2003).

Temperature

Temperature is one of the most important factors affecting the growth of microorganisms. When temperature rises, microbial growth rates increase due to the increase in the rates of chemical and enzymatic reactions in cells (Madigan et al. 2000). Dark fermentation metabolism takes place in a wide temperature range. H_2-producing dark fermentation reactors can be operated in various temperature ranges from of mesophilic (15-45°C) up to even of hyperthermophilic (more than 80°C) microorganisms. Most of the H_2 dark fermentation studies have been conducted at temperature range of mesophiles, between 35 and 40°C. Temperatures of the thermophiles may, however, offer several advantages as shown in Table 20.

Table 20. Advantages and disadvantages of hydrogen dark fermentation in temperature range of the thermophiles

Advantages	**Reference(s)**
+ Increase in the rates of chemical and enzymatic reactions	for reviews, see van Groenestijn et al. 2002; Hallenbeck 2005
+ Increase in thermodynamic favorability of H_2-producing reactions. H_2 production becomes less affected by partial pressure of H_2.	Lee and Zinder 1988; for reviews, see Conrad and Wetter 1990; Zinder 1990; Stams 1994; Schönheit and Schäfer 1995; Claassen et al. 1999; van Groenestijn et al. 2002; Levin et al. 2004; Hallenbeck 2005
+ Solubility of H_2 and CO_2 to water decreases	for a review, see Hawkes et al. 2002
+ Reactors are less prone to contamination by H_2-consuming organisms	for a review, see Claassen et al. 1999; van Groenestijn et al. 2002; de Vrije and Claassen 2003
+ Decreased diversity of side products	for a review, see Schönheit and Schäfer 1995
+ Some thermophiles excrete exoenzymes, which can hydrolyze biopolymers (e.g., starch, cellulose, xylan and peptides)	for reviews, see Schönheit and Schäfer 1995; Claassen et al. 1999
+ Suitable for direct processing high temperature waste waters	Yu et al. 2002b
+ Destruction of pathogens in the reactor effluent	Kotsopoulos et al. 2006
+ In general, easier to warm-up than cool bioreactors	Wiegel 1980
Disadvantages	
- Low cell densities achieved	for reviews, see de Vrije and Claassen 2003; Hallenbeck 2005
- Complex nutrient requirements	
- Energy need for heating	for reviews, see Hawkes et al. 2002, van Groenestijn et al. 2002; de Vrije and Claassen 2003

Medium pH

The optimal growth pH is highly dependent on the organism. The optimal pH of H_2 dark fermentation processes vary in the range of 4.0 to 9.0 (Fang and Liu 2002). Generally, weakly acidic pHs have been considered optimal for dark fermentative H_2 production. Control of pH and buffering of growth medium is essential in dark fermentation since organic acids produced which tend to decrease the pH. Low pH inhibits the activity of hydrogenases and can shift the metabolic pathways of dark fermentation microorganisms away from H_2 production. Probably the best known example of this is *C. acetobutylicum*, which changes metabolism from H_2 (+ acetate and butyrate) to the production of solvents (acetone and butanol) when the pH is decreased to less than 5.0 (Bahl et al. 1982). Alternatively, depending on the organism, low pH can shift the metabolism towards ethanol production (Li et al. 2007).

Change in the environmental pH affects the intracellular pH and the accumulation of organic acids within the cell (see Chapter "End product inhibition" below). These effects together with the necessity of generating ATP and recycling NADH affect the metabolic patterns of microorganisms (Li et al. 2007). Depending on the pH, different fermentation patterns have been proposed in mesophilic acidogenesis. Ethanol-acetate fermentation (Table 14) is considered to dominate at pH below 4.5 or 5.0 (Ren et al. 1997, 2007; Hwang et al. 2004). Stable H_2-production has been reported with ethanol-acetate fermentation (Ren and Gong 2006; Li et al. 2007; Ren et al. 2006, 2007).

Butyrate-acetate production dominates at pH < 5.5 and > 6.0, whereas propionate production is increased at pH 5.5 to 6.0 (Li et al. 2007). Butyrate-acetate type fermentation is favorable for H_2 production, where as propionate-type fermentation results in low H_2 production (Table 14). These fermentation patterns are also affected by other environmental (and process) factors, such as Redox-potential (Ren et al. 2007) and pH_2 (Ren et al. 1997; Li et al. 2007), and by the microorganisms (Ren et al. 2007).

Hydraulic retention time (HRT) and loading rate (LR)

Hydraulic retention time is inversely related to the substrate loading rate and the bacterial growth rate in bioreactors (Lay 2000). HRT and LR are the main optimization parameters of continuous H_2 dark fermentation bioprocesses. HRT and LR affect the metabolic balance of H_2 -fermenters (Dabrock et al. 1992; Ueno et al. 1996; Hawkes et al. 2002). Generally, short HRT (high loading rate) is considered to favor the H_2 fermentation metabolism (Chang et al. 2002), with optimal HRTs as short as 0.25 h reported in mesophilic dark fermentation reactors (Zhang et al. 2008a;b). On the other hand, too high loading rates (low HRT) or substrate concentrations may result in substrate inhibition effects, improper food to microorganism

(F/M) ratios to H_2 producers or washout of microorganisms (Lay 2001; Wu et al. 2003; Lee et al. 2003). These "shock loads" reduce the H_2 production metabolism through pH decrease, metabolite inhibition (accumulation of intermediates) and/or increased pH_2 (Van Ginkel et al. 2001).

Partial pressure of hydrogen (pH_2) and carbon dioxide (pCO_2)

Partial pressure of hydrogen (pH_2) in the liquid phase is a key factor in dark fermentative H_2 production (Hawkes et al. 2002; Levin et al. 2004). The pH_2 effects the hydrogen production pathways through end product inhibition (Levin et al. 2004; Nath and Das 2004). The re-oxidation of reduced ferredoxin and H_2-carrying coenzymes becomes less favorable when H_2 concentration in liquid increases and, therefore, a decrease in pH_2 should increase H_2 yields (Stams 1994; Hawkes et al. 2002). When pH_2 increases, metabolic pathways are directed towards more reduced organic acids and alcohols decreasing the H_2 yields. When temperature increases, H_2 production becomes less affected by pH_2 (Levin et al. 2004). In some microorganisms, e.g., *C. thermocellum* (Freier et al. 1988; Bothun et al. 2004) and *T. brockii* (Ben-Bassat et al. 1981), increased pH_2 can be used to increase ethanol production.

Despite several demonstrations of increased H_2 production with gas extraction techniques, the actual mechanisms of this improvement are not known and different theories exist (Kraemer and Bagley 2007). Hydrogen and CO_2 are substrates for the H_2-utilizing methanogens and acetogens. Decreasing pH_2 and pCO_2, may, therefore, limit the substrate availability for H_2-consumers and increase the H_2 yield (Hussy et al. 2003; Park et al. 2005; Kraemer and Bagley 2006). Further, CO_2 removal may enhance the fermentative H_2 production by increasing residual amounts of NADH, contributing to the H_2 production via so called NADH pathway (Tanisho et al. 1998). On the other hand, high CO_2-concentrations are inhibitory to bacteria, the reason why CO_2 is used as a preservation gas in food packing (Dixon and Kell 1989). Kim et al. (2006a) reported a decrease in microbial diversity and an increase in H_2 production during CO_2 sparging of H_2 fermentation bioreactor and suggested the CO_2 inhibition of acetogenic bacteria.

End product inhibition

Organic acids (such as acetate and butyrate) in their undissociated form can pass the cell membrane, and then dissociate within a cell (at higher pH than outside the cell), thus releasing a proton (van Ginkel and Logan 2005a). High concentrations of organic acids, therefore, uncouple the proton motive force (pH gradient) across the cell membrane resulting in metabolic inhibition (van Ginkel and Logan 2005a; for a review, see Jones and Woods 1986). At lower concentrations, organic acids may decrease the cell growth rate (Chin et al. 2003; for a review, see Jones and Woods 1986) and cause shifts in cell metabolism, e.g., from H_2 production (acetate and/or butyrate) production to the production of solvents or propionate (van Ginkel and Logan 2005a; Zheng and Yu 2004; Kyazze et al. 2006; for a review, see Jones and Woods 1986). The pH affects the dissociation of organic acids (van Ginkel and Logan 2005a), and the levels of undissociated organic acids increase when pH drops close to the pKa value (Hawkes et al. 2007). The inhibition in H_2 fermentation by undissociated organic acids, sets an upper limit on substrate concentration as a function of pH (van Ginkel and Logan 2005a). Hydrogen fermentation is generally affected more by undissociated butyric acid than acetic acid (Chin et al. 2003; van Ginkel and Logan 2005a).

Low ethanol tolerance of bacteria (especially thermophiles) is one of the major reasons limiting their use in commercial ethanol production (Lynd et al. 2001). Thermoanaerobes should be able to sustain ethanol concentrations above about 4 to 5 % (v/v) in order to obtain commercially viable separation of ethanol from bioprocess (Lynd et al. 2001; Sudha Rani and Seenayya, 1999). The mechanism of ethanol inhibition is considered to be related to changes

in membrane fluidity resulting in decreased cell growth (Herrero et al. 1980; Herrero and Gomez 1982), and blockage of glycolysis by inhibiting the enzymes involved (Herrero et al. 1985). Further, high ethanol concentration may cause changes in the end product formation (Lovitt et al. 1984) and cause imbalance in the NAD-recycling (Lovitt et al. 1988).

Thermophilic microorganisms are generally less tolerant to ethanol than mesophiles (Georgieva et al. 2007). High temperatures affect the synthesis of saturated fatty acids resulting in changes in membrane organization and increased membrane fluidity (Herrero and Gomez 1980; Georgieva et al. 2007). Thermophilic microorganisms have generally ethanol tolerance of only less than < 1 to 2%, v/v (Burdette et al. 2002; Lynd, 1989) and the highest ethanol tolerances for wild-type thermoanaerobes include 5.1% (v/v) for *Thermoanaerobacter* sp. strain A10 (Georgieva et al. 2007) and 5% (v/v) for *Clostridium thermocellum* sp. strain SS22 (Sudha Rani and Seenayya 1999), respectively. The ethanol tolerance of several thermoanaerobes has been successfully increased by batch or continuous cultivation at high ethanol concentrations resulting in adaptation or generation of ethanol-resistant mutants (Baskaran et al. 1995; Burdette et al. 2002; Sudha Rani and Seenayya 1999).

Other inhibitors

The pretreatment and hydrolysis of complex substrates (e.g., lignocellulosic materials) may release chemical compounds that are inhibitory to ethanol (or hydrogen) -producers (Klinke et al. 2001; for a review, see Zaldivar et al. 2001). The inhibitory compounds can be derived from degradation of sugars (furfurals, and organic acids), or lignin (lignols and other phenolic alcohols, acids and aldehydes), from acids used on pretreatment (acetic and formic acid), and from the pretreatment vessel (inorganics) (Zaldivar and Ingram 1999; Zaldivar et al. 1999, 2000; for a review, see Zaldivar 2001). These compounds may result in decreased viability, ethanol yields and ethanol productivity (for a review, see Zaldivar et al. 2001).

Some waste streams can contain significant amounts of heavy metals, which can inhibit hydrogen production (rate and yield), and cause metabolic shifts (Lin and Shei 2008). Li and Fang (2007b) studied the toxicity of several heavy metals on H_2 production by granular sludge and reported that toxicity was in the following order: Cu (most toxic)> Ni > Zn > Cr > Cd > Pb (least toxic).

Composition of growth media

The dark fermentation medium needs to be supplied with macronutrients including ammonium and phosphate ions, if not present in sufficient quantities in the feedstock (Heyndrickx et al. 1990; Lay et al. 2005). Sulfur (Lin and Chen 2006; Chen et al. 2008) and iron (Lee et al. 2001; Zhang et al. 2005; Zhang and Shen 2006) are important micronutrients in H_2 fermentation, as they are constituents of hydrogenases (for a review, see Vignais and Billoud 2007), and ferredoxin (Mistry and Cooney 1989). Iron limited hydrogenase activity may alter the fermentation pathways away from H_2 (plus acetate and butyrate) production towards the production of more reduced end products such as lactate, ethanol and butanol (Dabrock et al. 1992; Junelles et al. 1988; Lee et al. 2001; Peguin and Soucaille 1995; Zhang and Shen 2006). Iron limitation may also increase lactate production by decreasing ferredoxin formation and activity, and thereby decrease the formation of acetyl-CoA from pyruvate (Figure 3B) (Mistry and Cooney 1989). Other important minerals and trace elements include magnesium, zinc and sodium (Lin and Lay 2005).

Hydrogen dark fermentation systems require strong buffering of growth medium to resist the pH change caused by organic acids produced. Generally, carbonate buffers have been used in H_2 dark fermentation studies ($NaHCO_3$ and NH_4CO_3). However, carbonate buffers result in undesirable formation of additional CO_2 due to the interaction of HCO_3^- and acidic

metabolites, and therefore, the use of phosphate buffers may be desirable (Wang et al. 2006; Wang and Chang 2008).

7 BIOREACTOR PROCESSES FOR H_2 DARK FERMENTATIONS

Several bioreactor types have been applied for the dark fermentative H_2 production. The benefits and drawbacks of different H_2 dark fermentation bioreactor types are listed in Table 21.

Table 21. Benefits and drawbacks of bioreactors used for dark fermentative H_2 production

Reactor type	Benefits (+) and drawbacks (-)	References
Continuously stirred tank reactor (CSTR)	+ Simple process, easy to operate and control - Low biomass retention	Heydrickx et al. 1986; Taguchi et al. 1995a; Chang and Lin 1999, 2004; Chen et al. 2001; Kim et al. 2006a; Kyazze et al. 2006; Zhang et al. 2006b; for reviews, see Speece 1983; Li and Fang 2007a
Upflow anaerobic sludge blanket reactor (UASB)	+ Good retention of biomass - Slow development of granules (long start-up period)	Chang and Lin 2004,2006; Gavala et al. 2006; Kotsopoulos et al. 2006; Yu et al. 2002a,b; Mu and Yu 2006; Yu and Mu 2006; for a review, see Rajeshwari et al. 2000
Fluidized-bed reactor (FBR)	+ Good retention of biomass + Good mass transfer due to efficient mixing + No clogging - Instability of H_2 production - Volume occupied by carrier (less volume available for biomass) - Strong shear forces can detach biomass - Energy needed for biomass fluidization	Wu et al. 2003; Kim et al. 2005b; Lin et al. 2006a; Zhang et al. 2007b;2008a; Jeon et al. 2008; for reviews, see Rittman 1982; Speece 1983; Shieh and Keenan 1986; Marin et al. 1999; Rajeshwari et al. 2000; Liu et al. 2003
Packed-bed reactor (PBR)	+ No need for mechanical mixing + Good retention of biomass - Clogging - Lower mass transfer than in FBR - Gas hold-up - Volume occupied by carrier (less volume available for biomass)	Rachman et al. 1998; Kumar and Das 2001; Chang et al. 2002; Lee et al. 2003; for a review, see Rajeshwari et al. 2000
Tricking biofilter reactor (TBR)	+ Good biomass retention + High mass transfer between liquid and gas phase (reduced gas hold-up) - Clogging - Long start-up period	Oh et al. 2004b; Ahn et al. 2005; Zhang et al. 2006a; Jeon et al. 2008; for a review, see Cohen 2001
Granular bioreactors, e.g. carrier induced granular sludge bed bioreactor (CIGSB) and immobilized-cell-seeded anaerobic bioreactor (ICSAB)	+ Excellent biomass retention (allows very high loading and short HRT) + Rapid sludge granulation (short start-up time) + Maximized space available for biomass (no or low amount of carrier) - Mass transfer can be poor - Channeling of flow and formation of gas pockets (if no mixing)	Lee et al. 2004a, b, 2006a,b; Wu et al. 2006; Wang and Chang 2007
Membrane bioreactor (MBR)	+ Efficient retention of biomass + Disinfection and high quality of the treated water (no bacteria) + Low sludge volume - Fouling and clogging of membranes - High capital costs of the membrane - High energy requirements to push liquid through membranes	Oh et al. 2004a; for reviews, see Cicek et al. 1998; Defrance and Jaffrin 1999; Cohen 2001; Cicek 2003; Daigger et al. 2005; Li and Fang 2007a

7.1 Bioprocess parameters and configurations

Mass transfer

In H_2 production bioreactors, efficient mass transfer is especially important to enable good contact between microorganisms, substrates and nutrients (Lee et al. 2006b), and to enable efficient separation of gases from the system (Wu et al. 2003). Packed (carrier material) or granular bioreactors are prone to suffer from gas hold-up (Kumar and Das 2001) and from formation of gas pockets which result in decreased H_2 production (Lee et al. 2006b). Mass transfer can be increased by mixing and by proper bioreactor design. Efficient mixing can be achieved by mechanical stirring (Lamed et al. 1988; Lee et al. 2006b), recycling of gases (Kim et al. 2006a) or liquids (Wu et al. 2003; Lee et al. 2006b), or by gas purging (Mizuno et al. 2000a; Hussy et al. 2005; Kim et al. 2006a; Kraemer and Bagley 2006) depending on the reactor type and configuration. Mass transfer can be further enhanced by applying proper bioreactor shapes (Kumar and Das 2001), and by optimizing bioreactor dimensions such as the height-to diameter ratio (Lee et al. 2006b).

Biomass retention

High biomass concentration, enabling the use of high organic loading, is a prerequisite for high-rate H_2 production. However, the quality of bacteria is even more important. Different retainment strategies affect both the quality (Wu et al. 2008a) and quantity (Wu et al. 2005a; Lee et al. 2004b) of biomass. The cell retainment strategies applied for dark fermentative H_2 production are listed in Table 22.

Table 22. Cell retainment strategies used in H_2 dark fermentation

Strategy	Bioreactor	Comments	VSS (g L^{-1})	References
Biofilms on carrier material				
Porous glass beads	PBR	Higher H_2 production obtained than by agar entrapment	N.A.	Yokoi et al. 1997
Loofah sponge, expanded clay, activated carbon	PBR	Activated carbon resulted in the highest H_2 production rates	15*	Chang et al. 2002
Lignocellulosic materials; rice straw, bagasse and coir	FBR	The best cell retention and H_2 production rate obtained with coir	N.A.	Kumar and Das 2001
Urethane foam	PBR		N.A.	Tanisho and Ishiwata 1995
Synthetic commercial sponge	PBR		N.A.	Palazzi et al. 2000
Brick dust	Batch	Immobilization increased H_2 production. Higher H_2 production obtained with brick dust than with calcium-alginate entrapment	N.A.	Kumar et al. 1995
Activated carbon pellets	FBR	High biomass content in attached-growth phase (21.5 gVSS L^{-1}), no granulation observed	21.5	Zhang et al. 2007b
Polyvinyl alcohol	CSTR	Low H_2 production and process stability, likely due to mass transfer limitations with biofilm and carrier material	N.A.	Kim et al. 2005b
Cell entrapment within matrix				
Calcium alginate	Batch		N.A.	Kumar et al. 1995
Agar matrix	PBR		N.A.	Yokoi et al. 1997
Ethylene-vinyl acetate copolymer + AC powder	Batch	H_2 production stable in repeated batch assays	N.A.	Wu et al. 2005b
Sodium alginate + AC powder + polyurethane or acrylic latex/silicone	Batch	Cell entrapment increased H_2 production, acrylic latex/ silicone entrapment provided the best mechanical strength and durability in repeated batch assays	N.A.	Wu et al. 2002
Sodium alginate + AC powder + acrylic latex/silicone	FBR		N.A.	Wu et al. 2003
Granulation, induction method or self granulation				
Acid-treatment (pH 2, 24 h)	CSTR	Granules formed at HRT 2h within 5 d after acid treatment. No granules formed without acid treatment	32.2	Zhang et al. 2007c
Addition of cationic polyacrylamide and anionic silica sol	CSTR	Granulation occurred within 5 min. Higher H_2 production and better stability achieved than with biofilm reactor	N.A.	Kim et al. 2005b
Cylindrical AC pellets	FBR	Efficient cell granulation achieved, best H_2 production with lowest (70%) bed porosity (more space for granules). Granulation occurred at HRTs 2 to 4 h.	N.A.	Lee et al. 2003
Spherical or cylindrical AC, sand or filter sponge	CIGSB	Spherical AC most effective inducer, granulation occurred within 100 h at 2h HRT. Carrier type affected the time and HRT required for granulation.	26.1	Lee et al. 2004b
Cylindrical AC	CIGSB	Addition of $CaCl_2$ improved the mechanical strength of granules, liquid and gas refluxing increased H_2 production	~15	Lee et al. 2004a
Cylindrical AC	CIGSB	Applying agitation and optimization of reactor H/D-ratio increased biomass retainment and H_2 production.	~40	Lee et al. 2006b
Silicone+ AC powder-immobilized sludge	CSTR	Efficient granulation (35.4 g_{VSS} L^{-1}) and H_2 production achieved (15.09 L h^{-1} L^{-1})	34.5	Wu et al. 2006
Cylindrical AC or Silicone+ AC powder-immobilized sludge	CSTR	Granulation affected H_2 production through quantity and quality of microorganisms. Granular sludge reactors had higher biomass, less diverse community structure compared to a suspended-cell reactor	10.3	Wu et al. 2008a
Self-granulation	CMCR	Self-granulation was achieved at 10 h HRT. Organism *E. aerogenes*	N.A.	Rachman et al. 1998
Self-granulation	UASB	Self-granulation was achieved at 12 h HRT, formation of granules took 120 days.	3.1	Chang and Lin 2004
Self-granulation	UASB	Addition of calcium increased the size of granules, total biomass and H_2 production	~9	Chang and Lin 2006
Self-granulation	CSTR	Granules formed within 15 d at HRT 6 h	20	Fang et al. 2002a
Self-granulation	CMCR	Granules formed within 15 d at HRT of 2.2 h	N.A.	Wang and Chang 2008

*Calculated based on the information provided. AC = activated carbon; CSTR = completely-stirred tank reactor; CMCR = completely-mixed column reactor; CIGSB = carrier-induced granular sludge bed reactor; FBR = fluidized-bed reactor; H/D –ratio = height/diameter –ratio; PBR=packed bed reactor; UASB = up-flow sludge blanket bioreactor; N.A. = not available

Granulation

Granulation has been the most effective means of biomass retention in hydrogen dark fermentation bioprocesses (Lee et al. 2004b,2006b; Wu et al. 2005a, 2006a). Further, granulation can improve H_2 production by altering the microbial community structure (Wu et al. 2006;2008a). In H_2 fermentation bioreactors, granulation has been obtained through self-flocculation or through induction by the addition of entrapped cells or inert carriers (Table 22). The formation of methanogenic granular sludge has been studied extensively, but there are only few reports on the composition and characteristics of granules in H_2-producing bioreactors (Fang et al. 2002a; Zhang et al. 2004, 2007b; Mu and Yu 2006). H_2-producing granules have simpler (non-layered) structure (Fang et al. 2002a), higher extracellular polymeric substance (EPS) content (Fang et al. 2002a; Mu and Yu 2006), and higher carbohydrate content in the EPS (Mu and Yu 2006) than methanogenic granules.

The microbial granulation process is not fully understood and several granulation theories exist (Liu et al. 2003; Hulshoff Pol et al. 2004). Granulation is a complicated process involving physicochemical, biological and hydrodynamic factors (for reviews, see Liu et al. 2003; Hulshoff Pol et al. 2004; Zhang et al. 2008a) (Table 23). Microbial composition and characteristics (for a review, see Schmidt and Ahring 1996), EPS (Fang and Liu 2002; for a review, see Liu et al. 2004c) and hydrodynamic selection forces (for a review see Liu et al. 2003; Hulshoff Pol et al. 2004) likely play important roles in the granulation. EPS, metabolized and secreted by the cells, form a "sticky" layer in the cell surface and can alter the surface charge promoting the granule formation. (Schmidt and Ahring 1996; Liu et al. 2004c; Zhou et al. 2006). Hydraulic (and organic loading) pressure can trigger granulation and affect the characteristics of granules (Francese et al. 1998; Liu et al. 2003). Therefore, microbial granulation can be increased by optimizing bioreactor dimensions (e.g. H/D-ratio) to obtain proper up-flow velocity (Lee et al. 2006b), and by applying proper HRT and substrate LR (Zoutberg et al. 1989; van Ginkel and Logan 2005b; Lee et al. 2003, 2004a, 2006b). Since cell surface is negatively charged, the addition of positively charged ions (e.g. Al^{3+}, Ca^{2+}, Fe^{3+}, Mg^{2+}) can decrease the electronic repulsion between cells and trigger granulation (Figure 4A) (for a review, see Liu et al. 2003; Liu et al. 2004b; Zhou et al. 2006). Alternatively granulation can be enhanced by the addition of synthetic polymers through formation of bridges between the cells (Figure 4B) (Kim et al. 2005b; for a review see Liu et al. 2003).

Table 23. Factors affecting granulation (Lettinga 1995; Schmidt and Ahring 1996; Liu et al. 2003,2004a,b; Hulshoff Pol et al. 2004; Zhou et al. 2006)

Physicochemical
Gravitation force
Electrostatic forces (opposite change attraction)
Hydrodynamic shear forces
Surface tension
Diffusion
Van der Waals forces
Thermodynamic forces (Brownian motion)
Biological
Microbial community composition
Microbial morphology, physiology and genetic competence
Quality and quantity of extracellular polymeric substances excreted
Microbial signaling (quorum sensing)
Cell charges of microorganisms (cell hydrophobicity)
Cell mobility
Process conditions
Characteristics of waste water (substrates, nutrients, inhibitors)
Chemicals supplied (e.g. positively charged ions Al^{3+}, Ca^{2+}, Fe^{3+}, Mg^{2+})
Bioprocess design (reactor type, configuration and dimensions)
Hydrodynamic conditions and organic load
Temperature, pH, redox-state
Mixing

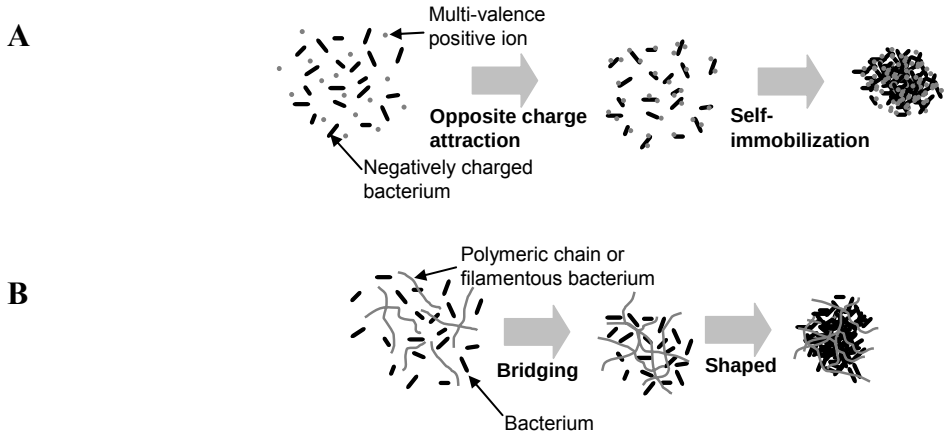


Figure 4. Biomass granulation models. A) Multi-valence positive ion bonding model (Adapted from Liu et al. 2003); B) Polymer or filament bonding model (Adapted from Liu et al. 2003).

Biofilms

In general, lower H_2 production rates have been obtained with biofilm reactors compared to granular reactors. In the high-rate H_2-producing FBRs, bacterial granulation have been observed, and the majority of biomass retained in granules compared to biofilms (Lee et al. 2003; Lin et al. 2006a; Zhang et al. 2008a). This phenomenon led to the development of carrier-induced sludge bed reactors (CISBR) with high H_2 production rates obtained (Lee et al. 2004a,b,2006a,b). Biofilm reactors may have lower stability in H_2 production than granular reactors (Kim et al. 2005b). Kim et al. (2005b) compared the performance of biofilm and granular bioreactors, and reported that biofilm reactor lower stability and performance of biofilm reactor due to the production of propionate. They suggested that the poor mass transfer within the biofilm and carrier material created optimal environment for propionate producers (high pH_2 and suitable pH). The change in propionate production was irreversible (Kim et al. 2005b). Optimization of carrier material is crucible in biofilm reactors. Jeon et al. (2008) reported the suitability of hydrophobic carrier material for biomass retention in a trickling-bed reactor.

Gas separation

Several gas separation methods have been applied to H_2 dark fermentation reactors in order to decrease the pH_2 (or pCO_2) in the system. Simplest means of enhancing biogas separation include gas sparging with inert gas (for reviews, see Hawkes et al. 2007; Li and Fang 2007a) or increased stirring (Lamed et al. 1988; Lay 2000). Significant enhancements in the H_2 production rate (and yield) and stability have been achieved by gas sparging with N_2 (Crabbendam et al. 1985; Mizuno et al. 2000a; Hussy et al. 2003, 2005; van Niel et al. 2003; Kyazze et al. 2006; Kraemer and Bagley 2006), argon (Tanisho et al. 1998), H_2 (Tanisho et al. 1998) or CO_2 (up to 118 %) (Kim et al. 2006a) or CH_4 (up to 88 %) (Liu et al. 2006). Highest increases in H_2 yield has been obtained with CO_2 sparging (120 %, Kim et al. 2006a), followed by CH_4 (88%, Liu et al. 2006) and N_2 sparging (68%, Mizuno et al. 2000a). Gas sparging, however, dilutes the H_2 concentration in the product biogas making the separation of gas components more demanding and expensive (Hawkes et al. 2007).

Hydrogen (and CO_2) concentrations in the liquid phase are supersaturated and it has been argued that gas sparging (using "practical" sparging rates) increase the H_2 production rather through decrease in H_2 consumption than through decreased *in vivo* pH_2 faced by the enzymes (Kraemer and Bagley 2006). Gas sparging reduces substrate (H_2 and/or CO_2) concentrations for the H_2-consuming acetogens or methanogens (Kramer and Bagley 2006). The results are, however, inconsistent since both H_2 (Tanisho et al. 1998) and CO_2 (Kim et al. 2006a) have been reported to increase the H_2 production. The effect of applying direct gas suction from reactor may be dependent on the organism and vacuum used. Kataoka et al. (1997) reported no increase in H_2 production by *C. butyricum* with 0.28 atm vacuum applied, while Mandal et al. (2006) reported 2 fold increase in H_2 yield by *E. cloacae* with 0.5 atm vacuum applied.

More advanced gas separation methods have included H_2 selective (Nielsen et al. 2001; Teplyakov et al. 2002; Belafi-Bako et al. 2006) or non-selective membranes (Liang et al. 2002; Oh et al. 2004a; Belafi-Bako et al. 2006). Liang et al. (2002) reported minor increases (10 % in H_2 production rate and 15 % increase in H_2 yield) by gas extraction through silicon rubber membranes. Selective membranes have been applied for the separation of gas components in product gas, not for gas extraction to decrease pH_2 (Nielsen et al. 2001; Teplyakov et al. 2002; Belafi-Bako et al. 2006). Biofilm build-up (biofouling of membranes), however, may reduce the efficiency of gas extraction through the membranes (Hawkes et al. 2002; Nath and Das 2004).

Hybrid processes

The effluent of H_2 dark fermentation process contains still plenty of chemical oxygen demand (COD) (organic acids and alcohols) and needs, in both environmental and economical point-of view, to be further treated (deVrije and Claassen 2003; Logan 2004). In hybrid processes, organic acids and alcohols produced by dark fermenters are converted to H_2 by photofermenters (Yokoi et al. 1998a,2001,2002; de Vrije and Claassen 2003) or microbial fuel cells (Logan 2004; Liu et al. 2005a,b; Oh and Logan 2005; Cheng and Logan 2007), or alternatively to CH_4 by methanogens (Hawkes et al. 2002; Han and Shin 2004b; Gavala et al. 2005; Kyazze et al. 2007; Ueno et al. 2007). The substrate range for photofermentors is wide meaning that low H_2 production efficiency in dark fermentation is compensated by a higher H_2 production in the consecutive photofermentation (de Vrije and Claassen 2003). The effluent from anaerobic digestion has high N and P content and could be used as a fertilizer, e.g., for energy crop production (Hawkes et al. 2002).

There are several examples of combining dark fermentation with photofermentation (Table 24) or methanogenesis (Table 25), but only a few reports, so far, on combining dark fermentation with microbial fuel cell (EAMC, or BAEMR) (Oh and Logan 2005). Gassanova

et al. (2006) proposed a three-stage process of using cyanobacteria in the 1st stage for production of biomass and consumption of CO_2, followed by 2nd stage methanogenesis and 3rd stage photofermentation for the production of fuel gases (CH_4 and H_2) from the cyanobacterial biomass.

Photo and dark fermentations can be combined within one reactor vessel (photo- and dark fermenters mixed), or in a two-stage process (dark fermentation in 1st stage, photofermentation in 2nd). Combination of photo and dark fermentations increase significantly the H_2 yields compared to dark fermentation only, with HYs of up to 7.2 mol-H_2 mol-hexose^{-1} (Yokoi et al. 2002) obtained in 2-stage dark and photo fermentation system (Table 24). The combined dark and photofermentation processes have been generally carried out by using pure cultures, but the process has been also been demonstrated using microbial communities (Fang et al. 2004).

In two-stage anaerobic digestion process, commercially extensively used at the moment, acidogenesis stage is followed by methanogenesis (Hawkes et al. 2007). This process resembles a combined H_2 dark fermentation and CH_4 production process. However, in two-stage anaerobic digestion the acidogenesis stage is not optimized for H_2 production (Hawkes et al. 2007). The two-stage anaerobic digestion process increases the stability and efficiency of the process (Liu et al. 2006; Hawkes et al. 2007). In combined H_2 dark fermentation and methanogenesis, the dark fermentation stage is carried out at significantly shorter retention time as the CH_4 production stage, and therefore, the H_2 production rate is generally higher than the CH_4 production rate (Table 25).

Table 24. Hydrogen production with processes combining dark and photofermentation

Reactor type	Microorganism	Conditions			Substrate (concentration [g/L])	H_2 production		Reference
		T (ºC)	pH	HRT (h)		HY (mol H_2 mol-substrate^{-1})	HPR (mmol h^{-1} L^{-1})	
ASBR	*Clostridium butyricum* + *Rhodobacter* sp.	30	6.5	N.A.	Starch (5)	6.6	N.A.	Yokoi et al. 1998a
Batch (two-stage)	*Clostridium butyricum* (I), *Rhodobacter* sp. (II).	30	N.A.	N.A.	Starch (5)	3.7	N.A.	Yokoi et al. 1998a
ASBR (two-stage)	*Clostridium butyricum* + *Enterobacter aerogenes* (I), *Rhodobacter* sp. (phase II).	37(I); 35 (II)	5.25 (I); 7.5 (II)	24 (I); 120 (II)	Sweet potato residue	7.0	N.A.	Yokoi et al. 2001
ASBR (two-stage)	*Clostridium butyricum* + *Enterobacter aerogenes* (I), *Rhodobacter* sp. (II).	37(I); 35 (II)	5.25 (I); 7.5 (II)	24 (I); 120 (II)	Sweet potato residue	7.2	N.A.	Yokoi et al. 2002
Batch	*Lactobacillus delbrueckii* + *Rhodobacter sphaeroides*	30	N.A.	-	Glucose	7.1	N.A.	Asada et al. 2006
CSTR (two-stage)	Mixed communities		5.5 (I)	6 (I)	Glucose and sucrose	3.8	N.A.	Fang et al. 2004
Batch	*Clostridium butyricum* + *Rhodobacter* sp	N.A.	8	-	Glucose	7.0	N.A.	Miyake et al. 1984
Batch (two-stage)	Community (I) + *Rhodobacter sphaeroides* (II)	38(I); 30 (II)	N.A.	-	Sucrose (18)	6.63	N.A.	Tao et al. 2007
Batch (two-stage)	Community (I) + *Rhodobacter sphaeroides* (II)	35 (I)	6.8 (I); 6.7 (II)	-	Olive mill wastewater	29 L_{H2} L_{waste}^{-1}	0.33	Eroğlu et al. 2007
Batch	*Cellulomonas* sp. + *Rhodopseudomonas capsulata* (mutant)	33	N.A.	-	Cellulose (5)	6.2	N.A.	Odom and Wal 1983
Batch	*Vibrio fluvialis* + *Rhodobium marinum*,	30	N.A.	N.A.	Starch (4.05)	~2.1	N.A.	Ike et al. 1999

HRT = hydraulic retention time; HPR = hydrogen production rate; HY = hydrogen yield

Table 25. Performance of mixed-culture processes combining hydrogen dark fermentation and methanogenesis

Reactor type	Conditions			Substrate (concentration [g/l])	H_2 and CH_4 production		VSS (g L^{-1})	Reference
	T (ºC)	pH	HRT (h)		Maximum yield	PR (mmol h^{-1} L^{-1})		
CSTR (two-phase)	55 (I, II)	N.A.	29 (I), 576 (II)	Olive pulp	1.6 $mmol_{H2}$ g_{TS}^{-1}; 19 mmol $_{CH4}$ g_{TS}^{-1}	**0.58** (H_2); 0.28 (CH_4)	N.A.	Gavala et al. 2005
ASBR	37 (I; II)	5.0-5.5 (I)	48 (I); 360 (II)	Household solid waste	1.8 $mmol_{H2}/g_{VS}^{-1}$ 20.8 mmol $_{CH4}$ g_{VS-1}^{-1}	**2.8** (H_2); 4.3 (CH_4)	N.A.	Liu et al. 2006
Leachin bed reactors (I); UASB (II)	37 (I; II)	N.A.	144 (I); 14.4 (II)	Food waste	12.9 $mmol_{H2}/g_{VS}^{-1}$ 8.8 mmol $_{CH4}$ g_{VS}^{-1}	**6.3** (H_2); 3.0 (CH_4)	N.A.	Han and Shin 2004b
CSTR (I); PBR (II)	55 (I); 60 (II)	5.9 (I); ~8 (II)	28.8 (I); 148.8 (II)	Food + paper waste	2.4 (6.6 L_{H2} g_{COD}^{-1}) (I)	**9.3** (H_2); 10.6 (CH_4)	N.A.	Ueno et al. 2007
CSTR (I); TRF (II)	35 (I; II)	5.2-5.3 (I)	12 (I); 48 (II)	Sucrose (20)	1.47 (0.172 L_{H2} g_{COD}^{-1}); 0.294 L_{CH4} g_{COD}^{-1})	**13.3** (H_2); 4.4 (CH_4)	3.0 (I)	Kyazze et al. 2007
CSTR (I); CSTR (II)	35 (I; II)	5.5 (I); 7.5 (II)	9.6 (I); 72.7 (II)	Glucose (60)	0.34 (H_2); 0.02 (CH_4)	**3.5** (H_2); 0.21 (CH_4)	N.A.	Cooney et al. 2007
CSTR (I); UASB (II)	35 (I); 28 (II)	5.5 (I); 6.9-7.2 (II)	10 (I); 64 (II)	Glucose (15)	0.115 gH_{2COD} g feed $_{COD}^{-1}$	**11.3** (H_2)[a] 3.2 (CH_4)[a]	0.8 (I) 0.9 (II)	Kraemer and Bagley 2005

HRT=hydraulic retention time; PR= production rate

[a] Calculated based on the information provided

[*] Conversion factors used in calculations; H_2 24.075 L mol^{-1}; CH_4 24.00 L mol^{-1}.

8 HYPOTHESES AND AIMS OF THE PRESENT WORK

The main objective of this study was to develop open system processes for dark fermentation of H_2 or ethanol+H_2 from carbohydrates. Moreover, the work aimed at understanding microbial community diversity and dynamics in open system bioreactors. FBRs are characterized by high retention of biomass on biofilm carrier and efficient mixing due to high recycle rates and carrier fluidization (Rittman 1982; Speece 1983; Shieh and Keenan 1986). It was hypothesized that high biomass retention in continuous-flow FBRs would allow high organic loading and high H_2 production rates, and that efficient mixing would improve mass transfer of gases allowing their efficient separation from the system (Paper I). There had been one successful demonstration of FBRs for H_2 dark fermentation (Wu et al. 2003). I was further hypothesized that applying a gas extraction system into the bioreactor recycle line would improve H_2 production by allowing better separation of gases from the system (Paper II).

There have been a limited number of studies on the dynamics of H_2-producing communities in mixed-culture bioprocesses. Majority of these studies have described community dynamics between different steady-states (e.g., at different HRTs), i.e., during stable H_2 production (Iyer et al. 2004; Wu et al. 2006; 2008a; Zhang et al. 2006b; Hung et al. 2007). In this work, it was hypothesized that analyzing microbial communities also during instable H_2 production, at transitional state and at steady-state will improve the understanding on the process microbiology, and aid in the process optimization (Paper I, III, IV, V). For example, propionate-production is a known concern in H_2 dark fermentation bioreactors resulting in decrease or cease in H_2 production (Cohen et al. 1985; Beeftinkt and van den Heuvel 1987; Hussy et al. 2003; Kim et al. 2005b; Cheong et al. 2007). The phenomena resulting in the induction of propionate production have been proposed (Cohen et al. 1985; Beeftinkt and van den Heuvel 1987; Kim et al. 2005b), but not disclosed.

After discovering that biofilms in FBR supported the growth of propionate-producers and resulted in instable H_2 production (Paper I), we hypothesized that improved H_2 production stability can be achieved by using a suspended-cell reactor. Therefore, a comparative study with a suspended-cell, completely-mixed column reactor (CMCR) with similar configuration, inoculum and feed composition, and comparable loading compared to the FBR was performed. Also keeping in mind the complex H_2 production behavior in the FBR, and fluctuation of several different metabolic patterns (i.e., acetate-butyrate, acetate-propionate and acetate-ethanol-lactate productions as dominant metabolisms) (Paper I), there was a need to better describe these transitions. Therefore, a Clustering Hybrid Regression (CHR) model was developed to better detect and visualize the metabolic transitions in order to increase the understanding on the process. Moreover, CHR was used to model the H_2 production rate based on the process data.

Even though several microorganisms produce substantial amounts of both ethanol and H_2 in fermentation (Tables 12 and 15), only a few studies have focused on continuous co-production of both ethanol and H_2 (Wu et al. 2007a,b). Two novel thermophiles, one with high H_2 (AK15) and other with high ethanol (AK17) production capability, with rather similar optimal temperatures and pHs were recently isolated (Orlygsson and Baldursson 2007). It was, therefore, hypothesized that continuous EtOH+H_2 co-production could be achieved and maintained by a co-culture of these strains (Paper III). For AK17, being a high EtOH-producer, it was important to determine the ethanol tolerance, as it is one of the most important characteristics of ethanol-producers.

Regarding the great diversity of geothermal spring environments in Iceland, and the relatively low attention paid to enriching thermophilic H_2-producers, it was hypothesized that a wide

diversity of H_2-producing microbial cultures, potentially including novel, efficient H_2-producers, could be obtained through extensive screening of Icelandic hot spring samples (Paper IV). Even though more thoroughly explored previously (e.g., Sonne-Hansen 1993; Ahring et al. 1996; Sommer et al. 2004), also thermophilic EtOH+H_2 –co-producing enrichments were aimed along with the H_2-producers. The H_2 (33HL, Paper IV and V) or EtOH+H_2 (9HG, Paper IV) production potentials of most promising enrichments were further characterized.

Even though thermophiles have been considered to possess higher H_2 production efficiencies (i.e., HY and HPR) compared to mesophiles, the continuous, thermophilic H_2 production processes have suffered from low H_2 production rates due to low cell densities (For reviews, see de Vrije and Claassen 2003; Hallenbeck 2005). It was therefore hypothesized, that if bacterial retention in reactors (e.g., by granulation) could be enhanced, high-rate thermophilic H_2 production could be achieved (Paper V). Based on the experience obtained with mesophilic H_2-producing granular-cell reactors, a reactor configuration suitable for obtaining self-granulation was used for thermophiles in this study (Paper V). Further, it was previously experienced that the H_2 production efficiency of 33HL was decreased when cultivated continuously compared to batch cultivations. It was hypothesized that applying a semi-continuous (ASBR) reactor would overcome this problem.

Keeping in mind the reasoning and hypotheses described above, the aims of this work included the following:

- Enrich mesophilic H_2-fermenting bacteria from anaerobic digester sludge and determine their H_2 yield from glucose in batch assays. (Paper I)
- Evaluate the suitability (performance and stability) and operation strategy of mesophilic FBR for H_2 production from glucose. (Paper I)
- Characterize the diversity and dynamics of attached- and suspended-growth microbial communities in FBR for H_2 dark fermentation. (Paper I)
- By the microbial community analyses, obtain insight into process microbiology and reasons for the instability of H_2 production in FBR. (Paper I)
- Operate a suspended-cell reactor in a comparative manner to the biofilm reactor (FBR)(Paper I) to obtain information on the effect of bioreactor type on H_2 production performance and stability (Paper II).
- Evaluate the effect of a gas extraction module on the continuous H_2 production performance in a completely-mixed column reactor (CMCR). (Paper II)
- To develop a computational approach which can be used to better detect and visualize prevailing metabolic patterns in the H_2 dark fermentation process dataset, and to model the H_2 production rate by using measured data (process performance and operational conditions). (Paper II)
- Determine H_2 and ethanol yields from glucose and xylose in batch assays by isolates AK15 and AK17. (Paper III)
- Determine ethanol tolerance for ethanol-producing strain AK17. (Paper III)
- Determine the effects of HRT and glucose loading on co-production of EtOH and H_2 by a co-culture of isolates AK15 and AK17 in the CMCR. (Paper III)
- Analyze the microbial community dynamics to reveal the fate of AK15 and AK17 in the open, suspended-cell (CMCR) bioreactor system. (Paper III)

- Enrich efficient, saccharolytic, thermophilic H_2 or EtOH+H_2 –producers with glucose and cellulose from Icelandic hot springs. (Paper IV)
- Determine temperature dependencies on H_2 and ethanol production by enrichments 33HL and 9HG. (Paper IV)
- Evaluate pH dependency of continuous EtOH+H_2 co-production by 9HG at 74 °C. (Paper IV)
- Determine the effects of HRT and glucose loading on H_2 production by enrichment 33HL at 58 °C in continuous and semi-continuous reactor systems (Paper V)
- Improve biomass retention of 33HL by bacterial granulation in continuous and semi-continuous reactor systems (Paper V)
- Characterize the microbial community diversity and dynamics in continuous and semi-continuous H_2 production processes maintained with enrichment 33HL (Paper V)

9 MATERIALS AND METHODS

9.1 Enrichment of microorganisms

Mesophilic H_2-producers were enriched from sludge samples of anaerobic digester treating municipal waste water sludge in Tampere, Finland (Table 26). Hydrogen and/or ethanol producing thermophiles were enriched from sediment samples of geothermal sprigs in Iceland (Table 26, Figure 5). The enrichments were done as series of batch incubations at the corresponding *in-situ* temperatures.

Table 26. Sources of hydrogen and/or ethanol producing microorganisms

Location	Source	Enrichment (or isolate)	T (°C)	Paper(s)
Tampere, Finland	Municipal waste water sludge digester	Mesophilic enrichment	37	I,II
Viti, Iceland	Geothermal spring sediment and water	AK15, AK17	60	III, Orlygsson and Baldursson 2007
Hveragerdi, Iceland	Geothermal spring sediment and water	9HG	78	IV
Hveravellir, Iceland	Geothermal spring sediment and water	33HL	60	IV, V

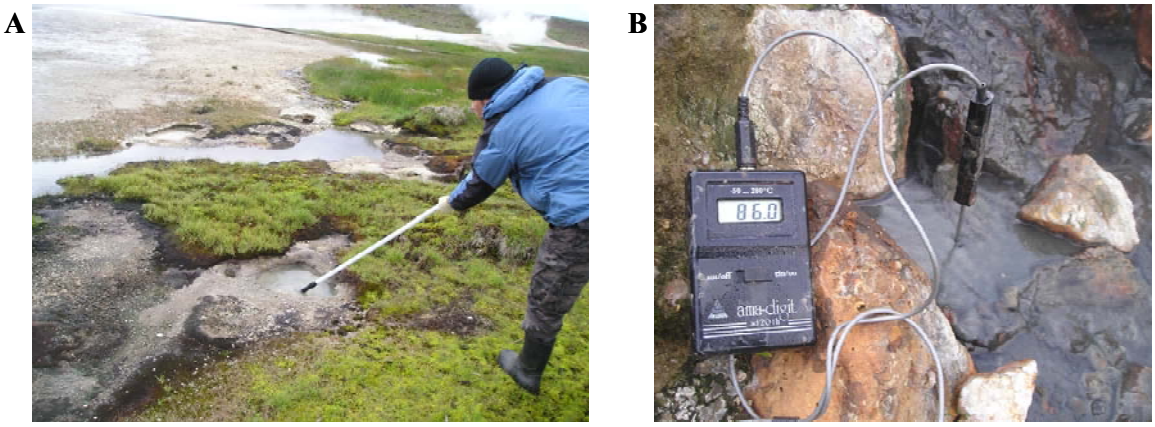


Figure 5. Sampling from geothermal springs in Iceland for the enrichment of H_2 and ethanol producing thermophiles. A) Hveravellir (Photo: T. Denecke); B) Hveragerdi (Photo: P. Koskinen).

9.2 Bioreactors

Hydrogen or ethanol+H_2 production was studied in suspended-cell, biofilm and granular-cell laboratory-scale bioreactor systems (Table 27a, Figures 6-9).

Table 27a. Continuous or semi-continuous bioreactor systems used for H_2 or ethanol+H_2 production.

Bioreactor (working volume [L])	T (°C)	Specifications	Parameters studied	Paper
FBR (0.8)	37	Biofilm-type. Carrier: Celite R-633, gas extraction	HRT, microbial community	I
CMCR (0.8)	37	Suspended-cell, mixing by re-circulation, gas extraction/no gas extraction	HRT	II
CMCR (0.3)	60	Suspended-cell, mixing by re-circulation	HRT, LR, microbial community	III
CMCR (0.45)	74	Suspended-cell, mixing by re-circulation	pH, microbial community	IV
CMCR (0.9)	58	Suspended-cell, mixing by re-circulation	HRT, microbial community	V
ASBR (2 to 4)	58	Granular-cell, mixing by magnetic stirrer	HRT, LR, microbial community	V
CSTR (0.9)	58	Granular-cell, mixing by mechanical stirring. Polymeric carrier: Horiba Ltd	HRT, LR, microbial community	V

ASBR = anaerobic sequencing batch reactor; CMCR = completely-mixed column bioreactor; CSTR = completely-stirred tank reactor; FBR= fluidized-bed bioreactor.

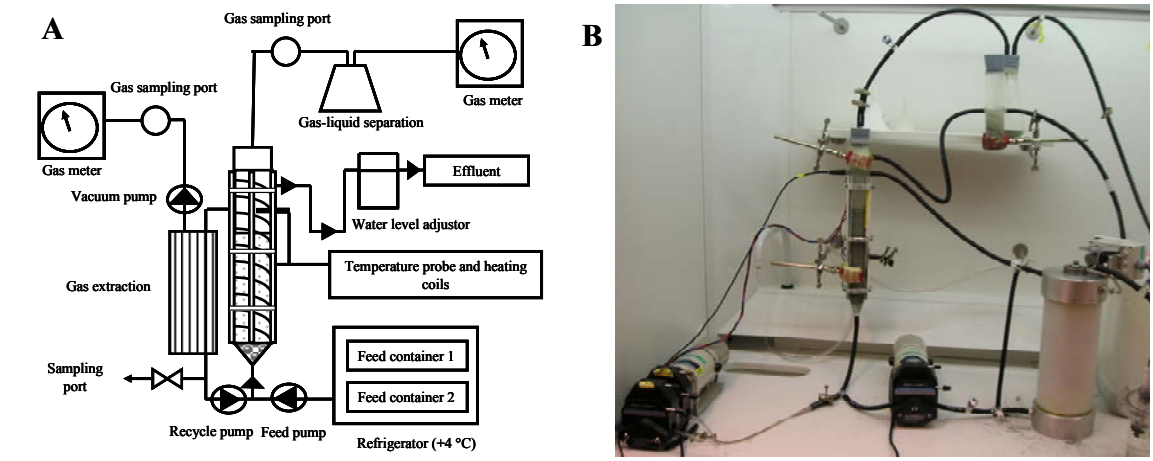


Figure 6. Fluidized-bed bioreactor (FBR) used in paper I A) configuration, B) photograph (Photo: P. Koskinen). In paper II similar reactor configuration was used, but reactor did not contain carrier.

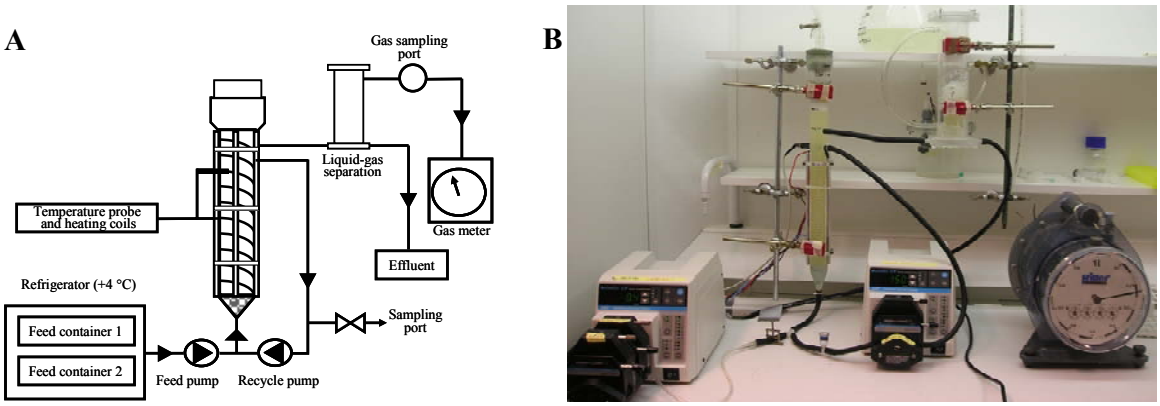


Figure 7. Completely-mixed column bioreactor (CMCR) used in paper IV A) configuration, B) photograph (Photo: P. Koskinen). In paper III, similar reactor configuration was used, but the reactor volume was 0.3 L.

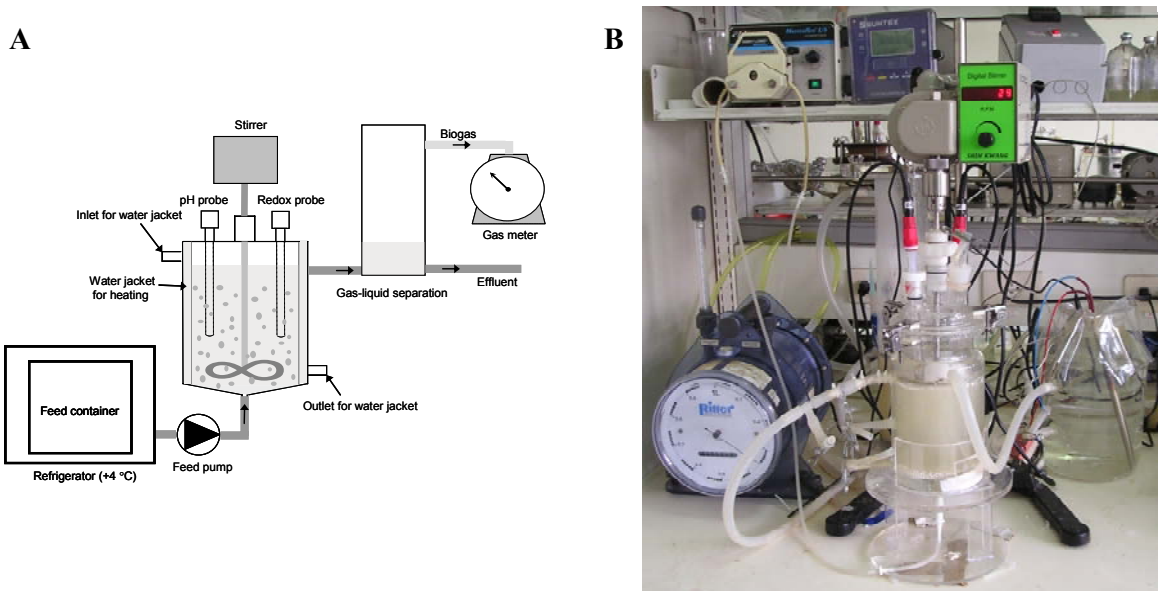
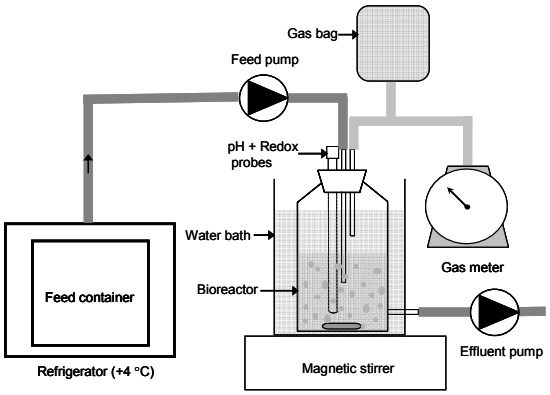


Figure 8. Continuous-flow, completely-stirred tank reactors (CSTR) used in paper V A) configuration, B) photograph (Photo: P. Koskinen).

A



B

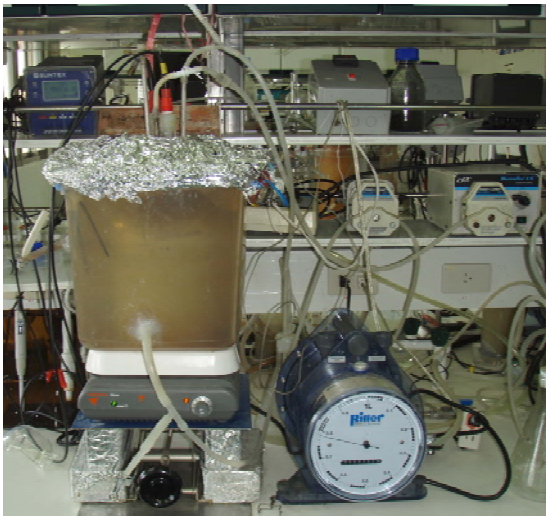


Figure 9. Anaerobic sequencing batch reactor (ASBR) used in paper V A) configuration, B) photograph (Photo: P. Koskinen).

9.3 Physicochemical analyses

The physicochemical analyses carried out in this work were as summarized in Table 27b.

Table 27b. Summary of physicochemical analyses performed in this study.

Analysis	Instrument(s)	Paper(s)
Composition of biogas (H_2, CO_2, CH_4)	Gas chromatograph with thermal conductivity detector	I,II,III,IV,V
Volume of biogas produced	Gas meter (bioreactor) or gas syringe (batch assays)	I,II,III,IV,V
Ethanol, butanol, acetate, propionate, butyrate, valerate, caproate	Gas chromatograph with flame ionization detector	I,II,III,IV,V
Glucose, formate, lactate	High performance liquid chromatograph	III, IV, V
Glucose	Spectrophotometer	I,II
Temperature	Temperature electrode or digital thermometer	I,II,III,IV,V
pH	pH electrode	I,II,III,IV,V
Oxidation-reduction potential	Redox electrode	IV,V
Volatile suspended solids (VSS)	Oven, balance	I,II,III,IV,V
Volatile solids (VS)	Oven, furnace, balance	I
Optical density	Spectrophotometer	III, IV, V

9.4 Microbiological analyses

The microbiological analyses carried out in this study were as summarized in Table 28.

Table 28. Summary of the microbiological analyses employed in this study.

	Method	Paper(s)
Diversity and dynamics	DNA extraction and purification	I, III, IV, V
	Polymerase chain reaction (PCR)	I, III, IV, V
	Denaturing gradient gel electrophoresis (DGGE)	I, III, IV, V
	DNA sequencing	I, III, IV, V
Physiological characteristics	Fermentation pattern in batch assays	I, III, IV, V
	Growth temperature range in temperature gradient incubator	IV
	Ethanol inhibition tests in batch assays	III
Visualization	Light microscopy	I,II,III,IV,V

Computer software used in the microbial community analyses were as summarized in Table 29.

Table 29. Software used in microbial community analyzes in this study.

Software	Purpose	Paper(s)	Reference/Source
Bioedit version 7.0.5.2	Analysis of chromatograms	I,III,IV,V	Hall 1999; http://www.mbio.ncsu.edu/BioEdit/bioedit.html
NCBI Nucleotide Blast	Identity search from GenBank database	I,III,IV,V	Altschul et al. 1990; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi
RDB-II Sequence Match	Identity search from RDB database	I,III,IV,V	Ribosomal database project II; http://rdp.cme.msu.edu/
Chimera_check, version 2.7	Identification of chimerical sequences	I,III,IV,V	Ribosomal database project II; http://rdp8.cme.msu.edu/cgis/chimera.cgi?su=SSU
ARB	Alignment of sequences, construction of phylogenetic trees	I, III, V	Ludwig et al. 2004; http://www.arb-home.de/
Treeview	Modification of phylogenetic trees	I, III, V	Page 1996; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html

9.5 Modelling methods

A Clustering Hybrid Regression (CHR) model was developed to better visualize and detect metabolic patterns in bioreactor experimental dataset, and to model H_2 production rate based on the data on process parameters and metabolites (Paper II). The schema of CHR approach is shown in Figure 10. The CHR modeling was performed with MATLAB v. 7.3 by using functions described in Table 30.

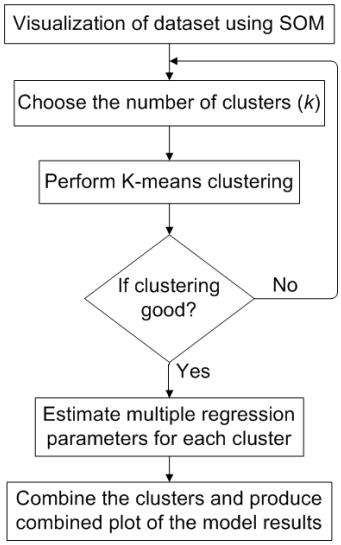


Figure 10. Schema for clustering hybrid regression model (CHR) (Adapted from Nikhil et al. 2008).

Table 30. Software used in Clustering Hybrid Regression model in Paper II.

MATLAB function/toolbox	Purpose
SOMPAK toolbox	Plot Self Organizing Maps (SOMs) and SOM trajectories. Determine the number (k) of significant clusters (metabolic patterns)
Kmeans function	Form 'k' clusters in the dataset
Silhoutte function	Evaluate quality of clusters formed in k-means clustering
Regstats function	Estimate multiple regression parameters of the model

10 RESULTS AND DISCUSSION

10.1. Enrichment of hydrogen- and ethanol-producing microorganisms

Enrichment of mesophiles

In this study, mesophilic H_2-fermenters were enriched from anaerobic digester treating municipal wastewater sludge (Paper I). The enrichment was performed as series of batch cultures, and the growth of methanogens was inhibited by using BESA. Several pre-treatment methods have been used to harvest H_2-fermenting microorganisms from anaerobic sludge including heat (Lay et al. 1999,2003; Oh et al. 2003; Kawagoshi et al. 2005; Cheong and Hansen 2006; Valdez-Vazquez et al. 2006; Mu et al. 2007; Venkata Mohan et al. 2008), acid (Chen et al. 2002; Oh et al. 2003; Kawagoshi et al. 2005; Cheong and Hansen 2006; Mu et al. 2007; Venkata Mohan et al. 2008), alkaline (Chen et al. 2002; Cai et al. 2004; Mu et al. 2007), BESA (Sparling et al. 1997; Cheong and Hansen 2006; Venkata Mohan et al. 2008), acetylene (Sparling et al. 1997; Valdez-Vazquez et al. 2006), freezing-thawing (Cheong and Hansen 2006) and dry-heating-desiccation (Cheong and Hansen 2006) treatments. Based on H_2 yields obtained with different pre-treatment methods, BESA-treatment can be considered as rather promising enrichment strategy (Table 31). Cheong and Hansen (2006) reported highest H_2 production from glucose with acid-treated and subsequently BESA -treated sludge while heat-desiccation resulted in the lowest H_2 production. Venkata Mohan et al. (2008) reported highest H_2-production from dairy wastewater by acid and BESA-treated sludge followed by BESA-only treated sludge while the lowest production was obtained with acid-only treated sludge. In both studies, heat-treatment resulted in average H_2-production.

The enrichment material (Kawagoshi et al. 2005) and pre-treatment method (Cheong and Hansen 2006; Mu et al. 2007; Venkata Mohan 2008) obviously affect the H_2 production efficiency of enrichment culture. The H_2 yield by mesophilic enrichment in this study (1.24 mol-H_2 mol-glucose^{-1}) is average compared to yields reported in the literature (Table 31). Higher H_2 yields have been reported by heat (Van Ginkel et al. 2001, Oh et al. 2003; Kawagoshi et al. 2005; Mu et al. 2007) and acid-treated (Mu et al. 2007) compost or anaerobic sludge. High concentrations of BESA (1M) have been suggested to inhibit the H_2 production by Clostridia (Wang et al. 2003a). The BESA did not affect the H_2 production in this study, since low BESA concentration (1 mM) was used. In fact, batch assays with low BESA concentration (1 mM) resulted in similar H_2 yield (1.24 mol-H_2 mol-glucose^{-1}) as compared with control assays without BESA (unpublished results). More importantly, the BESA-treatment does not select spore-forming bacteria only. The spore-formers include H_2-fermenting *Clostridia*, but also lactate-producers (e.g., *Bacillus racemilacticus* and *B. myxolacticus* [Iyer et al. 2004; Kim et al. 2006c]) or acetogens (e.g., *C. coccoides*, *C. magnum*, *C. scatologens*, *C. thermoaceticum*, *C. thermoautotrophicum* and *Sporomusa ovata* [Sung et al. 2002; Kim et al. 2006c]) unfavorable in H_2-production processes. However, the sporulation-treatment may decrease propionate production (Cohen et al. 1985) as the majority of saccharolytic propionate-producers are non-spore formers (Hawkes et al. 2007). It must be further noted that batch culturing may not reveal the H_2-production potential in continuous cultures. Microbial cultures for high rate H_2 production processes have been obtained by continuous acclimation of acid or heat-treated sludge (Lee et al. 2004a,b, 2006a,b; Wu et al. 2006).

Table 31. Comparison of H_2 yields (HYs) with different enrichment-methods for mesophilic H_2-fermenters.

Treatment method	Enrichment matrix	Electron donor	HY (mol-H_2 mol-hexose^{-1})	Reference
BESA-treatment (25mM, 10mM, 1mM)	Anaerobic digester sludge from municipal WWTP	Glucose	1.24	Paper I
Acid treatment (pH 3, 24 h) Alkaline treatment (pH 10, 24h)	Drying-bed sludge from municipal WWTP	Glucose	0.31* 0.18*	Chen et al. 2002
Acid treatment (pH 3, 48h) BESA-treatment (500 mM) Heat treatment (95°C, 20 min) Freezing and thawing (-10°C, 24h) Dry heat and desiccation (105°C 2h + 2 h desiccation)	Sludge from cattle manure treatment plant	Glucose	0.84* 0.72* 0.51* 0.34* 0.32*	Cheong and Hansen 2006
Heat treatment (104°C, 2h)	Compost	Sucrose	2.45	Van Ginkel et al. 2001
Heat treatment (100°C, 2h)	Anaerobic digester sludge from municipal WWTP	Glucose	~1.4	Kawagoshi et al. 2005
Heat treatment (104°C, 2h)	Anaerobic digester sludge from municipal WWTP	Glucose	1.64*	Oh et al. 2003
Heat treatment (102°C, 1.5h) Acid treatment (pH 3, 24h) Alkaline treatment (pH 12, 24h)	Sludge from anaerobic digester treating soybean-processing wastewater	Sucrose	2.00 1.30 0.48	Mu et al. 2007

* calculated based on information provided

Enrichment of thermophiles

Geothermal springs are potential sources for the enrichment of thermophilic, saccharolytic microorganisms – A variety of efficient H_2 and EtOH+H_2- producing organisms have been isolated from hot spring environments including e.g., *C. saccharolyticus* (Rainey et al. 1994), *Tbr. ethanolicus* (Wiegel and Ljungdahl 1981) and *Tbr. thermohydrosulfuricus* (formerly *C. thermohydrosulfuricum*) (Wiegel et al. 1979; Sonne-Hansen 1993). A variety of microorganisms capable of producing ethanol from hemicellulose components (xylan, xylose) have been previously enriched from Icelandic hot springs (Sonne-Hansen 1993; Ahring et al. 1996; Sommer et al. 2004). Further, an efficient xylose-degrading ethanol producer, *T. mathranii*, has been isolated from sediment samples of geothermal springs in Iceland (Larsen et al. 1997). Orlygsson and Baldursson (2007) isolated four H_2 or EtOH+H_2 -producing strains from Icelandic hot springs of which strains AK15 and AK17 were investigated in this study (Paper III).

In this study, several H_2 or EtOH+H_2 producing cultures from glucose, over a temperature range from 50 to 78 °C, were enriched from Icelandic geothermal springs using batch cultivations (Paper IV). Further, one culture produced H_2 directly from cellulose at 70 °C. Of the batch enrichments, culture 33HL (high H_2 production) (Paper V) and 9HG (high ethanol production) (Paper IV) were characterized further. The batch H_2 yield from glucose by 33HL, 3.2 mol-H_2 mol-glucose^{-1}, is among the highest reported for thermophiles (Table 32). Higher H_2 yields have been only reported for thermophilic pure cultures, cultivated with gas purging (van Niel et al. 2002). Culture 9HG had relatively high ethanol yield from glucose, 1.21 mol-EtOH mol-glucose^{-1}, and a H_2 yield of 0.68 mol-H_2 mol-glucose^{-1}.

Strains AK15 and AK17 used in this study, had a wide substrate utilization range (Orlygsson and Baldursson 2007), and a capability of utilizing a variety of sugar constituents found in lignocellulosic material hydrolysates (Paper III). Strain AK15 had relatively high H_2 production from glucose (up to 1.9 mol-H_2 mol-glucose^{-1}) and xylose (up to 1.1 mol-H_2 mol-xylose^{-1}) (Table 32, Paper III). Strain AK17 had high ethanol production from glucose (up to 1.6 mol-EtOH mol-glucose^{-1}) and xylose (1.1 mol-EtOH mol-xylose^{-1}) which are amongst the highest reported for thermoanaerobes (Table 33). Further, the ethanol tolerance of AK17 (up to 4%, v/v) was relatively high within wild type anaerobes (Paper III). Thermophiles, in general, tolerate less than 1 to 2%, v/v of ethanol (Lynd 1989; Burdette et al. 2002), while the

highest ethanol tolerance for wild-type thermoanaerobes of 5.1% has been reported for *Thermoanaerobacter* A10 (Georgieva et al. 2007).

Table 32. Batch hydrogen yields (HYs) of thermophilic enrichments or isolates

Culture	T (°C)	Electron donor	HY (mol-H_2 mol-hexose^{-1})	Reference
33HL	60	Glucose	3.2	PaperV
AK15	60	Glucose	1.9	Paper III
AK17	60	Glucose	1.2	Paper III
9HG	74	Glucose	0.68	Paper IV
Caldicellulosiruptor saccharolyticus	70	Glucose	3.3	van Niel et al. 2002
Thermotoga elfii	65	Glucose	3.3	van Niel et al. 2002
Thermotoga maritima	80	Glucose	1.56	Nguyen et al. 2008
Thermotoga neapolitana	75	Glucose	1.84	Nguyen et al. 2008
Thermoanaerobacterium thermosaccharolyticum	60	Sucrose	2.53	O-Thong et al. 2008
Clostridium thermocellum	60	Cellobiose	1.05	Islam et al. 2006
Clostridium uzonii	65	Glucose	0.67*	Krivenko et al. 1990
Mixed culture	75	Glucose	2.65	Yokoyama et al. 2007a
Mixed culture	60	Cellulose	2.00	Ueno et al. 2001a
Mixed culture	55	Glucose	0.32*	Cheong and Hansen 2007

* calculated based on information provided

Table 33. Batch ethanol yields of enrichments or isolates

Culture	T (°C)	EtOH yield hexose^{-1}	EtOH yield xylose^{-1}	Reference
AK15	60	0.8	0.4	Paper III
AK17	60	1.6	1.1	Paper III
9HG	74	1.21	N.D.	Paper IV
Thermoanaerobacter ethanolicus	~70	1.9	1.4	Wiegel and Ljungdahl 1981; Lacis and Lawford 1991
Thermoanaerobacter thermohydrosulfuricus	70	1.5	1.1	Wiegel et al. 1979; Cook and Morgan 1994
Thermoanaerobacter mathranii	70	N.A.	1.1	Larsen et al. 1997
Zymomonas mobilis	30	1.9	(1.57)[a]	Lawford and Rousseau 1999; for reviews, see Swings and De Ley 1977; Dien et al. 2003
Saccharomyces cerevisiae	30	1.9	(0.26)[a]	Kötter and Ciriacy 1993; for a review, see Kosaric and Vardar-Sukan 2001
Clostridium thermocellum	60	1.3	N.A.	Sudha Rani et al. 1997
Clostridium uzonii	65	0.76	N.A.	Krivenko et al. 1990
Thermoanerobacterium aciditolerans	55	0.9	N.A.	Kublanov et al. 2007
Thermoanaerobacterium polysaccharolyticum	65 - 68	1.08	N.A.	Cann et al. 2001
Thermoanaerobacterium thermosaccharolyticum	56	1.09	~1.3	Lee and Ordal 1967; Hill et al. 1993
Thermoanaerobacterium zeae	65 - 70	1.19	N.A.	Cann et al. 2001

[a]yield in brackets obtained through genetic engineering. N.A. = not available, N.D. not determined.

In continuous-flow enrichment with glucose (100 mM) at 78 °C, the bioreactor (CSTR-type, volume of 400 ml) was directly inoculated with 8 samples collected from Icelandic hot springs (unpublished results). The reactor was operated with a constant HRT of 7 h. In the reactor, ethanol was the main soluble metabolite with ethanol yield of 1.07 ± 0.19 mol-EtOH mol-glucose^{-1} (unpublished results). Hydrogen production rates in the reactor were low, 1.59 ± 0.46 mmol h^{-1} L^{-1}, corresponding to a H_2 yield of 0.20 ± 0.04 mol-H_2 mol-glucose^{-1} (unpublished results).

10.2. Diversity and dynamics of microbial communities

Rittmann (2006) defines environmental biotechnology as "managing microbial communities to provide services to society". Therefore, for optimization of bioprocesses, it is essential to know the composition of microorganisms and to understand their behavior in the process (Brionis and Raskin 2003; Rittmann 2006). Little attention has been paid to analyzing the diversity of H_2-fermenting microbial communities and even less to assessing community dynamics in continuous, H_2 dark fermentation processes (Iyer et al. 2004; Wu et al. 2006; 2008a; Zhang et al. 2006b; Hung et al. 2007a; Jo et al. 2007). Table 34 shows the main organisms detected in the H_2- or EtOH+H_2 -producing cultures in this study.

Table 34. Dominant hydrogen and ethanol-producing microorganisms based on PCR-DGGE and 16S rRNA gene sequence analyses.

Culture	Enrichment method	Origin	T (°C)	Dominant organisms	Reference(s)
Mesophilic enrichment	Batch	WWTP, Finland	35	*C. butyricum* (99.8-100%), *E. coli* (100%)	Paper I
	FBR	Mesophilic enrichment	35	*C. butyricum* (99.8-100%), *E. coli* (100%), and several others	Paper I
	CMCR	Mesophilic enrichment	35	*C. butyricum* (99.8-100%), *E. coli* (100%), *B. circulans* (99.6%) and others	Paper II, Tolvanen et al. 2008
Isolates AK15 and AK17	CMCR	Hot spring, Iceland	60	AK17 (*Tbm. aciditolerans* [99.2%])	Paper III
9HG	Batch	Hot spring, Iceland	78	*Tbr. thermohydrosulfuricus* (100%)	Paper IV
	CMCR	9HG	74	*Tbr. thermohydrosulfuricus* (100%)	Paper IV
33HL	Batch	Hot spring, Iceland	60	*Thermobrachium celere* 100%)	Paper V
	ASBR, CSTR	33HL	58	*Tbm. aotearoense* (98.5–99.6 %)	Paper V

Mesophilic processes (Papers I, II)

The mesophilic enrichment was dominated by bacteria closely affiliated with *C. butyricum* and *E. coli* (Paper I). These organisms were also detected in the mesophilic FBR (Paper I) and CMCR (Tolvanen et al. 2008), inoculated with the enrichment culture, throughout the experiments. New organisms were enriched in the FBR (Paper I) and CMCR (Tolvanen et al. 2008) communities rapidly, after 2 days of the start-up of continuous operation, seen as new bands in DGGE gels. The mesophilic FBR culture (17 different OTUs detected) was more diverse than the CMCR culture (9 different OTUs). The microbial community analyses indicated that the instability of H_2 production in mesophilic reactors was due to changes in microbial community compositions, i.e., enrichment of H_2-consuming or H_2 non-producing organisms (Paper I, Tolvanen et al. 2008). During the highest H_2 production in FBR and CMCR, microbial community was dominated by *C. butyricum* indicated by high intensity of *C. butyricum* affiliated bands in DGGE gels and simple community structure, and by the high butyrate production. Further, in the CMCR, the HPR followed the same trend with quantities of *C. butyricum* determined with quantitative, real-time PCR (qrt-PCR) (Tolvanen et al. 2008). During low H_2 production, microbial community was more diverse. Unfortunately, majority of the organisms enriched on FBR and CMCR were either distantly affiliated with any known organisms or affiliated with organisms with unknown H_2 production characteristics. This hindered the determination of possible functions of these organisms in the bioprocesses.

C. butyricum is one of the most studied mesophilic H_2-producing organisms and is often detected in mixed-culture H_2-fermentation processes (Chen et al. 2005; Lin et al. 2006b; Kim et al. 2006a,c; Hung et al. 2008; Jan et al. 2008; Wu et al. 2008a,b). *C. butyricum* has relatively high H_2 yield (up to 2.3 mol-H_2 mol-glucose^{-1} [Kataoka et al. 1997]), and utilizes a variety of carbohydrates including starch and pectin (Hippe et al. 1992). *C. pasteurianum* does not utilize starch (Hippe et al. 1992), but has higher H_2 production rate from glucose and sucrose than *C. butyricum* (Heyndrickx et al. 1990). In fact, *C. pasteurianum* has been the dominant H_2-producer in the high-rate, granular, H_2-producing bioreactors utilizing sucrose (Wu et al. 2006, 2008a) or glucose (Fang et al. 2002a; Hung et al. 2007). *C. butyricum* has been the dominant H_2-producer in xylose-utilizing granular bioreactors (Wu et al. 2008b). Both *C. butyricum* (van Andel et al. 1985; Crabbendam et al. 1985; Zoutberg et al. 1989) and *C. pasteurianum* (Hippe et al. 1992) can excrete extracellular proteins that contribute to the formation of granules (Zoutberg et al. 1989). The self-granulation of *C. butyricum* is favored by high substrate loading and high concentrations of butyric acid (Zoutberg et al. 1989). Further, the formation of *Clostridia*-rich, H_2-producing granules may be enhanced by other bacteria present. By performing fluorecent *in situ* hybridization (FISH) analyses, Hung et al.

(2007) suggested that *Steptococcus* affiliated organisms acted as a seed for the for formation *Clostridia*-rich granules.

In the mesophilic, H_2-producing, mixed-culture bioreactors, obligately anaerobic *Clostridia* are commonly accompanied with facultatively anaerobic H_2-producers belonging to the genera *Citrobacter* (Fang et al. 2002b; Iyer et al. 2004), *Escherichia* (Hung et al. 2005), *Enterobacter* (Iyer et al. 2004) or *Klebsiella* (Wu et al. 2006, 2008a,b; Hung et al. 2007). The facultative anaerobes remove the traces of O_2 and create anaerobic environment for obligately anaerobic H_2-producers (Yokoi et al. 1998b; Hung et al. 2007). *E. coli* was likely the main facultative anaerobe in the mesophilic mixed cultures in this study (Paper I; Tolvanen et al. 2008). In general, wild-type strains of *E. coli* produce H_2 yields from glucose of less than 1 mol-H_2 mol-glucose^{-1} (Blackwood et al. 1956; Turcot et al. 2008), but the yields have been increased by genetic engineering (Chittibabu et al. 2006; Yoshida et al. 2006). The H_2 production by *E. coli* is highly strain-depended, including strains that do not produce H_2 (Mishra et al. 2004), and on the culturing conditions. Some *E. coli* strains produce high H_2 yields (up to 2.55 mol-H_2 mol-glucose^{-1}) under carbon or nutrient limitation at low dilution rates (= long HRTs) (Turcot et al. 2008).

Thermophilic ethanol and H_2 co-production (Papers III, IV)

The diversity (Ueno et al. 2001a; Zhang et al. 2003; Ahn et al. 2005; Shin and Youn 2005; Yokoyama et al. 2007a,b) and dynamics of thermophilic H_2-producing bioreactors has not been thoroughly explored. Thermophilic mixed communities in this study (Papers III, IV, V) were less diverse compared to the mesophilic communities (Paper I; Tolvanen et al. 2008).

Strain AK17, affiliated with *Tbm. aciditolerans* (99.2 %) became dominant thermophile in the ethanol- and H_2-producing CMCR inoculated with a co-culture of isolates AK15 and AK17. The ethanol and H_2 production performance of CMCR corresponded to that of batch cultures of AK17 (Table 33 and 37). Ethanol production by AK17 was substantially higher (up to 1.6 mol-H_2 mol-glucose^{-1}) than reported for *Tbm. aciditolerans* (0.9 mol-H_2 mol-glucose^{-1}) (Table 33). The strain AK15, affiliated with *C. uzonii* (98.8%), disappeared from the community profile within three weeks from the start-up of continuous operation indicating that the environmental and/or hydrodynamic conditions in the reactor did not favor AK15 in the CMCR.

The batch enrichment 9HG was dominated by bacteria closely affiliated with *Tbr. thermohydrosulfuricus* (100%). Similarly, *Tbr. thermohydrosulfuricus* affiliated strain dominated the ethanol- and H_2-producing CMCR. *Tbr. thermohydrosulfuricus* is a rather extensively studied ethanol producer. The 9HG culture, behaved similarly as characterized strains of *Tbr. thermohydrosulfuricus*. Wiegel et al. (1979) reported H_2 yields from 0.5 to 1.5 mol-H_2 mol-glucose^{-1} by *Tbr. thermohydrosulfuricus*, depending mainly on the culture pH. The decreased H_2 yield was due to increased lactate production (Wiegel et al. 1979), alike in the CMCR in this study (Paper IV). Further, as observed in the CMCR in this study, decreased pH did not favor ethanol production by *Tbr. thermohydrosulfuricus* (Wiegel et al. 1979; Cook and Morgan 1994). Unlike *C. thermocellum* (Freier et al. 1988; Sudha Rani et al. 1997; Bothun et al. 2004) and *Tbr. brockii* (Ben-Bassat et al. 1981), increased pH_2 does not generally increase ethanol production by *Tbr. thermohydrosulfuricus* (Lovitt et al. 1988; Cook and Morgan 1994), but this behavior is strain-dependent (Lovitt et al. 1988).

Thermophilic H_2 production (Paper V)

Thermophilic batch enrichment 33HL, producing efficiently H_2 from glucose through acetate, was dominated by bacteria closely affiliated with *Thermobrachium celere* (100%). *Tbh. celere* affiliated strains, however, did not thrive in the open system bioreactors. Instead,

Thermoanaerobacterium aotearoense (98.5 – 99.6%) affiliated strains, producing H_2 along with butyrate and acetate, dominated the reactor (ASBR and CSTR) cultures. However, *Tbm. aotearoense* does not produce butyrate in the glucose fermentation. Instead, another close relative of the strains detected in the bioreactors, *Thermoanaerobacterium thermosaccharolyticum* (previously *Clostridium thermosaccharolyticum*) (98.3 – 98.9 %), is a thermophilic, saccharolytic, butyrate- and hydrogen-producer (Sjolander 1937). *Tbm. thermosaccharolyticum* has been dominant organism in several thermophilic, H_2-producing mixed culture processes utilizing glucose (Ueno et al. 2001b; Ahn et al. 2005), food waste (Shin and Youn 2005) or garbage slurry (Ueno et al. 2006). Further, *Tbm. thermosaccharolyticum* has been detected in H_2 production processes utilizing starch (Zhang et al. 2003) or cellulose (Ueno et al. 2001a). *Tbm. thermosaccharolyticum* has a wide substrate utilization spectrum including e.g., xylose, glycogen, cellobiose, starch and pectin (Hollaus and Sleytr 1972; Hippe et al. 1992).

The 33HL ASBR culture (*Tbm. aotearoense* dominated culture) incubated in batch utilized glucose, galactose, xylose, sucrose, mannose, fructose, arabinose and starch, while cellulose and lignin did not support growth (Mäkinen A., unpublished results). From the above mentioned, 33HL batch enrichment (*Tbh. celere* dominated culture) did not utilize arabinose, cellulose or lignin (Mäkinen A., unpublished results). The substrate utilization spectrum of 33HL ASBR culture includes the main sugar monomers of lignocellulosic material (see Chapter 5).

10.3. Performances of continuous H_2 or ethanol+H_2 production processes

Performance of mesophilic, continuous H_2 production (Papers I, II)

Mesophilic H_2 production was studied in two continuous-flow bioreactor systems, a fluidized-bed bioreactor (FBR) (Paper I) and a suspended-cell, completely-mixed column bioreactor (CMCR) (Paper II). The H_2 yields obtained during the highest H_2 production 1.90 (FBR) and 1.70 (CMCR) mol-H_2 mol-glucose^{-1} are relatively high compared to those reported in the literature for mesophilic processes (Table 35). However, the hydrogen production in mesophilic reactors was instable and high H_2 yields were obtained only momentarily. In FBR, propionate production ceased the H_2 production rapidly (within few days) after the start-up (Paper I). A semi-continuous operation pattern, an intermittent batch operation, allowed a momentary recovery of the H_2 production. In suspended-cell reactor (Paper II), H_2 production decreased substantially (H_2 production rates between 1 and 6 mmol h^{-1} L^{-1}) by the increased production of acetate, suggesting acetogenic pathways, and by increased ethanol production. Further, after 150 d of continuous operation, the hydrogen production finally ceased due to the production of propionate. Installation of gas-extraction module in the CMCR increased the H_2 production slightly. The highest H_2 production rate in suspended-cell CMCR-system (18.8 mmol h^{-1} L^{-1}) is average compared with other suspended-cell systems reported in the literature. The H_2 production rate observed in the FBR (28.8 mmol h^{-1} L^{-1}) is lower than generally reported for biofilm systems (Table 35). The H_2 production rates observed in the mesophilic reactors in this study, however, remain far less compared to the highest rates obtained with granular (Lee et al. 2003, 2004b, 2006a,b; Wu et al. 2005a, 2006a; Zhang et al. 2008a,b) or biofilm reactors (Jeon et al. 2008) (Table 35) operated with high substrate loading (i.e., low HRT and high substrate concentrations) (Table 35). Highest H_2 production rates have been obtained with sucrose as the substrate (Wu et al. 2006; Jeon et al. 2008), rather than glucose used in this study.

Table 35. Performances of mesophilic hydrogen dark fermentation bioreactors with model compounds as electron donors

Reactor type	Microorganism	Conditions			Electron donor (concentration [gL^{-1}])	H_2 production		VSS ($g\ L^{-1}$)	Reference
		T (ºC)	pH	HRT (h)		Max. HY (mol H_2 mol-substrate^{-1})[a]	Max. HPR (mmol h^{-1} L^{-1})		
Suspended cell –reactors									
CMCR	Mixed culture	35	~6	1.9	Glucose (5)	**1.70**	**18.8**	0.20	**Paper II**
CSTR	Mixed culture	35	5.7	6	Glucose (15)	**1.7**	**30**	1.3	Lin and Chang 1999
CSTR	Mixed culture	35	6.7	8	Glucose (18.8)	**N.A.**	**26**	N.A.	Chen et al. 2001
CSTR	*Clostridium butyricum*	37	5.8	4.5	Sucrose (48.6 g/(l×h))	**3.5**	**21.7**	N.A.	Heydrickx et al. 1986
CSTR	*Clostridium* sp.	36	6.0	1.04	Glucose (10 g/l)	**2.36 (HRT 4.8h)**	**21.0**	N.A.	Taguchi et al. 1995a
CSTR	*Clostridium* sp.	36	6.0	0.87	Xylose (3.0 g/l)	**2.06 (HRT 5.6h)**	**20.4**	N.A.	Taguchi et al. 1995a
CSTR	Mixed culture	35	5.3	12	Sucrose (40)	**1.15**	**20.4**	6.4	Kyazze et al. 2006
CSTR	Mixed culture	35	6.8	4	Sucrose(17.8)	**3.5**	**17**	3.02	Lin et al. 2006c
CSTR	Mixed culture	30-34	6.2	6	Glucose (3.0 g/l)	**1.42 (HRT 12h)**	**15.0**	1.7	Lin and Chang 2004
CSTR	Mixed culture	37	5.5	6	Glucose (10)	**1.88**	**13.4**	0.8	Zhang et al. 2006b
CSTR	Mixed culture	35	5.4	12	Sucrose (26.7)	**2.44**[*]	**13.2**[*]	N.A.	Kim et al. 2006b
CSTR	Mixed culture	25	5.3	12	Sucrose (17.8)	**3.08**[*]	**9.8**	0.77	Kim et al. 2006a
CSTR	Mixed culture	32	N.A.	4	Glucose	**N.A.**	**9.3**[*]	N.A.	Maijzat et al. 1997
CSTR	Mixed culture	35	6.0	8.5	Glucose (10 g/l)	**1.43**	**8.7**[*]	N.A.	Mizuno et al. 2000a
CSTR	Mixed culture	35	6.7	13.3	Sucrose	**4.45**	**7.0**[*]	N.A.	Chen and Lin 2001
CSTR	Mixed culture	35	7.1	12	Xylose (18.8)	**0.7**	**4.2**	N.A.	Lin and Cheng 2006
CSTR	Mixed culture	37	5.2	17	Starch (6.0 kg/(m^3×d))	**1.29 L $g_{starchCOD}^{-1}$**	**2.6**[b]	2.08	Lay 2000
CSTR	Mixed culture	36	N.A.	2	Glucose (11.7 g/l)	**N.A.**	**2.0**[*]	N.A.	Nakamura et al. 1993
Biofilm reactors									
FBR	Mixed culture	35	~6	1.8	Glucose (5)	**1.9**	**28.8**	N.A.	**Paper I**
TBR	Mixed culture	40	5.5	N.A.	Sucrose (40)	**N.A.**	**436**[*]	N.A.	Jeon et al. 2008
FBR	Mixed culture	37	4.0	1	Glucose (10)	**1.16**	**98**	21.5	Zhang et al. 2007b
FBR	*Enterobacter cloacae*	37	N.A.	1.1	Glucose (10)	**N.A.**	**77.3**	N.A.	Kumar and Das 2001
PBR	Community	35	6.7	1	Sucrose (17.8)	**N.A.**	**54.0**[*]	15.0[*]	Chang et al. 2002
CSTR + carrier	*Clostridium butyricum +Enterobacter aerogenes*	36	5.2	1	Starch (20)	**2.6**[a] $molH_2$ mol-hexose^{-1}	**53**	N.A.	Yokoi et al. 1998b
CSTR+ carrier	*Enterobacter aerogenes*	37	N.A.	1	Glucose (10)	**0.73**	**38**	N.A.	Yokoi et al. 1997
FBR	Mixed culture	35	5.8-6.8	2	Sucrose (17.8)	**2.67**	**38**[*]	N.A.	Wu et al. 2003
PBR	*Enterobacter aerogenes*	40	5.5	10	Starch hydrolysates	**1.5**[a]	**10.2**	N.A.	Palazzi et al. 2000
CSTR + carrier	Mixed culture	37	5.0	20	Glucose (20)	**N.A.**	**8.3**	N.A.	Kim et al. 2005b
Granular reactors									
CSTR + SIC + granules	Mixed culture	40	6.6	0.5	Sucrose (35.6)	**3.17**	**627**[*]	35.4	Wu et al. 2006
CIGSB	Mixed culture	35	6.7	0.5	Sucrose (17.8)	**3.91**	**387**[*]	~40	Lee et al. 2006b
CIGSB	Mixed culture	40	6.7	0.5	Sucrose (17.8)	**3.88 (HRT 1h)**	**318**[*]	30-40	Lee et al. 2006a
FBR + granules	Mixed culture	37	5.5	0.25	Glucose (10)	**1.7**	**316**	~37	Zhang et al. 2008a
CSTR + SIC+ granules	Mixed culture	40	6.6	0.5	Glucose (18.8)	**1.54**	**312**[*]	39.9	Wu et al. 2005a
FBR + granule	Mixed culture	37	5.5	0.25	Glucose (10)	**1.71**	**311**[*]	37.3	Zhang et al. 2008b
CIGSB	Mixed culture	35	6.7	0.5	Sucrose (17.8)	**3.03**	**304**	26.1	Lee et al. 2004b
CSTR+AC powder+granules	Mixed culture	40	6.6	0.5	Glucose (18.8)	**1.52**	**303**[*]	79.4	Wu et al. 2005a
PBR + granules	Mixed culture	35	6.7	0.5	Sucrose (17.8)	**3.9 (HRT 4h)**	**302**[*]	N.A.	Lee et al. 2003
CIGSB	Mixed culture	35	6.7	0.5	Sucrose (17.8)	**3.11**	**285**	~37	Lee et al. 2006b
CSTR + granules	Mixed culture	37	5.5	0.25	Glucose (10)	**1.7**	**274**	~35	Zhang et al. 2008a
CIGSB	Mixed culture	35	6.7	0.5	Sucrose (17.8)	**2.19**	**212**	~15	Lee et al. 2004a
CSTR + granules	Mixed culture	35	~5.3	0.5	Starch (15)	**1.43 (HRT 5.2 h)**	**171**	N.A.	Wang and Chang 2008

CSTR + granules	Mixed culture	37	5.5	0.5	Glucose (10)	**1.81**	**133**	32.2	Zhang et al. 2007c
FBR + granules	Mixed culture	40	N.A.	2.2	Sucrose (35.6)	**2.92**	**94**	N.A.	Lin et al. 2006a
Cylindrical reactor	*Enterobacter aerogenes*	37	~6.0	1.5	Glucose (10 g/l)	**1.3**	**58**	N.A.	Rachman et al. 1998
CIGSB	Mixed culture	40	5.8	2	Xylose (18.8)	**0.8**	**44.2**	8.0	Wu et al. 2007
CSTR + granules	Mixed culture	26	5.5	6	Sucrose (17.8)	**3.9**	**22**	20	Fang et al. 2002a
UASB	Mixed culture	35	6.7	8	Sucrose (17.8)	**2.9**	**21.3**	6.2	Chang and Lin 2005
UASB	Mixed culture	35	4.4	2	Glucose (10)	**0.69***	**19.05**	0.44	Gavala et al. 2006
CSTR+ granules	Mixed culture	37	5.0	20	Glucose (20)	**N.A.**	**15.8**	N.A.	Kim et al. 2005b
UASB	Mixed culture	35	6.7	8	Sucrose (17.8)	**1.6**	**11.3**	3.1	Chang and Lin 2004
UASB	Mixed culture	38	4.4	N.A.	Sucrose	**1.44**	**N.A.**	N.A.	Yu and Mu 2006b
Membrane bioreactors									
MBR	Mixed culture	N.A	5.5	5	Glucose (10)	**1.0**	**8.0***	5.8	Oh et al. 2004a
MBR	Mixed culture	35	~7.0	N.A.	Glucose (2.5)	**N.A.**	**1.3**	0.12	Liang et al. 2002
Entrapped cells									
CSTR+PMMA-entrapped cells	Mixed culture	35	6.0	6	Sucrose (17.8)	**2.0**	**74.8**	N.A.	Wu and Chang 2007
Semi-continuous processes									
ASBR	Mixed culture	34.5	5.7	8	Glucose (21.3)	**0.64***	**9.5***	16.7	Cheong et al. 2007

[a] Theoretical maxima: glucose, 4 mol-H_2 mol-glucose^{-1}; sucrose 8 mol-H_2 mol-sucrose^{-1}; xylose 3.33 mol-H_2 mol-xylose^{-1}* calculated based on the information provided

AC= activated carbon; ASBR = anaerobic sequencing batch reactor; CIGSB = carrier-induced granular sludge bed reactor; CMCR = completely-mixed column reactor; CSTR = completely-stirred tank reactor; FBR = fluidized-bed reactor; HY = hydrogen yield; HPR = hydrogen production rate; N.A. = not available; MBR = membrane bioreactor; PBR = packed-bed reactor; PMMA= polymethyl methacrylate; SIC=silicone-immobilized cells; TBR = trickling-bed bioreactor; UASB = up-flow anaerobic sludge blanket reactor.

Table 36 Performances of thermophilic hydrogen dark fermentation bioreactors with model compounds as substrates

Reactor type	**Microorganism**	**Conditions**			**Electron donor (concentration [g/l])**	**H_2 production**		**VSS (g l^{-1})**	**Reference**
		T (ºC)	**pH**	**HRT (h)**		**Max. HY (mol H_2 mol-substrate^{-1})[a]**	**Max. HPR (mmol h^{-1} L^{-1})**		
Suspended cell –reactors									
CMCR	AK15, AK17	60	~6	3.1	Glucose (4.5)	**0.80**	**6.1**	0.26	**Paper III**
CMCR	Mixed culture, 9HG	74	~6.8	19	Glucose (18)	**0.32**	**1.15**	0.66	**Paper IV**
CSTR	Mixed culture	55	5.5	2	Glucose (10)	**1.08***	**6.9***	0.093	Gavala et al. 2006
CSTR	*Caldicellulosiruptor saccharolyticus*	72	6.7	2.9	Glucose (4.4)	**3.0**	**12.4**	0.42	de Vrije et al. 2007
CSTR	*Clostridium thermolacticum*	58	7.0	17.8	Lactose (10)	**1.5**	**2.6**	N.A.	Collet et al. 2004
CSTR	Mixed culture	60	6.4	72	Cellulose powder (5.0)	**2.0**[b]	**1.2***	N.A.	Ueno et al. 2001a
Biofilm reactors									
TBR	Mixed culture	60	5.5	2	Glucose (20.6)	**1.11**	**43.8***	18-24	Oh et al. 2004b
Granular reactors									
CSTR (CSTR2)	Mixed culture, 33HL	58	~6	3	Glucose (18)	**1.54**	**45.8**	1.23	**Paper V**
CSTR (CSTR1)	Mixed culture, 33HL	58	~6	5	Glucose (9)	**0.74**	**6.5**	0.96	**Paper V**
UASB	Mixed culture	70	5.5	24	Glucose (4.5)	**2.47**	**2.1***	N.A.	Kotsopoulos et al. 2006
Semi-continuous processes									
ASBR	Mixed culture, 33HL	58	~6	8	Glucose (18)	**2.23**	**19.7**	1.9	**Paper V**
ASBR	Mixed culture	55	5	N.A.	Xylose (2)	**1.5**	**2.5***	N.A.	Calli et al. 2008
ASBR	Mixed culture	55	5.3	N.A.	Lactose (2)	**3.2**	**2.4***	N.A.	Calli et al. 2008
ASBR	Mixed culture	75	N.A.	48	Glucose (6.25)	**2.65**	**1.6**	0.33	Yokoyama et al. 2007a

[a] Theoretical maxima: glucose, 4 mol-H_2 mol-glucose^{-1}; lactose 8 mol-H_2 mol-lactose^{-1}; xylose 3.33 mol-H_2 mol-xylose^{-1}; [b] mol- H_2 mol hexose^{-1}

* calculated based on the information provided

ASBR = anaerobic sequencing batch reactor; CMCR = completely-mixed column reactor; CSTR = completely-stirred tank reactor; HY = hydrogen yield; HPR = hydrogen production rate; N.A. = not available; TBR = trickling-bed bioreactor; UASB = up-flow anaerobic sludge blanket reactor.

Thermophilic, continuous H_2 or ethanol+H_2 production (Papers III, IV,V)

Thermophilic hydrogen or EtOH + H_2 production was studied in semi-continuous -flow reactor (ASBR) (Paper V), and continuous –flow, suspended- (CMCR) (Papers III, IV) or granular-cell bioreactors (Paper V). There have been only a limited number of studies of continuous, thermophilic dark H_2 fermentation (Table 36).

High H_2 production rates were obtained with granular CSTR systems with enrichment 33HL (Paper V). The rates obtained (up to 45.8 mmol h^{-1} L^{-1}, ~ 1.1 L h^{-1} L^{-1}) were higher than previously reported for thermophiles (Table 36). Comparable H_2 production rates (43.8 mmol h^{-1} L^{-1}) were reported by Oh et al. (2004b) using a trickling-bed reactor maintained with a thermophilic mixed culture, dominated by *Tbm. thermosaccharolyticum* (Ahn et al. 2005). In a semi-continuous process (ASBR) with enrichment 33HL, lower H_2 production rates, but higher H_2 yields were obtained compared to the granular CSTR-system (Table 36). The H_2 yields obtained in the ASBR (2.51 mol-H_2 mol-glucose^{-1} at HRT 24 h, and 2.23 at 8h) are comparable with the highest obtained by thermophilic mix cultures (Table 36).

A supplementation of 6 mg L^{-1} of $FeSO_4 \bullet 7H_2O$ provided a stable and improved H_2 production in granular ASBR and CSTR (CSTR2) systems compared to the CSTR (CSTR1) without $FeSO_4$ supplementation (Paper V). The H_2 production by *Tbm. aotearoense* affiliated strains, dominating the reactor cultures, was, therefore, clearly improved by the addition of 6 mg L^{-1} of $FeSO_4 \bullet 7H_2O$ to the feed. Mistry and Cooney (1989) reported that $FeSO_4$ addition decreased the lactate production, and increased acetate and ethanol production by *Tbm. thermosaccharolyticum*. They suggested that $FeSO_4$ supplementation provided iron for the sufficient formation of ferredoxin resulting increased production of acetyl-CoA (rather than lactate) from pyruvate, and therefore, increased acetate and ethanol production (Figure 3).

The H_2 production rates and yields in continuous co-production of ethanol+H_2 (Papers III, IV) were low due to the direction of electrons to ethanol production rather than H_2 production. The hydrogen production rate obtained in cylindrical CMCR with strains AK15 and AK17 (Paper III), is however, comparable with the majority of H_2 production processes with thermophiles (Table 36). The co-production of ethanol and H_2 with enrichment 9HG resulted in low H_2 production rates (Paper IV).

There have been only a few reports on the co-production of ethanol and H_2 (Wu et al. 2007a,b). Table 37 shows performances, and energy production rates of continuous ethanol or EtOH+H_2 production systems. The ethanol production (and energy generation) rates obtained in this study (Papers III, IV) remain far less than those obtained by commercially utilized *S. cerevisiae* and *Z. mobilis*, or by mesophilic mixed cultures (Table 37). However, the ethanol yields in cylindrical CMCR with strains AK15 and AK17 (Paper III) are comparable with continuous processes with *S. cerevisiae*. Further, the CMCR with AK15 and AK17 had higher ethanol and H_2 yields, and therefore, higher energy yield than reported by mesophilic mixed cultures (Table 37).

The ethanol and H_2 production by 9HG (dominated by *Tbr. thermohydrosulfuricus*) in CMCR was low. The H_2 and ethanol yields in CMCR remained substantially lower than obtained in batch due to increased lactate production. The reasons for this are unclear. It may be related to iron limitation, as observed with 33HL with similar feed.

In dark fermentation, H_2 and ethanol production are competing reactions, both of which can not be maximized at the same time – production of one will consume reducing equivalents from the production of the other. High ethanol-producing thermophilic isolate AK17 produces substantial amounts of H_2 along with ethanol. If the H_2 separation from the product gas is not

feasible, the energy content of H_2 in the product gas could be utilized at least by incineration to obtain heat for reactor heating. However, in the case of high H_2-producing enrichment 33HL, the produced ethanol would be likely too low (< 4%) for commercially viable separation of ethanol. In this case, the ethanol and organic acids in the dark fermentation effluent could be potentially further converted to biogas by anaerobic digestion, to hydrogen by photofermentation or by EAMFCs, or to electricity by microbial fuel cells.

Table 37. Performance of continuous ethanol or ethanol and H_2 production systems

Culture	T (°C)	Electron donor	HY (mol-H_2 mol-hexose^{-1})	EtY (mol-EtOH mol-hexose^{-1})	HPR (mmol h^{-1} L^{-1})	EtPR (mmol h^{-1} L^{-1}) [g h^{-1} L^{-1}]	Energy generation rate (kJ h^{-1} L^{-1})[a]	Energy yield (kJ mol hexose^{-1})[b]	Reference
CMCR AK15+AK17	60	Glucose	0.63	1.35	5.31	11.4 [0.53]	17.1	2023	Paper III
CMCR 9HG	74	Glucose	0.16	0.65	0.75	3.24 [0.15]	4.64	958	Paper IV
Thermoanaerobacterium. thermosaccharolyticum	N.A.	Xylose	0.32*[c]	0.78*[c]	9.5	22.1* [1.02]	32.9*	N.A.	Mistry and Cooney 1989
Zymomonas mobilis	N.A.	Glucose	N.D.	1.71*	N.D.	2605 [120]	5870*	N.A.	for a review, see Kosaric and Vardar Sukan 2001
Saccharomyces cerevisiae	N.A.	Glucose	N.D.	1.35*	N.D.	2171 [100]	5041*	N.A.	Cheryan and Mehaia 1984
Mixed culture	35	Fructose	0.56	0.65	33	378 [17.4]*	526	1048	Wu et al. 2007a
Mixed culture	35	Sucrose	~0.01	0.9	2.5*	84.1 [3.9]*	116	1235	Wu et al. 2007b

[a] Energy generation rate = H_2 production rate (mol h^{-1} L^{-1}) × 286 kJ mol H_2^{-1} + EtOH production rate (mol h^{-1} L^{-1}) × 1366 kJ mol $EtOH^{-1}$(Wu et al. 2007a). [b] Energy yield = (mol H_2 produced × 286 kJ mol H_2^{-1} + mol EtOH produced × 1366 kJ mol $EtOH^{-1}$)/mol hexose consumed. [c] mol mol-xylose^{-1}. * calculated based on the information provided. CMCR = completely-mixed column reactor; N.A.= not available

10.4. Efficiency of H_2 production

Table 38 compares the hydrogen production efficiencies from glucose by meso- and thermophilic dark fermentation processes. For the comparison of results, H_2 production at comparable HRTs was included along with the rates obtained at optimal HRTs (Table 38, Wu et al. 2005a, Zhang et al. 2007b,c). Thermophiles are considered to have higher H_2 production efficiency than mesophiles (van Groenestijn et al. 2002; Hallenbeck 2005) (see chapter 6.5), i.e., higher H_2 yield and higher specific H_2 production rate (SHPR = H_2 production rate vs g biomass). The results obtained in this and in some other studies (Valdez-Vazquez et al. 2005; Gavala et al. 2006) are in accordance with this theorem. The hydrogen yield (and H_2 percentage) in the ASBR and SPHR in the CSTR2 (Paper V) were superior compared to the mesophilic granular, suspended-cell or biofilm processes reported in the literature (Table 38). Even higher SPHR, but lower HPR, was reported by Gavala et al. (2006) in CSTR by a thermophilic mixed culture. Mesophilic CSTR culture had a very high SHPR during the highest H_2 production, but this was only momentarily (Paper II). Otherwise, the SHPR were comparable with those reported for mesophilic H_2 production systems (Table 38).

The hydrogen production rates in the thermophilic reactors ASBR and CSTR2 were below the highest rates reported for mesophilic systems operated at very short retention times of 0.25 to 0.5 h (Wu et al. 2005a; Zhang et al. 2007b,c) (Table 38). The hydrogen production rate of CSTR2 at 3h was, however, higher than those obtained with mesophilic cultures operated at comparable HRTs of 2 h (Wu et al. 2005a; Zhang et al. 2007b,c) (Table 38).

Table 38. Comparison of hydrogen production efficiencies from glucose by meso- and thermophilic dark fermentation processes

Reactor type	T (°C)	HRT (h)	VSS (g L^{-1})	H_2 (%)	HY(mol-H_2 mol-glucose^{-1})	HPR (mmol h^{-1} L^{-1})	SHPR (mmol h^{-1} $gVSS^{-1}$)	Reference
Mesophilic processes								
CMCR	**35**	1.7-2.7 (1.9)	~ 0.2-1.2 (0.20)[a]	~ 15-40 (48.1)[a]	~0.1-0.5 (1.70)[a]	~1-6 (18.8)[a]	**~2-14 (96.3)[a]**	**Paper II**
CSTR + SIC + granule	**40**	2	2.5	30.8	0.76	29.9[*]	**12.0**	Wu et al. 2005a
CSTR + SIC + granule	**40**	0.5	39.9	42.5	1.54	312[*]	**7.8**[*]	Wu et al. 2005a
CSTR + granule	**37**	2	29.8	53.0	1.81	39.0[*]	**1.3**[*]	Zhang et al. 2007c
CSTR + granule	**37**	0.5	32.1	63.5	1.81	133[*]	**4.1**[*]	Zhang et al. 2007c
FBR	**37**	2	~16	~59	1.12	~37	**~3.4**	Zhang et al. 2007b
FBR	**37**	0.5	21.5	57.2	1.16	92[*]	**4.2**	Zhang et al. 2007b
FBR + granule	**37**	0.25	37.3	N.A.	1.71	311[*]	**9.0**	Zhang et al. 2008b
CSTR	**37**	6	0.85	N.A.	1.88	13.3[*]	**15.6**	Zhang et al. 2008b
CSTR	**35**	6	1.3	43.1	1.71[b]	29.6[*]	**19.0**[*]	Lin and Chang 1999
CSTR	**35**	2	0.44	33.6	1.18[*]	7.8[*]	**17.8**[*]	Gavala et al. 2006
ASBR	**34.5**	8	16.7	50.9	0.64[*]	9.5[*]	**0.57**[*]	Cheong et al. 2007
Thermophilic processes								
CMCR AK15+AK17	**60**	3.1	0.26	39.2	0.80	6.1	**23.5**	**Paper III**
CMCR 9HG	**74**	19	0.66	32.3	0.32	1.15	**1.73**	**Paper IV**
ASBR + granule 33HL	**58**	8	1.9	67.5	2.23	19.7	**10.3**	**Paper V (ASBR)**
CSTR + granule 33HL	**58**	3	1.23	51.4	1.54	45.8	**37.1[a]**	**Paper V (CSTR2)**
CSTR	**55**	2	0.093	36.8	1.08[*]	6.9[*]	**74.2**[*]	Gavala et al. 2006
CSTR	**72**	2.9	0.42	N.A.	3.0	12.4	**30**	De Vrije et al. 2007
TRB	**60**	2	18-24	53	1.11	43.8[*]	**1.8 - 2.4**[*]	Oh et al. 2004b
ASBR	**75**	48	0.33	N.A.	2.65	1.6[*]	**4.9**[*]	Yokoyama et al. 2007a

[a] Calculated based on the suspended biomass; [*] Calculated based on the information provided; HY = hydrogen yield; HPR = hydrogen production rate; SHPR = specific hydrogen production rate; CMCR = completely-mixed column reactor; CSTR = completely-stirred tank reactor; ASBR = anaerobic sequencing batch reactor; TRB = trickling-bed reactor; CSTR+SIC = completely-stirred tank reactor + silicone-immobilized cells; FBR = fluidized-bed reactor; N.A. = not available; UASB = up-flow anaerobic sludge blanket reactor.

10.5. Stability of continuous H_2 or ethanol+H_2 production

In this study, mesophilic and thermophilic H_2 production was studied in several bioreactor systems. The bioreactors included suspended-cell (Paper II, III, IV), biofilm (Paper I) and granular-cell systems (Paper V). More stable H_2 production was obtained in thermophilic suspended- (EtOH+H_2, Paper III) and granular-cell (Paper V) processes compared to mesophilic processes (Papers I, II). The better stability was related to more stable and less diverse microbial communities in the thermophilic systems compared to mesophilic systems.

Mesophilic biofilm (FBR) and suspended-cell (CMCR) bioreactor systems were of similar configuration, feed composition and inoculum, and operated with comparable LRs. Hydrogen production was very instable in the FBR ceasing within 8 days after the start-up of continuous operation due to propionate production. Intermittent batch operation recovered H_2 production in the FBR momentarily. In the mesophilic CMCR, H_2 production was more stabile, and continuous H_2 production was observed for 150 d. It is, therefore, clear that the biofilm development did not favor continuous H_2 production in this study. However, in mesophilic suspended-cell system, the H_2 production was decreased by changes in community metabolism from the initial butyrate-acetate, first to ethanol-acetate, and then to acetate-dominated metabolism. After an increase in HRT, the metabolism was finally changed to propionate production which ceased H_2 production (Paper II). These transitions were successfully detected and visualized by self-organizing maps (SOMs).

Cohen et al. (1985) reported that propionate-acetate production took over the initial butyrate-acetate and H_2 production at low loading rates in a suspended-cell, glucose-utilizing, mixed-culture reactor. They suggested that low loading induced sporogenesis and, therefore, caused a shift in community metabolism from butyrate-acetate to propionate-acetate production. Propionate production did not occur when heat-treated microbial culture was used suggesting that the propionate producers were non-sporeforming organisms. These results are in accordance with the results obtained in the mesophilic CMCR, where H_2 production was

ceased after decreased loading. Based on the DGGE profiles (Tolvanen et al. 2008), *C. butyricum* lost it's dominance during this period, which may be a sign of sporulation. In fact, microscopy observations revealed that rod-shaped organisms carried spores during the transition to propionate production, while several non-sporulating curved rods were seen, affiliated possibly with propionate-producers (Cohen et al. 1985) (Figure 11).

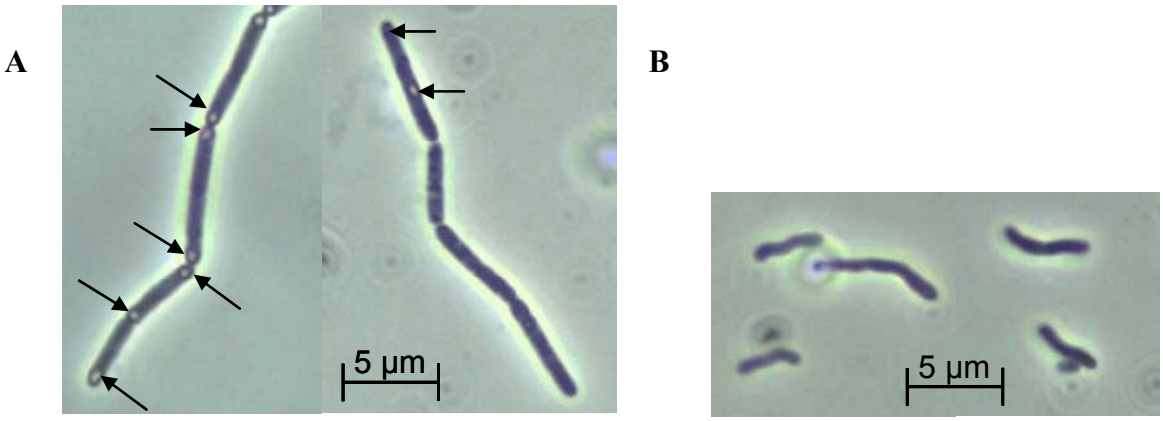


Figure 11. Phase-contrast micrographs from mesophilic suspended-cell (CMCR) reactor during transition to propionate-acetate metabolism. A) Sporulating rod shaped cells, B) Curved rod shaped cells.

Lower stability of H_2 production in biofilm compared to granular-cell process was previously reported by Kim et al. (2005b). As in this study, they reported that this instability in biofilm systems was due to irreversible propionate production. They suggested that the poor mass transfer within the biofilm and carrier material micro pores created optimal environment for propionate producers (high pH_2 and suitable pH). Beeftinkt and van den Heuvel (1987) suggested that the propionate production was due to efficient adhesion of propionate producers on the biofilm. Alternatively, Jeon et al. (2008) reported that the utilization of hydrophobic carrier enabled prolonged, high rate H_2 production in a biofilm (FBR) reactor. The carrier hydrophobicity may improve mass transfer of H_2 from carrier biofilm and micro pores. Surface hydrophobicity also affects the attachment of organisms on the carrier (for a review, see van Loosdrecht and Zehnder 1990) resulting in, generally, higher cell adhesion with increasing surface hydrophobicity (for a review, see Qureshi et al. 2005). The adhesion properties and hydrophobicity of cells is, however, highly dependent on the bacterial strain and on growth conditions (van Loosdrecht et al. 1987; for a review see, van Loosdrecht and Zehnder 1990). The hydrogen-producers have been reported to be hydrophilic when in suspension, but become more hydrophobic when attached to granules due to the formation of EPS, which alter the surface charges (Mu et al. 2006; Zhang et al. 2007c). The carrier used in this study, Celite R-633, is a non-charged carrier with limited (or no) hydrophobicity (Cullere et al. 2001). Although proven successful for biomass retention in FBRs in biodegradation of chlorophenols (Melin et al. 1998) and in the generation of ferrous iron for bioleaching (van der Meer et al. 2007), the results of this study indicate that Celite R-633 was not suitable biocarrier for dark fermentative H_2 production.

10.6. Bioreactor types for continuous H_2 production

Stable, high rate H_2 production from carbohydrates (glucose, sucrose, xylose, starch) has been achieved using mesophilic granular-cell bioreactors (Lee et al. 2004a,b,2006a,b; Wu et al. 2005a,2006, 2007; Zhang et al. 2007c;2008a,b; Wang and Chang 2008). In this study, high-rate, thermophilic H_2 production with granular cells was demonstrated for the first time (Paper V). The thermophilic granular-cell processes provided higher H_2 production efficiency (i.e., higher H_2 yield and higher SHPR) than obtained with mesophilic systems.

There have been few reports on comparing performances of granular- and suspended-cell reactors. Mesophilic granular-cell processes have better H_2 production stability (Gavala et al. 2006) and performance (HPR and/or HY) (Gavala et al. 2006; Wu et al. 2008a) compared to suspended-cell processes. Further, it has been shown that within the same reactor, the formation of granules improve H_2 production (Lee et al. 2004a; Wu et al. 2005a,2006a) by altering the microbial community structure (Wu et al. 2006). There have been some reports on high-rate H_2 production with mesophilic (Kumar and Das 2001; Zhang et al. 2007b; Jeon et al. 2008) and thermophilic (Oh et al. 2004b) biofilm processes, but their long-term stability has not been proven. Further, the H_2-producing biofilm (FBRs, PBRs, and TRBs) (Kumar and Das 2001; Oh et al. 2004b; Jeon et al. 2008) and membrane processes (Oh et al. 2004a) have been reported to suffer from clogging or gas build-up problems due to excessive build-up of biomass. In the granular-cell reactors, mechanical agitation can be used to decrease the gas hold-up and to increase the mass-transfer efficiency (Lee et al. 2006b).

Thermophilic H_2 fermentation processes have been considered to suffer from low cell densities (Hallenbeck 2005) disabling the process operation with high organic loading. In this study (Paper V), the thermophilic culture dominated by *Tbm. aotearoense* affiliated strains readily formed granules in the bioreactors when operated at 6 to 8 h HRT in CSTRs and at 24 h HRT in the ASBR (Figure 12). The good biomass retention by the formation of granules enabled high organic loading resulting in high HPR. This shows that the culture does not suffer from the low cell density limitation of thermophilic processes. The self-flocculation by *Tbm. aotearoense* affiliated strains differs from the findings in granular CSTRs operated at the temperature range of mesophiles. In general, in mesophilic systems the granulation, triggered by hydrodynamic and organic load pressure, has been obtained at shorter HRTs of 0.5 to 4 h (Lee et al. 2004a,b,2006a; Wu et al. 2005a, 2006a; Zhang et al. 2007c). The granulation of the thermophilic hot spring enrichment at long HRTs is an indication of good self-flocculation capability

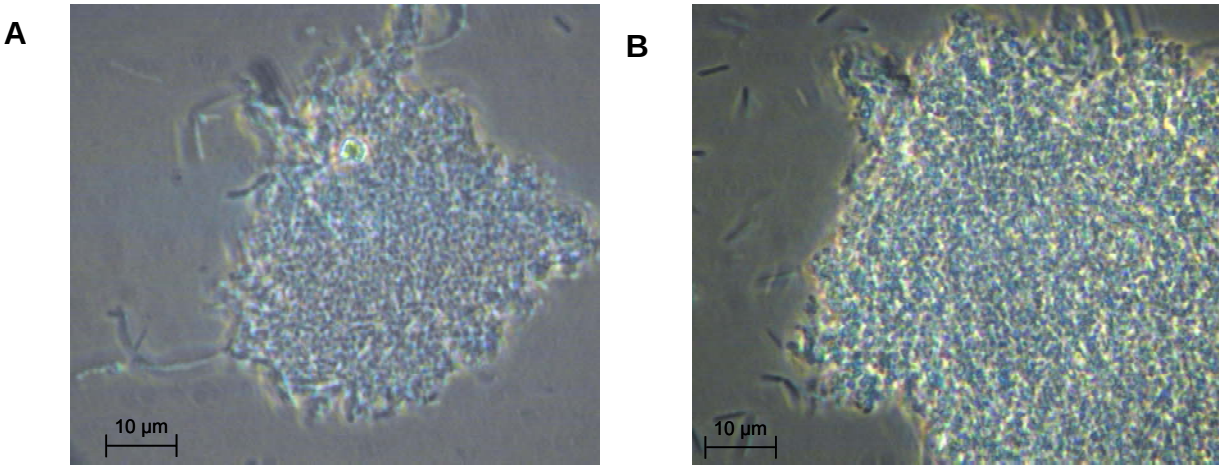


Figure 12. Phase-contrast micrographs of microgranula in ASBR (A) and CSTR2 (B) systems (Paper V).

10.7 Fermentation pathways

Reduction degree balances (Oh et al. 2008b) were applied to describe the distribution of electrons in fermentation products of meso- and thermophilic cultures used in this study. Reduction degree balance analysis describes the distribution of transferable electrons in the fermentation products. In dark fermentation, electrons from electron donor are distributed between H_2, organic acids and alcohols, and biomass.

In the mesophilic batch and bioreactor (during highest H_2 production) cultures, the majority of the electrons were directed to butyrate production (Figure 13A, B, C; Table 39). The molar

butyrate to acetate –ratio (B/A –ratios) were 4.1, 0.92 and 2 for the mesophilic enrichment, FBR and CMCR, respectively (Papers I, II). B/A –ratio has been considered as a performance indicator of mesophilic H_2 dark fermentation (Khanal et al. 2004) and, in general, optimal H_2 production performance is obtained with molar B/A –ratios higher than 1.5 (Chen and Lin 2003; Hussy et al. 2003; Khanal et al. 2004; Chang and Lin 2006; Kim et al. 2006a,c; Lin et al. 2006b; Wu et al. 2006). The B/A- ratios from 1.1. to 1.9 have been reported for *C. butyricum* (Heyndrickx et al. 1990; Kataoka et al. 1997; Lin et al. 2007b).

In the glucose fermentation by the isolate AK15, hydrogen production was associated with the production of acetate (Paper III). The electrons from glucose were mainly directed to ethanol, acetate and H_2 (Figure 13 D; Table 39). Similarly, strain AK17 produced H_2 along with acetate. The majority of electrons in the batch cultures of AK17, and in the CMCR dominated by the AK17, were directed to the production of ethanol (Figure 13E,F, Table 39).

The enrichment 9HG produced H_2 along with acetate, while the majority of electrons were directed to the production of ethanol followed by lactate (Figure 13G, Table 39). Similarly, in the CMCR with 9HG electrons were mainly distributed to ethanol and lactate, but the ethanol yield was less than in the batch (Figure 13H, Table 39). Further, a substantial fraction of the electrons were missing from the reduction degree balance, attributed possibly to the products such as, n-propanol or iso-propanol that can be potentially produced by Tbr. *thermohydrosulfuricus* (Wiegel et al. 1979), but that were not determined.

The H_2 production by batch enrichment 33HL (dominated by *Tbh. celere* affiliated strain), was associated with the production of acetate. The electrons in 33HL were mainly directed to acetate, followed by H_2 and ethanol (Figure 13I, Table 39). However, in the continuous (CSTR2) and semi-continuous reactors (ASBR) (dominated by *Tbm. aotearoense* affiliated strains), hydrogen production occurred along with acetate and butyrate. In the ASBR, butyrate was the main electron sink followed by H_2, biomass and acetate (Figure 13K, Table 39). In the CSTR, however, lactate and ethanol were the main electron sinks followed by butyrate, H_2 and acetate (Figure 13K, Table 39). Increased lactate and ethanol production in the CSTR decreased the H_2 yields in the CSTR compared to ASBR.

Table 39. Reduction degree balances for batch and continuous-flow bioreactor cultures used in this study. The reduction degree balances describe the distribution of transferable electrons in the fermentation products, directed from the electron donor (glucose) in fermentation. The balances were calculated per 1 mole of glucose (Oh et al. 2008b).

	Mesophilic enrichment	mesophilic FBR	mesophilic CMCR	AK15	AK17	CMCR AK15+AK17	9HG	CMCR 9HG	33HL	ASBR 33HL	CMCR2 33HL
Paper	**I**	**I**	**II**	**III**	**III**	**III**	**IV**	**IV**	**V**	**V**	**V**
Cultivation[a]	B	C	C	B	B	C	B	C	B	SC	C
Glucose (mM)	27.8	27.8	25.2	20	20	25.2	100	100	33.3	100	100
Glucose LR ($mmol\ h^{-1}\ L^{-1}$)	-	15.6	11	-	-	8.1	-	5.3	-	8.8	33.9
HRT (h)	-	1.8	2.3	-	-	3.1	-	19	-	8	3
Electron donor											
Glucose	24	24	24	24	24	24	24	24	24	24	24
Product											
Ethanol	N.D.	0.99	1.20	8.34	19.02	15.58	12.52	8.24	4.41	1.49	5.46
Acetate	0.96	3.42	1.74	6.73	4.36	3.51	3.75	2.80	10.44	2.73	2.36
Butyrate	12.50	7.81	8.29	N.D.	N.D.	0.05	N.D.	0.24	N.D.	8.73	4.32
Butanol	N.D.	0.08	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.40
Propionate	N.D.	0.45	0.12	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.16	0.25
Lactate	N.A.	0.72	N.A.	0.80	N.D.	1.21	5.08	3.86	N.D.	1.48	6.26
Formate	N.A.	N.A.	N.A.	N.D.	N.D.	N.D.	N.D.	0.04	N.D.	0.07	0.00
Biomass	N.A.	N.A.	1.49	N.A.	N.A.	1.90	N.A.	2.03	N.A.	3.36	2.83
H_2	2.48	3.85	3.72	3.83	0.76	1.59	1.36	0.63	6.31	4.47	2.99
Total products	15.94	17.32	16.55	19.70	24.14	23.85	22.70	18.08	21.16	22.47	24.88
Balance	0.66	0.72	0.69	0.82	1.01	0.99	0.95	0.75	0.88	0.94	1.04

[a] Cultivation type: B = batch; C = continuous; SB = semi-continuous. N.A. = not available; N.D. = not detected.

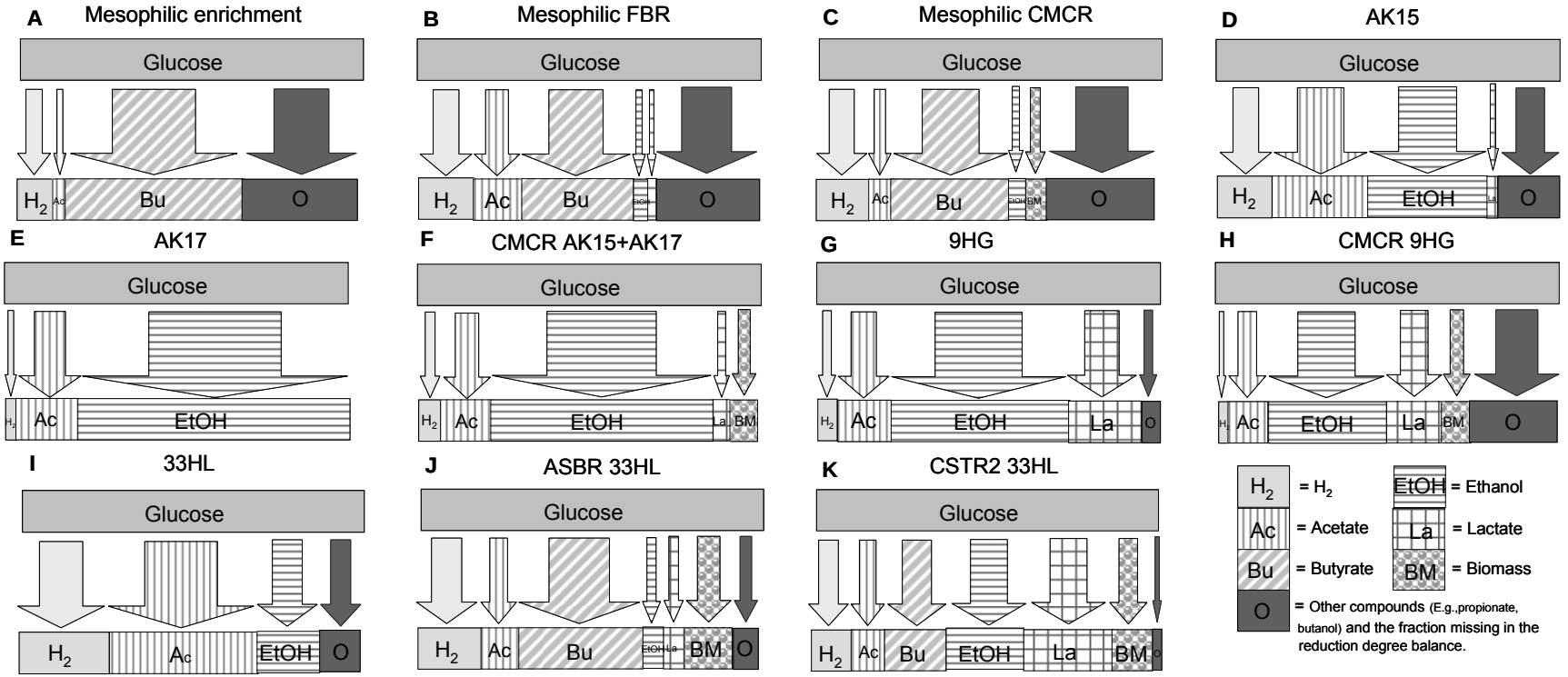


Figure 13. Electron distribution diagrams of batch or continuous-flow bioreactor cultures used in this study. The diagram illustrates the transfer of electrons from electron donor (glucose) to fermentation products, and describes the distribution of transferable electrons present in the fermentation products. The diagram is based on reduction degree balance analysis (Table 39). A) Batch culture of mesophilic enrichment (Paper I); B) Mesophilic FBR (Paper I); C) Mesophilic CMCR (Paper II); D) Batch culture of thermophilic isolate AK15 (Paper III); E) Batch culture of thermophilic isolate AK17 (Paper III); F) Thermophilic CMCR with a co-culture of AK15 and AK17 (Paper III); G) Batch culture of thermophilic enrichment 9HG (Paper IV); H) Thermophilic CMCR with enrichment 9HG (Paper IV); I) Batch culture of thermophilic enrichment 33HL (Paper V); J) Thermophilic ASBR with enrichment 33HL (Paper V); Thermophilic CSTR (CSTR2) with enrichment 33HL (Paper V). Ac = Acetate; Bu = Butyrate; EtOH = Ethanol; La = Lactate; BM = Biomass; O = Other compounds (e.g., propionate, valerate, butanol) and the fraction missing in the reduction degree balance (Electrons included in electron donor – Electrons in Total products determined).

11 CONCLUSIONS

This study demonstrated the hydrogen and ethanol+H_2 production potential by dark fermentation bioprocesses. Meso and thermophilic dark-fermenting microorganisms were enriched, and continuous bioreactor processes developed and studied. Efficient H_2 or ethanol+H_2 –producing cultures were obtained and further characterized. High-rate, thermophilic H_2 production with granular cells was demonstrated. This study also contributed to the increased understanding on microbial diversity of dark fermenters and their dynamics in continuous-flow bioprocesses. Microbial community analyses indicated rapid changes in community structures, which were linked to those in reactor performance. Based on the study, the following conclusions can be drawn:

- Batch enrichment with BESA of mesophilic H_2-producers from anaerobic digester sludge resulted in enrichment culture with average H_2 production efficiency (1.24 mol-H_2 mol-glucose^{-1}) in batch. Hydrogen production in FBR, inoculated with the batch-enrichment culture, was very instable due to rapid change from butyrate-acetate (and H_2) production to propionate-acetate production. Intermittent batch (semi-continuous) operation allowed a momentary recovery of H_2 production in the FBR. During the highest H_2 production, relatively high H_2 yield of 1.90 mol-H_2 mol-glucose^{-1}, and a HPR of 28.8 mmol h^{-1} L^{-1}, was obtained. (Paper I)
- Mesophilic FBR microbiology was characterized by diverse microbial communities and rapid changes in the community structure. The changes in microbial communities corresponded to those in bioreactor performance. Initially simple microbial community structure was rapidly (within one week) developed into diverse attached- and suspended-growth microbial communities. This enrichment resulted in the instability of the H_2 production in the FBR. Propionate-producing organisms were enriched in the reactor which altered the community metabolism from butyrate-acetate (and H_2 production) to propionate-acetate production ceasing the H_2 production. (Paper I)
- Mesophilic CMCR, compared to the FBR, provided more prolonged H_2 production, for over five months. Hydrogen production in CMCR was decreased by shifts in microbial community metabolism from initial butyrate-acetate, first to ethanol-acetate, than to acetate-dominated metabolism, and finally to propionate-dominated metabolism which ceased H_2 production. Installation of gas (H_2 and CO_2) extraction unit into the recycle-line of cylindrical CMCR improved H_2 production slightly. Improved H_2 production may be due to improved mass transfer of H_2 and CO_2 from liquid phase. The gas extraction did not alter community metabolism in the process. (Paper II)
- The transitions in dominant metabolism in the mesophilic CMCR were successfully detected and visualized by self-organizing maps (SOMs). Developed Clustering Hybrid Regression (CHR) -model can be a useful tool in data mining of meaningful phenomena from complex datasets. The model can increase understanding on the H_2 dark fermentation process for its optimization. CHR also performed very well in modeling the hydrogen production rate in process based on process parameters (pH and HRT) and metabolites (organic acids and alcohols, and CO_2) data. (Paper II)
- *C. butyricum* was the main H_2-producing organism in mesophilic bioreactors inoculated with enrichment from anaerobic digester sludge. The changes in quantities of *C. butyricum* corresponded roughly to those in hydrogen production rate. Low loading may have caused sporulation of *C. butyricum* giving a rise to change in

community metabolism and a decrease in H_2 production (Papers I, II and Tolvanen et al. 2008)

- Rapid community changes were observed in the mesophilic FBR and CMCR. 16S ribosomal RNA gene –based microbial community analyses provided insight into processes microbiology. Biofilm reactor (FBR) supported the growth of more diverse microbial community than observed in suspended-cell reactor (CMCR). Many of the organisms enriched in bioreactors were either distantly affiliated with any known organisms or affiliated with organisms with unknown H_2 production characteristics.
- The instability of H_2 production in mesophilic reactors were likely related to the following reasons: Improper biocarrier in FBR (low mass transfer of H_2, good adhesion of propionate-producers); presence of propionate-producers in microbial community (BESA treatment in enrichment, no selection of spore-formers); too low bioreactor loading (causing sporulation of *C. butyricum* and favoring growth of propionate-producers). (Papers I and II)
- Thermophilic isolate AK15 (*C. uzonii*, 98.8%) produced in batch assays H_2 as the main fermentation product from glucose, up to 1.9 mol-H_2 mol-glucose^{-1} (48% of theoretical maximum), and xylose, up to 1.1 mol-H_2 mol-xylose^{-1} (33%). The ethanol yields in batch by the AK15 were 0.8 mol-EtOH mol-glucose^{-1} and 0.4 mol-EtOH mol-xylose^{-1}.
- Thermophilic isolate AK17 (*Tbm. aciditolerans*, 99.2%) produced ethanol as the main fermentation product with ethanol yields up to 1.6 mol-EtOH mol-glucose^{-1} (80% of theoretical maximum) and 1.1 mol-EtOH mol-xylose^{-1} (66%), respectively. Hydrogen yield in batch by AK17 were 1.2 mol-H_2 mol-glucose^{-1} and 1.0 mol-H_2 mol-xylose^{-1}. Thermophilic isolate AK17 is a very promising co-producer of ethanol and H_2 with a wide substrate utilization spectrum, relatively high ethanol tolerance (up to 4%, v/v), and ethanol yields from glucose (up to 1.6 mol-EtOH mol-glucose^{-1}) and xylose (up to 1.1 mol-EtOH mol-xylose^{-1}) among the highest reported for thermoanaerobes. Research on lignocellulosic material hydrolysates is required to evaluate the potential of the culture for practical applications. (Paper III)
- Long-term, stable maintenance of ethanol and hydrogen co-production activity by thermophilic pure cultures (co-culture of AK15 and AK17) was demonstrated in an open system CMCR at 60 °C. The strain AK17 replaced AK15 and became the dominant thermophile in the process. Promising EtOH yield (1.35 mol-EtOH mol-glucose^{-1}[68% of theoretical maximum]) and HPR (6.1 mmol h^{-1} L^{-1}) from glucose was obtained in the CMCR at HRT of 3.1 h and glucose LR of 8.1 mmol h^{-1} L^{-1}. EtOH production rates remain far less than those obtained in commercially utilized organisms. The advantages of AK17 may be related to the co-production of EtOH and H_2, and a potential of utilizing the main sugar residues found in hydrolysates of lignocellulosic material. (Paper III)
- Extensive screening of Icelandic hot spring samples with glucose resulted in several hydrogen- and/or EtOH -producing enrichment cultures, over a temperature range of 50–78 °C. One enrichment produced H_2 directly from cellulose at 70 °C. Icelandic hot springs possess a great diversity of saccharolytic, thermophilic organisms capable of producing H_2 and/or ethanol. (Paper IV)
- Thermophilic enrichment 9HG, dominated by bacteria closely affiliated with *Tbr. thermohydrosulfuricus* (100%), produced relatively high amounts of ethanol from glucose in batch, 1.21 mol-EtOH mol-glucose^{-1}, at the ethanol distillation temperature 78 °C. The ethanol and H_2 yields by 9HG increased with increasing temperature. Decreased EtOH+H_2 production by 9HG was observed in continuous-flow bioreactor

at 74 °C compared to batch cultivations, possibly due to iron limitation. The ethanol production behavior of 9HG were similar than that of characterized *Tbr. thermohydrosulfuricus* strains, i.e., pH dependent and favored at the pH of 6.5 to 7.1 (Paper IV).

- The hydrogen yield from glucose in batch at 60 °C, 3.2 mol-H_2 mol-glucose^{-1} (80% of theoretical maximum), by hot spring enrichment 33HL was among the highest reported for thermoanaerobes. The batch 33HL produced H_2 along with acetate. The dominant bacteria in the batch 33HL, *Thermobrachium celere* (100%) affiliated strains, did not thrive in continuous or semi-continuous open system reactor systems. (Paper V)
- Continuous or semi-continuous reactor cultures with thermophilic enrichment 33HL were dominated by bacteria closely affiliated with *Tbm. aotearoense* (98.5 – 99.6%). The culture produced H_2 along with acetate and butyrate. Hydrogen production by *Tbm. aotearoense* was iron and/or sulfur limited – A supplementation of 6 mg L^{-1} of $FeSO_4 \cdot 7H_2O$ stabilized and improved H_2 production in granular, continuous or semi-continuous reactor systems. (Paper V)
- High hydrogen yield by thermophilic 33HL, 2.51 mol-H_2 mol-glucose (63% of theoretical maximum), was obtained in semi-continuous reactor (ASBR) at the HRT of 24 h and glucose LR of 300 mmol d^{-1} at 58 °C. Highest hydrogen production rate from glucose, 45.8 mmol h^{-1} L^{-1}, was obtained in continuous-flow reactor by 33HL at the HRT of 3h and LR of 33.1 mmol h^{-1} L^{-1}. Hydrogen production by 33HL was characterized by higher H_2 production efficiency (i.e., higher H_2 yield or specific H_2 production rate) than reported for mesophilic cultures. (Paper V)
- The thermophilic 33HL had good self-flocculation capability as it readily formed granules in the continuous (at HRTs 6 and 8 h) and semi-continuous reactor (at HRT 24 h) systems. The culture is not likely to suffer from the low cell density limitation of thermophilic processes. (Paper V) Possessing good self-granulation, wide substrate utilization range and high hydrogen production efficiency, the 33HL is considered very suitable for thermophilic H_2 fermentation from carbohydrates. (Paper V)
- Better stability and higher H_2 production was obtained by thermophilic processes compared to mesophilic processes. The better stability was related to more stable and less diverse microbial communities in the thermophilic systems compared to mesophilic systems. (Papers I,II,III,V)

12 RECOMMENDATIONS FOR FUTURE RESEARCH

This study demonstrated the H_2 and ethanol+H_2 production efficiency and potential from simple sugars (glucose and xylose) by thermophiles. Considering practical applications, H_2 or ethanol+H_2 production potential from real waste materials should be determined. Continuous H_2 production from sucrose, present in several crops and food industry waste waters (Chang and Lin 2004), by thermophilic enrichment 33HL should be studied. Further, considering wide substrate utilization ranges of 33HL and AK17 cultures, the continuous H_2 or ethanol +H_2 production from sugar residues of hemicellulose (e.g., xylose), and ultimately, from actual lignocellulose hydrolysates should be studied.

In the continuous ethanol+H_2 production by thermophilic isolates AK15 and AK17, low organic loading (low substrate concentration) was used. Higher organic loading, and bioreactor system suitable for granule-formation, should be studied to analyze critical organic loads, and ethanol+H_2 production capability of AK17. Thermoanaerobes should be able to sustain ethanol concentrations above about 4 % (v/v) in order to obtain commercially viable separation of ethanol from bioprocess (Sudha Rani and Seenayya 1999; Lynd et al. 2001). The ethanol tolerance of AK17 may be increased by culture adaptation (Baskaran et al. 1995; Burdette et al. 2002; Sudha Rani and Seenayya 1999).

In this study, the high H_2 production potential by 33HL was shown in short-term continuous-flow experiments. Longer term experiments with gradual increases in LR, allowing better adjustment and retention of microorganisms, may achieve substantially higher H_2 production (HY and HPR). Further, immobilization strategies of H_2-producers in silicone-matrixes with high mechanistic strength have been developed (Wu et al. 2002). Efficient biomass retention at low HRTs and induced granulation have been obtained with mesophilic processes using silicone-immobilized H_2-producers (Wu et al. 2005a; 2006a). These immobilization strategies should be applied to thermophilic cultures. The methods can potentially improve substantially the H_2 production performance by thermophiles at high organic loading.

In the production of H_2 or ethanol+H_2, the effluent contains high amounts of COD, attributed to organic acids and alcohols, which need to be treated. The effluent treatment technologies should be studied. Organic acids and alcohols can be converted to H_2 by photofermenters (de Vrije and Claassen 2003) or by EAMFCs (Oh and Logan 2005), to CH_4 by methanogens (Hawkes et al. 2002), or to electricity by microbial fuel cells.

In this work, CHR model was developed for knowledge mining of essential biological features from complex datasets. By incorporating microbial community information in the CHR along with data on process operation parameters and metabolites may provide further insights into and understanding on the complex behavior of H_2 dark fermentation. This information can be potentially utilized in the process control and optimization.

In this study, several promising enrichments were obtained from Icelandic hot springs, but only cultures 33HL and 9HG were studied in detail. Hydrogen and EtOH+H_2 production potential from sugar residues found in lignocellulosic materials by the other enrichments obtained should be determined.

It has been shown that combination of culturing and molecular methods allow more comprehensive detection of species and process microbiology than either of the methods alone (Kaksonen et al. 2004). The isolation of H_2 or EtOH+H_2 –producing organisms from mixed cultures, and studying their characteristics may provide tools for improving process performance. Especially, in batch enrichment 33HL, very efficient H_2-producer affiliated with

Tbh. celere should be attempted to isolate. The reasons disfavoring the growth and/or retention of *Tbh. celere* in continuous cultures should be revealed.

In this study, microbial communities were monitored by PCR-DGGE of 16S rRNA genes allowing the detection of most dominant organisms in the processes and their dynamics. Species-specific quantitative analyses of main microorganisms e.g., by quantitative real-time (qrt) PCR (Tolvanen et al. 2008) or FISH (Hung et al. 2007), would give better understanding on their behavior in the process. Quantification, targeting functional genes (e.g., hydrogenase) and their expression (Chang et al. 2006,2007) would provide information not only on the presence, but also on the hydrogen production activity of organisms in the process. High-throughput quantification of microorganisms and/or their expression profiles by using e.g., qrt-PCR (Tolvanen et al. 2008) or microarrays, would provide adequate amounts of data required for modeling.

13 REFERENCES

Abengoa 2008. BCyl biomass plant. Internet document, accessed 29.7.2008, http://www.abengoabioenergy.com/NewTechnologies/index.cfm?page=10.

Abrini J, Naveau H, Nyns EJ. 1994. *Clostridium autoethanogenum*, sp. nov., an anaerobic bacterium that produces ethanol from carbon monoxide. Archives of Microbiology 161:345-351.

Agnolucci P. 2007. Economics and market prospects of portable fuel cells. International Journal of Hydrogen Energy 32:4319-4328.

Ahn Y, Park EJ, Oh YK, Park S, Webster G, Weightman AJ. 2005. Biofilm microbial community of a thermophilic trickling biofilter used for continuous biohydrogen production. FEMS Microbiology Letters 249:31–38.

Ahring BK, Jensen K, Nielsen P, Bjerre AB, Schmidt AS. 1996. Pretreatment of wheat straw and conversion of xylose and xylan to ethanol by thermophilic anaerobic bacteria. Bioresource Technology 58:107-113.

Alterthum F, Ingram LO. 1989. Efficient ethanol-production from glucose, lactose, and xylose by recombinant *Escherichia coli*. Applied and Environmental Microbiology 55:1943–1948.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. Journal of Molecular Biology 215:403-410.

van Andel JG, Zoutberg GR, Crabbendam PM, Breure AM. 1985. Glucose fermentation by *Clostridium butyricum* grown under a self generated gas atmosphere in chemostat culture. Applied Microbiology and Biotechnology 23:21-26.

Angenent LT. 2007. Energy biotechnology: beyond the general lignocellulose-to-ethanol pathway. Current Opinion on Biotechnology 18:191–192.

Angenent LT, Karim K, Al-Dahhan MH, Wrenn BA, Domiguez-Espinosa R. 2004. Production of bioenergy and biochemicals from industrial and agricultural wastewater. Trends in Biotechnology 22:477-485.

Anonymous. 2006. Clean Urban Transport for Europe (CUTE). Detailed summary of achievements. A hydrogen fuel cell bus project in Europe 2001-2006. Internet document, accessed 1.3.2008, available at: http://www.fuel-cell-bus-club.com/modules/UpDownload/store_folder/Publications/DETAILED_SCREEN.pdf.

Anonymous 2008a. European Hydrogen and Fuel Cell Technology Platform. Internet document, accessed 9.1.2008, available at: http://www.infotools.hfpeurope.org/hfp_pro/.

Anonymous 2008b. California fuel cell partnership. Internet document, accessed 26.2.2008, available at: http://www.fuelcellpartnership.org/fuel-vehl_map.html.

Antoni D, Zverlov VV, Schwarz WH. 2007. Biofuels from microbes. Applied Microbiology and Biotechnology 77:23–35.

Antonopoulou G, Gavala HN, Skiadas IV, Angelopoulos K, Lyberatos G.2008. Biofuels generation from sweet sorghum: Fermentative hydrogen production and anaerobic digestion of the remaining biomass. Bioresource Technology 99:110-119.

Aristidou AA, San K-Y, Bennett GN. 1999. Metabolic flux analysis of *Escherichia coli* expressing the *Bacillus subtilis* acetolactate synthase in batch and continuous cultures. Biotechnology and Bioengineering 63:737-749.

Asada Y, Mijake Y. 1999. Photobiological hydrogen production. Journal of Bioscience and Bioengineering 88:1-6.

Asada Y, Tokumoto M, Aihara Y, Oku M, Ishimi K, Wakayama T, Miyake J, Tomiyama M, Kohno H. 2006. Hydrogen production by co-cultures of *Lactobacillus* and photosynthetic bacterium, *Rhodobacter sphaeroides* RV. International Journal of Hydrogen Energy 31:1509-1513.

Asther M, Khan AW. 1985. Improved fermentation of cellobiose, glucose, and xylose to ethanol by *Zymomonas anaerobia* and a high ethanol tolerant strain of *Clostridium saccharolyticum*. Applied Microbiology and Biotechnology 21:234-237.

Atkinson A, Ellwood DC, Evans CG, Yeo RG. 1975. Production of alcohol by *Bacillus stearothermophilus*. Biotechnology and Bioengineering 17:1375-1377.

Australian Government 2003. National hydrogen study. Australian government, department of industry, tourism and resources. ACIL Tasman Pty Ltd. 154 p. Internet document, accessed 7.11.2007, available at: http://www.industry.gov.au/content/itrinternet/cmscontent.cfm?objectID=60DCC756-C6C5-45CB-9F446E905D1B0DF2.

Avci A, Dönmez S. 2006. Effect of zinc on ethanol production by two *Thermoanaerobacter* strains. Process Biochemistry 41:984–989.

Bahl H. Andersch W, Braun K, Gottschalk G. 1982. Effect of pH and butyrate concentration on the production of acetone and butanol by *Clostridium acetobutylicum* grown in continuous culture. European Journal of Applied Microbiology and Biotechnology 14:17-20.

Barnett JA. 2003. Beginnings of microbiology and biochemistry: the contribution of yeast research. Microbiology149:557-567.

Baskaran S, Ahn H-J, Lynd LR. 1995. Investigation of the ethanol tolerance of *Clostridium thermosaccharolyticum* in continuous culture. Biotechnology Progress 11:276-281.

Becker J, Boles E. 2003. A modified *Saccharomyces cerevisiae* strain that consumes L-arabinose and produces ethanol. Applied and Environmental Microbiology 69:4144–4150.

Beeftink HH, van den Heuvel JC. 1987. Novel anerobic gas lift reactor (AGLR) with retention of biomass: Start-up routine and establishment of hold up. Biotechnology and Bioengineering 30:233–238.

Bélafi-Bakó K, Búcsú D, Pientka Z, Bálint B, Herbel Z, Kovács KL, Wessling M. 2006. Integration of biohydrogen fermentation and gas separation processes to recover and enrich hydrogen. International Journal of Hydrogen Energy 31:1490-1495.

Ben-Bassat A, Lamed R, Zeikus JG. 1981. Ethanol production by thermophilic bacteria: Metabolic control of end product formation in *Thermoanaerobium brockii*. Journal of Bacteriology 146:192-199.

Benemann J. 1996. Hydrogen biotechnology: Progress and prospects. Nature Biotechnology 14:1101-1103.

Benemann J. 1997. Feasibility analysis of photobiological hydrogen production. International Journal of Hydrogen Energy 22:979-987.

Biniwale RB, Rayalu S, Devotta S, Ichikawa M. 2008. Chemical hydrides: A solution to high capacity hydrogen storage and supply. International Journal of Hydrogen Energy 33:360-365.

Blackwood AC, Neish AC, Ledingham GA. 1956. Dissimilation of glucose at controlled pH values by pigmented and non-pigmented strains of Escherichia coli. Journal of Bacteriology 72:497-499.

Bockris JO'M. 2002. The origin of ideas on a hydrogen economy ad its solution to the decay of the environment. International Journal of Hydrogen Energy 27:731-740.

Bond DR, Holmes DE, Tender LM, Lovley DR. 2002. Electrode-reducing microorganisms that harvest energy from marine sediments. Science 295 483–485.

Bond DR, Lovley DR. 2003. Electricity production by *Geobacter sulfurreducens* attached to electrodes. Applied and Environmental Microbiology 69:1548–1555.

Bothast RJ, Schlicher MA. 2005. Biotechnological processes for conversion of corn into ethanol. Biomass and Bioenergy 67:19-25.

Bothun GD, Knutson BL, Berberich JA, Strobel HJ, Nokes SE. 2004. Metabolic selectivity and growth of *Clostridium thermocellum* in continuous culture under elevated hydrostatic pressure. Applied Microbiology and Biotechnology 65: 149–157.

Bououdina M, Grant D, Walker G. 2006. Review on hydrogen absorbing materials—structure, microstructure, and thermodynamic properties. International Journal of Hydrogen Energy 31:177-182.

Boynton ZL, Bennett GN, Rudolph FB. 1996. Cloning, sequencing, and expression of genes encoding phosphotransacetylase and acetate kinase from *Clostridium acetobutylicum* ATCC 824. Applied and Environmental Microbiology 62:2758– 2766.

Brionis A, Raskin L. 2003. Diversity and dynamics of microbial communities in engineered environments and their implications for process stability. Current Opinion in Biotechnology 14:270–276.

Brosseau JD, Zajic JE. 1982. Hydrogen-gas production with *Citrobacter intermedius* and *Clostridium pasteurianum*. Journal of Chemical Technology and Biotechnology 32:496-502.

Burdette DS, Jung S-H, Shen G-J, Hollingsworth RI, Zeikus JG. 2002. Physiological function of alcohol dehydrogenases and long-chain (C30) fatty acids in alcohol tolerance of *Thermoanaerobacter ethanolicus*. Applied Microbiology and Biotechnology 68:1914-1918.

Busby RL. 2005. Hydrogen and fuel cells: A comprehensive guide. 1st edition. PennWell Corporation, Tulsa, U.S. 445 p.

Cai M, Liu J, Wei Y. 2004. Enhanced biohydrogen production from sewage sludge with alkaline pretreatment. Environmental Science and Technology 38:3195-3202.

Calli B, Schoenmaeker K, Vanbroekhoven K, Diels L. 2008. Dark fermentative H_2 production from xylose and lactose—Effects of on-line pH control. International Journal of Hydrogen Energy 33:522-530.

Canale-Parola E. 1977. Physiology and evolution of Spirochetes. Bacteriological Reviews 41:181-204.

Cann IKO, Stroot PG, Mackie KR, White BA, Mackie RI. 2001. Characterization of two novel saccharolytic, anaerobic thermophiles, *Thermoanaerobacterium polysaccharolyticum* sp. nov. and *Thermoanaerobacterium zeae* sp. nov., and emendation of the genus *Thermoanaerobacterium*. International Journal of Systematic and Evolutionary Microbiology 51:293-302.

Cardona CA, Sanchez OJ. 2007. Fuel ethanol production: Process design trends and integration opportunities. Bioresource Technology 98:2415–2457.

Chan E-C, Ueng PP, Eder KL, Chen LF. 1989. Integration and expression of the *Escherichia coli* xylose isomerase gene in *Schizosaccharomyces pombe*. Journal of Industrial Microbiology 4:409-418.

Chang F-Y, Lin C-Y. 2004. Biohydrogen production using an up-flow anaerobic sludge blanket reactor. International Journal of Hydrogen Energy 29:33-39.

Chang F-Y, Lin C-Y. 2006. Calcium effect on fermentative hydrogen production in an anaerobic up-flow sludge blanket system. Water Science and Technology 54(9): 105-112.

Chang J-J, Chen W-E, Shih S-Y, Yu S-J, Lay J-J, Wen F-S, Huang C-C. 2006. Molecular detection of the clostridia in an anaerobic biohydrogen fermentation system by hydrogenase mRNA-targeted reverse transcription-PCR. Applied Microbiology and Biotechnology 70:598–604.

Chang J-J, Chou C-H, Hsu P-C, Yu SJ, Chen W-E, Lay J-J, Huang C-C, Wen F-Y. 2007. Flow-FISH analysis and isolation of clostridial strains in an anaerobic semi-solid bio-hydrogen producing system by hydrogenase gene target. Applied Microbiology and Biotechnology 74:1126–1134.

Chang J-S, Lee K-S, Lin P-J. 2002. Biohydrogen production with fixed-bed bioreactors. International Journal of Hydrogen Energy 27:1167-1174.

Cheryan M, Mehaia MA. 1984. Ethanol production in a membrane recycle bioreactor. Conversion of glucose using *Saccharomyces cerevisiae*. Process Biochemistry 19:204-208.

Chen C-C, Chen H-P, Wu J-H, Lin C-Y. 2008. Fermentative hydrogen production at high sulfate concentration. International Journal of Hydrogen Energy 33:1573-1578.

Chen C-C, Lin C-Y. 2001. Start-up of anaerobic hydrogen producing reactors seeded with sewage sludge. Acta Biotechnologica 21:371-379.

Chen CC, Lin CY. 2003. Using sucrose as a substrate in an anaerobic hydrogen-producing reactor. Advances in Environmental Research 7:695–699.

Chen C-C, Lin C-Y, Chang J-S. 2001. Kinetics of hydrogen production with continuous cultures utilizing sucrose as the limiting substrate. Applied Microbiology and Biotechnology 57:56-64.

Chen C-C, Lin C-Y, Lin M-C. 2002. Acid-base enrichment enhances anaerobic hydrogen production process. Applied Microbiology and Biotechnology 58:224-228.

Chen W-H, Chen S-Y, Kumar Khanal S, Sung S. 2006a. Kinetic study of biological hydrogen production by anaerobic fermentation. International Journal of Hydrogen Energy 31:2170–2178.

Chen W-M, Tseng Z-J, Lee K-S, Chang J-S. 2005. Fermentative hydrogen production with *Clostridium butyricum* CGS5 isolated from anaerobic sewage sludge. International Journal of Hydrogen Energy 30:1063-1070

Chen X, Sun Y, Xiu Z, Li X, Zhang D. 2006b. Stoichiometric analysis of biological hydrogen production by fermentative bacteria. International Journal of Hydrogen Energy 31:539-549.

Cheng S, Logan BE. 2007. Sustainable and efficient biohydrogen production via electrohydrogenesis. PNAS 104: 18871–18873.

Cheong D-Y, Hansen CL, Stevens DK. 2007. Production of bio-hydrogen by mesophilic anaerobic fermentation in an acid-phase sequencing batch reactor. Biotechnology and Bioengineering 96:421-432.

Cheong D-Y, Hansen CL. 2007. Feasibility of hydrogen production in thermophilic fermentation by natural anaerobes. Bioresource Technology 98:2229–2239.

Cheong D-Y, Hansen CL. 2006. Acidogenesis characteristics of natural, mixed anaerobes converting carbohydrate-rich synthetic wastewater to hydrogen. Process Biochemistry 41:1736–1745.

Chin H-L, Chen Z-S, Chou C.P. 2003. Fedbatch operation using *Clostridium acetobutylicum* suspension culture as biocatalyst for enhancing hydrogen production. Biotechnology Progress 19:383-388.

Chinn MS, Nokes SE, Strobel HJ. 2006. Screening of thermophilic anaerobic bacteria for solid substrate cultivation on lignocellulosic substrates. Biotechnology Progress 22:53-59.

Chinn MS, Nokes SE, Strobel HJ. 2007. Influence of process conditions on end product formation from *Clostridium thermocellum* 27405 in solid substrate cultivation on paper pulp sludge. Bioresource Technology 98:2184–2193.

Chinn MS, Nokes SE, Strobel HJ. 2008. Influence of moisture content and cultivation duration on *Clostridium thermocellum* 27405 end-product formation in solid substrate cultivation on Avicel. Bioresource Technology 99: 2664-2671.

Chittibabu G, Nath K, Das D. 2006.Feasibility studies on the fermentative hydrogen production by recombinant *Escherichia coli* BL-21. Process Biochemistry 41:682–688.

Cicek N. 2003. A review of membrane bioreactors and their potential application in the treatment of agricultural wastewater. Canadian Biosystems Engineering 45:6.37-6.49.

Cicek N, Winnen H, Suidan MT, Wrenn BE, Urbain V, Mane, J. 1998. Effectiveness of the membrane bioreactor in the biodegradation of high molecular weight compounds. Water Research 32:1553-1563.

Chou C-H, Wang C-W, Huang C-C, Lay J-J. 2008. Pilot study of the influence of stirring and pH on anaerobes converting high-solid organic wastes to hydrogen. International Journal of Hydrogen Energy 33:1550-1558.

Claassen PAM, de Vrije T, Budde MAW, Koukios EG, Glynos A, Réczey K. 2004. Biological hydrogen production from sweet sorghum by thermophilic bacteria. Proceedings of the 2nd World Conference on Biomass for Energy, Industry and Climate Protection, 10-14 May 2004, Rome, Italy. 1522-1525 p.

Claassen PAM, van Lier JB, Lopez Contreras AM, van Niel EWJ, Sijtsma L, Stams, AJM, de Vries SS, Weusthuis RA. 1999. Utilisation of biomass for the supply of energy carriers. Applied Microbiology and Biotechnology 42:741-755.

Cohen A, Distel B, van Deursen A, van Andel JG. 1985. Role of anaerobic spore-forming bacteria in the acidogenesis of glucose; changes induced by discontinuous or low-rate feed supply. Antonie van Leeuwenhoek Journal of Microbiology 51:179–192.

Cohen Y. 2001. Biofiltration - the treatment of fluids by microorganisms immobilized into the filter bedding material: a review. Bioresource Technology 77:257-274.

Collet C, Adler N, Schwitzguebel J-P, Peringer P. 2004. Hydrogen production by *Clostridium thermolacticum* during continuous fermentation of lactose. International Journal of Hydrogen Energy 29:1479-1485.

Collet C, Schwitzguébel J-P, Péringer P. 2003. Improvement of acetate production from lactose by growing *Clostridium thermolacticum* in mixed batch culture. Journal of Applied Microbiology 95:824–831

Conrad R, Wetter B. 1990. Influence of temperature on energetics of hydrogen metabolism in homoacetogenic, methanogenic, and other anaerobic bacteria. Archives of Microbiology 155:94- 98.

Cook GM, Morgan HW. 1994. Hyperbolic growth of *Thermoanaerobacter thermohydrosulfuricus* (*Clostridium thermohydrosulfuricum*) increases ethanol production in pH-controlled batch culture. Applied Microbiology and Biotechnology 41:84-89.

Cooney M, Maynard N, Cannizzaro C, Benemann J. 2007. Two-phase anaerobic digestion for production of hydrogen–methane mixtures. Bioresource Technology 98:2641-2651.

Crabbendam PM, Neijssel OM, Tempest DW. 1985. Metabolic and energetic aspects of the growth of *Clostridium butyricum* on glucose in chemostat culture. Archives of Microbiology 142:375–382.

Cullere J, Durany O, Busquets M, Manresa A. 2001. Biotransformation of oleic acid into (E)-10-hydroxy-8-octadecenoic acid and (E)-7,10-dihydroxy-8-octadecenoic acid by *Pseudomonas* sp. 42A2 in an immobilized system. Biotechnology Letters 23:215–219.

Cysewski GR and Wilke CR. 1978. Process design and economic studies of alternative fermentation methods for the production of ethanol. Biotechnology and Bioengineering 20:1421−1444.

Dabrock B, Bahl H, Gottschalk G. 1992. Parameters affecting solvent production by *Clostridium pasteurianum*. Applied and Environmental Microbiology 58:1233-1239.

Daigger GT, Rittman BE, Adham S, Andreottola G. 2005. Are membrane bioreactors ready for widespread application? Environmental Science and Technology October 1st: 399A-406A.

Das D, Veziroğlu TN. 2001. Hydrogen production by biological processes: a survey of literature. International Journal of Hydrogen Energy 26:13-28.

Das LM. 1996. On-board hydrogen storage systems for automotive application. International Journal of Hydrogen Energy 21:789-800.

Deanda K, Zhang M, Eddy M, Picataggio S. 1996. Development of an arabinose-fermenting *Zymomonas mobilis* strain by metabolic pathway engineering. Applied and Environmental Microbiology 62: 4465–4470.

Defrance L, Jaffrin MY. 1999. Comparison between filtrations at fixed transmembrane pressure and fixed permeate flux: application to a membrane bioreactor used for wastewater treatment. Journal of Membrane Science 152:203-210.

Delgenes JP, Moletta R, Navarre JM. 1986. The effect of aera tion on D-xylose fermentation by *Pachysolen tannophilus*, *Pichia stipitis*, *Kluyveromyces marxianus*, and *Candida shehatae*. Biotechnology Letters 8:897-900.

Dien BS, Cotta MA, Jeffries TW. 2003. Bacteria engineered for fuel ethanol production: current status. Applied Microbiology and Biotechnology 63:258-266.

Dietrich G, Weiss N, Winter J. 1988. *Acetothermus paucivorans*, gen. nov., sp. nov., a strictly anaerobic, thermophilic bacterium from sewage sludge, fermenting hexoses to acetate, CO_2 and H_2.S. Systematic and Applied Microbiology 10:174-179.

Dincer I. 2002. Technical, environmental and exergetic aspects of hydrogen energy systems. International Journal of Hydrogen Energy 27:265-285.

Dixon NM, Kell DB. 1989. The inhibition by CO_2 of the growth and metabolism of microorganisms. Journal of Applied Bacteriology 67:109–136.

Dunn S. 2002. Hydrogen futures: toward a sustainable energy system. International Journal of Hydrogen Energy 25: 235–264.

Eroĝlu E, Eroĝlu I, Gündüz U, Türker L, Yücel M. 2007.Biological hydrogen production from olive mill wastewater with two-stage processes. International Journal of Hydrogen Energy 31:1527-1535.

EurObserv'ER. 2007a. Biofuel barometer. Systemes Solaires – Le Journal des Energies Renouvelables 179:63-75.

EurObserv'ER. 2007b. Biogas barometer. Systemes Solaires – Le Journal des Energies Renouvelables 179:51-60.

European Commission. 1997. Communication from the Commission. Energy for the future: Renewable sources of energy. White paper for a community strategy and action plan. Internet document, accessed 1.3.2008, available at: http://ec.europa.eu/energy/library/599fi_en.pdf.

European Commission. 2003a. Directive 2003/30/EC of the European Parliament and of the council of May 2003 on the promotion of the use of biofuels or other renewable fuels for transport. Internet document, accessed 1.3.2008, available at: http://ec.europa.eu/energy/res/legislation/doc/biofuels/en_final.pdf.

European Commission. 2003b. Hydrogen and fuel cells: A vision for our future. European commission , Directorate-General for Research, Directorate-General for Energy and Transport, EUR 20719 EN. Internet document, accessed 19.12.2007, available at: https://www.hfpeurope.org/uploads/59/hydrogen-vision-report_HLG_2003_en.pdf.

European Commission. 2006a. Communication from the commission. An EU Strategy for biofuels. (SEC[2006] 142). Internet document, accessed 1.3.2008, available at: http://ec.europa.eu/agriculture/biomass/biofuel/com2006_34_en.pdf.

European Commission. 2006b. Green paper. A European strategy for sustainable, competitive and secure Energy (SEC[2006] 317). Internet document, accessed 1.3.2008, available at: http://ec.europa.eu/energy/green-paper-energy/doc/2006_03_08_gp_document_en.pdf.

European Commission. 2006c. Innovation and technological development in energy. State of renewable energies in Europe. Internet document, accessed 1.3.2008, available at: http://ec.europa.eu/energy/res/index_en.htm.

European Commission. 2008. Environment: Transport and environment. Internet document, accessed 26.2.2008, available at: http://ec.europa.eu/environment/air/transport.htm.

Evvyernie D, Morimoto K, Karita S, Kimura T, Sakka K, Ohmiya K. 2001. Conversion of chitinous wastes to hydrogen gas by *Clostridium paraputrificum* M-21, Journal of Bioscience and Bioengineering 91:339–343.

Evvyernie D, Yamazaki S, Morimoto K, Karita S, Kimura T, Sakka K, Ohmiya K. 2000. Identification and characterization of *Clostriduim paraputrificum* M-21, a chitinolytic, mesophilic and hydrogen-producing bacterium. Journal of Bioscience and Bioengineering 89:596-601.

Fabiano B, Perego P. 2002. Thermodynamic study and optimization of hydrogen production by *Enterobacter aerogenes*. International Journal of Hydrogen Energy 27:149-156.

Fakioğlu E, Yürüm Y, Veziroğlu TN. 2004. A review of hydrogen storage systems based on boron and its compounds. International Journal of Hydrogen Energy 29: 1371-1376.

Fan K-S, Chen Y-Y. 2004. H_2 production through anaerobic mixed culture: effect of batch S0/X0 and shock loading in CSTR. Chemosphere 57:1059-1068.

Fan K-S, Kan N-R, Lay JJ. 2006a. Effect of hydraulic retention time on anaerobic hydrogenesis in CSTR. Bioresource Technology 97:84-89.

Fan Y, Li C, Lay J-J, Hou H, Zhang G. 2004. Optimization of initial substrate and pH levels for germination of sporing hydrogen-producing anaerobes in cow dung compost. Bioresource Technology 91:189-193.

Fan Y-T, Zhang G-S, Guo X-Y, Xing Y, Fan M-H. 2006b. Biohydrogen-production from beer lees biomass by cow dung compost. Biomass and Bioenergy 30:493-496.

Fan Y-T, Zhang Y-H, Zhang G-S, Hou H-W, Ren B-Z. 2006c. Efficient conversion of wheat straw wastes into biohydrogen gas by cow dung compost. Bioresource Technology 97:500-505.

Fang HHP, Li C, Zhang T. 2006. Acidophilic biohydrogen production from rice slurry. International Journal of Hydrogen Energy 31:683-692.

Fang HHP, Liu H, Zhang T. 2002a. Characterization of a hydrogen producing granular sludge. Biotechnology and Bioengineering 78:44-52.

Fang HHP, Zhang T, Liu H. 2002b. Microbial diversity of a mesophilic hydrogen-producing sludge. Applied Microbiology and Biotechnology 58:112–118.

Fang HHP, Liu H, Zhang T. 2004. Bio-hydrogen production from wastewater. Water Science and Technology: Water supply 4:77-85.

Fang HHP, Liu H. 2002. Effect of pH on hydrogen production from glucose by a mixed culture. Bioresource Technology 82:87-93.

Fang HHP, Yu H. 2002. Mesophilic acidification of gelatinaceous wastewater. Journal of Biotechnology 93:99–108.

Fang HHP, Yu HQ. 2001. Acidification of lactose in wastewater. Journal of Environmental Engineering, September:825-831.

Fedorov AS, Kosourov S, Ghirardi ML, Seibert M. 2005. Continuous hydrogen photoproduction by *Chlamydomonas reinhardtii*. Applied Biochemistry and Biotechnology 121:403–412.

Fedorov AS, Tsygankov AA, Rao KK, Hall DO. 1998. Hydrogen photoproduction by *Rhodobacter sphaeroides* immobilized on polyurethane foam. Biotechnology Letters 20:1007-1009.

Feldmann S, Sprenger GA, Sahm H.1989. Ethanol production from xylose with a pyruvate-formate-lyase mutant of *Klebsiella planticola* carrying a pyruvate-decarboxylase gene from *Zymomonas mobilis*. Applied Microbiology and Biotechnology 31:152-157.

Florin NH, Harris AT. 2007. Hydrogen production from biomass coupled with carbon dioxide capture: The implications of thermodynamic equilibrium. International Journal of Hydrogen Energy 32: 4119-4134.

Francese A, Cordoba P, Duran J, Sineriz F. 1998. High upflow velocity and organic loading rate improves granulation in upflow anaerobic sludge blanket reactors. World Journal of Microbiology and Biotechnology 14:337-341.

Freier D, Mothershed CP, Wiegel J. 1988. Characterization of *Clostridium thermocellum* JW20. Applied and Environmental Microbiology 54:204-211.

Gaffron H, Rubin J. 1942. Fermentative and photochemical production of hydrogen in algae. Journal of General Physiology 26: 219–240.

Galbe M, Zacchi G. 2002. A review of the production of ethanol from softwood. Applied Microbiology and Biotechnology 59:618-628.

Gassanova LG, Netrusov AI, Teplyakov VV. Modigell M. 2006. Fuel gases from organic wastes using membrane bioreactors. Desalination 198:56–66.

Gavala HN, Skiadas IV, Ahring BK, Lyberatos G. 2005. Potential for biohydrogen and methane production from olive pulp. Water Science and Technology 52(1/2):209-215.

Gavala HN, Skiadas IV, Ahring BK. 2006. Biological hydrogen production in suspended and attached growth anaerobic reactor systems. International Journal of Hydrogen Energy 31:1164–1175.

Georgieva TI, Ahring BK. 2007. Evaluation of continuous ethanol fermentation of dilute-acid corn stover hydrolysate using thermophilic anaerobic bacterium *Thermoanaerobacter* BG1L1. Applied Microbiology and Biotechnology 77:61–68.

Georgieva TI, Skiadas IV, Ahring BK. 2007. Effect of temperature on ethanol tolerance of a thermophilic anaerobic ethanol producer *Thermoanaerobacter* A10: modeling and simulation. Biotechnology and Bioengineering 98:1161-1170.

Ghirardi ML, Zhang L, Lee JW, Flynn T, Seibert M, Greenbaum E, Melis A. 2000. Microalgae: green source of renewable H_2. Trends in Biotechnology 18:506-511.

van Ginkel S, Logan BE. 2005a. Inhibition of biohydrogen production by undissociated acetic and butyric acids. Environmental Science and Technology 39: 9351-9356.

van Ginkel S, Sung S, Lay JJ. 2001. Biohydrogen production as a function of pH and substrate concentration. Environmental Science and Technology 35:4726-4730.

van Ginkel SW, Logan BE. 2005b. Increased hydrogen production with reduced organic loading. Water Research 39:3819-3826.

van Ginkel SW, Oh S-E, Logan BE. 2005. Biohydrogen gas production from food processing and domestic wastewaters. International Journal of Hydrogen Energy 30:1535-1542.

Gnansounou E, Dauriat A, Wyman CE. 2005. Refining sweet sorghum to ethanol and sugar: economic trade-offs in the context of North China. Bioresource Technology 96:985–1002.

Gosselink JW. 2002. Pathways to a more sustainable production of energy: sustainable hydrogen – a research objective for Shell. International Journal of Hydrogen Energy 27:1125-1129.

Gray KA, Zhao L, Emptage M. 2006. Bioethanol. Current Opinion in Chemical Biology 10:141–146.

Green EM, Bennett GN. 1998. Genetic manipulation of acid and solvent formation in *Clostridium acetobutylicum* ATCC 824. Biotechnology and Bioengineering 58:215– 221.

Green EM, Boynton ZL, Harris LM, Rudolph FB, Papoutsakis ET, Bennett GN. 1996. Genetic manipulation of acid formation pathways by gene inactivation in *Clostridium acetobutylicum* ATCC 824. Microbiology 142:2079-2086.

van Groenestijn JW, Hazewinkel JHO, Nienoord M, Bussmann PJT. 2002. Energy aspects of biological hydrogen production in high rate bioreactors operated in the thermophilic temperature range. International Journal of Hydrogen Energy 27:1141-1147.

de Groot, A. 2003. Methane and hydrogen: on the role of end-use technologies in shaping the infrastructure. In: Bio-methane & Bio-hydrogen: Status and perspectives of biological methane and hydrogen production. Ed: Reith, J.H., Wiffels, R.H., Barten, H. Dutch Biological hydrogen Foundation, The Netherlands. 29-57 p. Available on the Internet: http://www.biohydrogen.nl/publicfiles/16_20804_2_Bio_methane_and_Bio_hydrogen_2003.pdf (Accessed 7.1.2008).

Hahn-Hägerdal B, Galbe M, Gorwa-Grauslund MF, Liden G, Zacchi G. 2006. Bio-ethanol – the fuel of tomorrow from the residues of today. Trends in Biotechnology 24:549-556.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41:95-98.

Hallenbeck PC. 2005. Fundamentals of fermentative production of hydrogen. Water Science and Technology 52(1-2):21-29.

Hallenbeck PC, Benemann JR. 2002. Biological hydrogen production; fundamentals and limiting processes. International Journal of Hydrogen Energy 27:1185-1193.

Hamelinck CN, van Hooijdonk G, Faaij APC. 2005. Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. Biomass and Bioenergy 28:384–410.

Han S-K, Shin H-S. 2004a. Biohydrogen production by anaerobic fermentation of food waste. International Journal of Hydrogen Energy 29:569-577.

Han S-K, Shin H-S. 2004b. Performance of an innovative two-stage process converting food waste to hydrogen and methane. Journal of Air and Waste Management Association 54:242–249.

Hao Z, Zhou M, Yu H, Shen Q, Lei L. 2006. Effect of sodium ion concentration on hydrogen production from sucrose by anaerobic hydrogen-producing Granular Sludge. Chinese Journal of Chemical Engineering 14:511-517.

Harris J, Mulder R, Kell DB, Walter RP, Morris JG. 1986. Solvent production by *Clostridium pasteurianum* in media of high sugar content. Biotechnology Letters 8:889-892.

Hawkes FR, Dinsdale R, Hawkes DL, Hussy I. 2002. Sustainable fermentative hydrogen production: challenges for process optimization. International Journal of Hydrogen Energy 27:1339-1347.

Hawkes FR, Hussy I, Kyazze G, Dinsdale R, Hawkes DL. 2007. Continuous dark fermentative hydrogen production by mesophilic microflora: Principles and progress. International Journal of Hydrogen Energy 32:172–184.

Hellman HL, van den Hoed R. 2007. Characterising fuel cell technology: Challenges of the commercialisation process. International Journal of Hydrogen Energy 32:305-315.

Henstra AM, Sipma J, Rinzema A, Stams AJM. 2007. Microbiology of synthesis gas fermentation for biofuel production. Current Opinion in Biotechnology 18:200–206.

Herrero AA, Gomez RF, Roberts MF. 1982. Ethanol-induced changes in the membrane lipid composition of *Clostridium thermocellum*. Biochimica et Biophysica Acta 693:195–204.

Herrero AA, Gomez RF, Roberts MF. 1985. 31P NMR studies of *Clostridium thermocellum*. The Journal of Biological Chemistry 260:7442–7451.

Herrero AA, Gomez RF. 1980. Development of ethanol tolerance in *Clostridium thermocellum*: Effect of growth temperature. Applied and Environmental Microbiology 40:571–577.

Heyndrickx M, De Vos P, De Ley J. 1990. H_2 production from chemosat fermentation of glucose by *Clostridium buryricum* and *Clostridium pasteurianum* in ammonium and phosphate limitation. Biotechnology Letters 12:731-736.

Heyndrickx M, De Vos P, Thibau B, Stevens P, De Ley J. 1987. Effect of various external factors on the fermenative production of hydrogen gas from glucose by *Clostridium buryricum* strains in batch culture. Systematic and Applied Microbiology 9:163-168.

Heyndrickx M, Vansteenbeeck A, De Vos P, De Ley J. 1986. Hydrogen gas production from continuous fermentation of glucose in a minimal medium with *Clostridium buryricum* LMG 1213t1. Systematic and Applied Microbiology 8:239-244.

Hill PW, Klapatch TR, Lynd LR. 1993. Bioenergetics and end-product regulation of C*lostridium thermosaccharolyticum* in response to nutrient limitation. Biotechnology Bioengineering 43:873–883.

Hillmer P, Gest H. 1977. H_2 metabolism in the photosynthetic bacterium *Rhodopseudomonas capsulate*: H_2 production by growing cultures. Journal of Bacteriology 129:724:731.

Hippe H, Andreesen JR, Gottschalk G. 1992. The genus Clostridium—nonmedical. In: Balows A, Trüber HG, Dworkin M, Harder W, Schkeifer K-H, editors. The Prokaryotes. 2nd edn, vol II. New York: Springer-Verlag. p 1800–1866.

Hirscher M, Becher M, Haluska M, von Zeppelin F, Chen X, Dettlaff-Weglikowska U, Roth S. 2003. Are carbon nanostructures an efficient hydrogen storage medium? Journal of Alloys and Compounds 356-357:433–437.

Hoffman P. 2002.Tomorrow's energy: Hydrogen, fuel cells, and the prospects for a cleaner planet. The MIT press, London, U.K. 289 p.

Hollaus F, Sleytr U. 1972. On the taxonomy and fine structure of some hyperthermophilic saccharolytic clostridia. Archives of Mikrobiology 86:129-146.

Homann PH. 2003. Hydrogen metabolism of green algae: discovery and early research – a tribute to Hans Gaffron and his coworkers. Photosynthesis Research 76:93-103.

Horiuchi J-I, Shimizu T, Tada K, Kanno T, Kobayashi, M. 2002. Selective production of organic acids in anaerobic acid reactor by pH control. Bioresource Technology 82:209-213.

Hu R, Li J, Wang Z. 2005. China's renewable energy law and biomass energy. Industrial Biotechnology 1: 222-227.

Hulshoff Pol LW, de Castro Lopes SI, Lettinga G, Lens PNL. 2004. Anaerobic sludge granulation. Water Research 38:1376–1389.

Hung C-H, Chen H-W, Chen C-H, Cheng L-H. 2005. Combining fluorescence *in situ* hybridization and denaturing gradient gel electrophoresis on mentoring the anaerobic hydrogen production bacterial community. Proceedings of The 2005 Biohydrogen Technology Symposium and the 3rd International Workshop on Innovative Anaerobic Technology, 9 -11th December 2005, Taichung, Taiwan, 54-60 p

Hung C-H, Lee K-S, Cheng L-H, Huang Y-H, Lin P-J, Chang J-S. 2007. Quantitative analysis of a high-rate hydrogen-producing microbial community in anaerobic agitated granular sludge bed bioreactors using glucose as substrate. Applied Microbiology and Biotechnology 75:693–701.

Hung C-H, Cheng C-H, Cheng L-H, Liang C-M, Lin C-Y. 2008. Application of Clostridium-specific PCR primers on the analysis of dark fermentation hydrogen-producing bacterial community. International Journal of Hydrogen Energy 33:1586-1592.

Hussy I, Hawkes FR, Dinsdale R, Hawkes DL. 2003. Continuous fermentative hydrogen production from a wheat starch co-product by mixed microflora. Biotechnology and Bioengineering 84:619-626.

Hussy I, Hawkes FR, Dinsdale R, Hawkes DL. 2005. Continuous fermentative hydrogen production from sucrose and sugarbeet. International Journal of Hydrogen Energy 30:471-483.

Hwang MH, Jang NJ, Hyum SH, Kim IS. 2004. Anaerobic biohydrogen production from ethanol fermentation: the role of pH. Journal of Biotechnology 111:297-309.

Iannotti EI, Kafkewitz D, Wolin MJ, Bryant MP. 1973. Glucose fermentation products of *Ruminococcus albus* grown in continuous culture with *Vibrio succinogenes*: changes caused by interspecies transfer of H_2. Journal of Bacteriology 1231-1240.

Ike A, Murakawa T, Kawaguchi H, Hirata K, Miyamoto K. 1999. Photoproduction of hydrogen from raw starch using a halophilic bacterial community. Journal of Bioscience and Bioengineering 88:72-77.

Ingram LO, Aldrich HC, Borges ACC, Causey TB, Martinez A, Morales F, Saleh A, Underwood SA, Yomano LP, York SW, Zaldivar J, and Zhou S. 1999. Enteric bacterial catalysts for fuel ethanol production. Biotechnology Progress 15:855-866.

International Energy Agency. 2006. IEA Agreement on the Production and Utilization of Hydrogen – Annual report 2006. Eds: de Valladares MR. Internet document, accessed 17.12.2007, available at: http://www.ieahia.org/page.php?s=d&p=annual.

International Energy Agency. 2007. World energy outlook 2007. Executive summary. China and India insights. Internet document, accessed 1.3.2008, available at:http://www.iea.org/Textbase/npsum/WEO2007SUM.pdf.

IPCC. 2007. Intergovernmental Panel on Climate Change fourth assessment report. Climate change 2007: Synthesis report. Summary for policymakers. Internet document, accessed 1.3.2008, available at: http://www.ipcc.ch/pdf/assessment-report/ar4/syr/ar4_syr_spm.pdf.

Islam R, Cicek N, Sparling R, Levin D. 2006. Effect of substrate loading on hydrogen production during anaerobic fermentation by *Clostridium thermocellum* 27405. Applied Microbiology and Biotechnology 72:576-583.

Ito T, Nakashimada Y, Kakizono T, Nishio N. 2004. High-yield production of hydrogen by *Enterobacter aerogenes* mutants with decreased α-acetolactate synthase activity. Journal of Bioscience and Bioengineering 97:227-232.

Iyer P, Bruns MA, Zhang H, Van Ginkel S, Logan BE. 2004. H_2-Producing bacterial communities from a heat-treated soil inoculum. Applied Microbiology and Biotechnology 66:166–173.

Janssen PH, Morgan H.1992. Glucose catabolism by *Spirochaeta thermophila* RI 19.B1. Journal of Bacteriology 174:2449-2453.

Jan T-W, Adav SS, Lee DJ, Wu RM, Su A, Tay J-H. 2007. Hydrogen fermentation and methane production from sludge with pretreatments. Energy&Fuels 22:98–102.

Jeon B-S, Um YS, Lee S-M, Lee SY, Kim H-J, Kim YH, Gu MB, Sang B-I. 2008. Performance analysis of a proton exchange membrane fuel cell (PEMFC) integrated with a trickling bed bioreactor for biological high-rate hydrogen production. Energy&Fuels 22:83–86.

Jo JH, Jeon CO, Lee DS, Park JM. 2007. Process stability and microbial community structure in anaerobic hydrogen-producing microflora from food waste containing kimchi. Journal of Biotechnology 131:300–308.

Jones DT, Woods DR. 1986. Acetone-butanol fermentation revisited. Microbiological Reviews 50:484-524.

Junelles AM, Janati-Idrissi R, Petitdemange H, Gay R. 1988. Iron effect on acetone-butanol fermentation. Current Microbiology 17:299-303.

Kadar Z, de Vrije T, Budde MAW, Szengyel Z, Reczey K, Claassen PAM. 2003. Hydrogen production from paper sludge hydrolysate. Applied Biochemistry and Biotechnology 105-108:557-566.

Kadar Z, de Vrije T, van Noorden GE, Budde MAW, Szengyel Z, Reczey K, Claassen PAM. 2004. Yields from glucose, xylose, and paper sludge hydrolysate during hydrogen production by the extreme thermophile *Caldicellulosiruptor saccharolyticus*. Applied Biochemistry and Biotechnology 113–116:497-508.

Kaksonen AH. 2004. The performance, kinetics and microbiology of sulfidogenic fluidized-bed reactors treating acidic metal- and sulfate-containing wastewater. DTech Thesis. Tampere University of Technology. Publication 489.

Kalia VC, Jain SR, Kumar A, Joshi AP. 1994. Fermentation of biowaste to H_2 by *Bacillus licheniformis*. World Journal of Microbiology and Biotechnology 10:224-227.

Kalia VC, Joshi AP. 1995. Conversion of waste biomass (pea-cells) into hydrogen and methane through anaerobic digestion. Bioresource Technology 53:165-168.

Kanai T, Imanaka H, Nakajima A, Uwamori K, Omori Y, Fukui T, Atomi H, Imanaka T. 2005. Continuous hydrogen production by the hyperthermophilic archaeon, *Thermococcus kodakaraensis* KOD1. Journal of Biotechnology 116:271-282.

Kapdan IK, Kargi F. 2006. Bio-hydrogen production from waste materials. Enzyme and Microbial Technology 38:569-582.

Karhumaa K, Wiedemann B, Hahn-Hägerdal B, Boles E, Gorwa-Grauslund MF. 2006. Co-utilization of L-arabinose and D-xylose by laboratory and industrial *Saccharomyces cerevisiae* strains. Microbial Cell Factories 5:18.

Karube I, Urano N, Matsunaga T, Suzuki S. 1982. Hydrogen production from glucose by immobilized growing cells of *Clostridium butyricum*. European Journal of Applied Microbiology and Biotechnology 16:5-9.

Kataoka N, Miya A, Kiriyama K. 1997. Studies on hydrogen production by continuous culture system of hydrogen-producing anaerobic bacteria. Water Science and Technology 36(6-7):41-47.

Kawagoshi Y, Hino N, Fujimoto A, Nakao M, Fujita Y, Sugimura S, Furukawa K. 2005. Effect of inoculum conditioning on hydrogen fermentation and pH effect on bacterial community relevant to hydrogen production. Journal of Bioscience and Bioengineering 100:524–530.

Kengen SWM and Stams AJM: 1994. Formation of L-alanine as a reduced end product in carbohydrate fermentation by the hyperthermophilic archaeon *Pyrococcus furiosus*. Archives of Microbiology 161: 168-175.

Khan AW, Murray WD. 1982. Single step conversion of cellulose to ethanol by a mesophilic culture. Biotechnology Letters 4:177-180.

Khanal SK, Chen W-H, Li L, Sung S. 2004. Biological hydrogen production: effects of pH and intermediate products. International Journal of Hydrogen Energy 29:1123-1131.

Kim D-H, Han S-K, Kim S-H, Shin H-S. 2006a. Effect of gas sparging on continuous fermentative hydrogen production. International Journal of Hydrogen Energy 31:2158–2169.

Kim D-H, Kim S-H, Han S-K ,Shin H-S. 2005a. Performance of pilot-scale H_2-fermenter: C/N –ratio effect & alkaline shock treatment. Proceedings of The 2005 Biohydrogen Technology Symposium and the 3rd International Workshop on Innovative Anaerobic Technology, 9 -11th December 2005, Taichung, Taiwan, 68-74 p.

Kim IS, Hwang MH, Jang NJ, Hyun SH, Lee ST. 2004a. Effect of low pH on the activity of hydrogen utilizing methanogen in bio-hydrogen processes. International Journal of Hydrogen Energy 29:1133-1140.

Kim JO, Kim YH, Ryu JY, Song BK, Kim IH, Yeom SH. 2005b. Immobilization methods for continuous hydrogen gas production biofilm formation versus granulation. Process Biochemistry 40:1331-1337.

Kim JO, Kim YH, Yeom SH, Song BK, Kim IH. 2006b. Enhancing continuous hydrogen gas production by the addition of nitrate into an anaerobic reactor. Process Biochemistry 41:1208–1212.

Kim S-H, Han S-K, Shin, H-S. 2004b. Feasibility of biohydrogen production by anaerobic co-digestion of food waste and sewage sludge. International Journal of Hydrogen Energy 29:1607-1616.

Kim S-H, Han S-K, Shin, H-S. 2006c. Effect of substrate concentration on hydrogen production and 16S rDNA-based analysis of the microbial community in a continuous fermenter. Process Biochemistry 41:199-207.

Kleerebezem R, van Loosdrecht MCM. 2007. Mixed culture biotechnology for bioenergy production. Current Opinion in Biotechnology 18:1-6.

Klinke HB, Tomsen AB, Ahring BK. 2001. Potential inhibitors from wet oxidation of wheat straw and their effect on growth and ethanol production by *Thermoanaerobacter mathranii*. Applied Microbiology and Biotechnology 57:631–638.

Koroneos C, Dompros A, Roumbas G, Moussiopoulos N. 2004. Life cycle assessment of hydrogen fuel production processes. International Journal of Hydrogen Energy 29:1443-1450.

Kotay SM, Das D. 2007. Microbial hydrogen production with *Bacillus coagulans* IIT-BT S1 isolated from anaerobic sewage sludge. Bioresource Technology 98:1183-1190.

Kötter P, Ciriacy M. 1993. Xylose fermentation by *Saccharomyces cerevisiae*. Applied Microbiology and Biotechnology 38:776–783.

Kosaric N, Vardar-Sukan F. 2001. Potential source of energy and chemical products. Ed: Roehr M. Wiley-WCH, Weinheim, Germany. 89-226 p.

Kotsopoulos TA, Raymond JZ, Angelidaki I. 2006. Biohydrogen production in granular up-flow anaerobic sludge blanket reactors with mixed cultures under hyper-thermophilic temperature (70 °C). Biotechnology Bioengineering 94:296-302.

Kovács KL, Marótia G, Rákhely G. 2006. A novel approach for biohydrogen production. International Journal of Hydrogen Energy 31:1460-1468.

Kraemer JT, Bagley DM. 2006. Supersaturation of dissolved H_2 and CO_2 during fermentative hydrogen production with N_2 sparging. Biotechnology Letters 28:1485–1491.

Kraemer JT, Bagley DM. 2005. Continuous fermentative hydrogen production using a two-phase reactor system with recycle. Environmental Science and Technology 39:3819–3825.

Kraemer JT, Bagley DM. 2007. Improving the yield from fermentative hydrogen production. Biotechnology Letters 29:685–695.

Krivenko VV, Vadachloriya RM, Chermykh NA, Mityushina LL, Krasil'nikova EN. 1990. *Clostridium uzonii* sp. nov., an anaerobic thermophilic glycolytic bacterium isolated from hot springs in the Kamchatka Peninsula. Microbiologiya 59:1058-1066.

Kruse O, Rupprecht J, Bader KP, Thomas-Hall S, Schenk PM, Finazzi G, Hankamer B. 2005. Improved photobiological H_2 production in engineered green algal cells. Journal of Biological Chemistry 280:34170–34177.

Kublanov IV, Prokofeva MI, Kostrikina NA, Kolganova TV, Tourova TP, Wiegel J, Bonch-Osmolovskaya EA. 2007. *Thermoanaerobacterium aciditolerans* sp. nov., a moderate thermoacidophile from a Kamchatka hot spring. International Journal of Systematic and Evolutionaty Microbiology 57:260-264.

Kuhn M, Steinbüchel A, Schlegel HG. 1984. Hydrogen evolution by strictly aerobic hydrogen bacteria under anaerobic conditions. Journal of Bacteriology 159:633-639.

Kumar N, Das D. 2000. Enhancement of hydrogen production by *Enterobacter cloacae* IIT-BT 08. Process Biochemistry 35:589–593.

Kumar N, Das D. 2001a. Continuous hydrogen production by immobilized *Enterobacter cloacae* IIT-BT 08 using lignocellulosic materials as solid matrices. Enzyme and Microbial Technology 29:280-287.

Kumar N, Das D. 2001b. Electron microscopy of hydrogen producing immobilized *E. cloacae* IIT-BT 08 on natural polymers. International Journal of Hydrogen Energy 26:1155-1163.

Kumar N, Ghosh A, Das D. 2001. Redirection of biochemical pathways for the enhancement of H2 production by *Enterobacter cloacae*. Biotechnology Letters 23:537–541.

Kumar N, Jain SR, Sharma AP, Joshi AP, Kalia VC. 1995. Increased H_2 production by immobilized microorganisms. World Journal of Microbiology and Biotechnology 11:156-159.

Kumar N, Monga PS, Biswas AS, Das D. 2000. Modeling and simulation of clean fuel production by *Enterobacter cloacae* IIT-BT 08. International Journal of Hydrogen Energy 25:945-952.

Kyazze G, Dinsdale R, Guwy AJ, Hawkes FR, Premier GC, Hawkes DL. 2007.Performance characteristics of a two-stage dark fermentative system producing hydrogen and methane continuously. Biotechnology and Bioengineering 97:759-770.

Kyazze G, Martinez-Perez N, Dinsdale R, Premier GC, Hawkes FR, Guwy AJ, Hawkes DL. 2006. Influence of substrate concentration on the stability and yield of continuous biohydrogen production. Biotechnology and Bioengineering 93:971-979.

Lacis LS, Lawford H. 1988. Ethanol production from xylose by *Thermoanaerobacter ethanolicus* in batch and continuous culture. Archives of Microbiology 150:48-55.

Lacis LS, Lawford H. 1991. *Thermoanaerobacter ethanolicus* growth and product yield from elevated levels of xylose or glucose in continuous cultures. Applied and Environmental Microbiology 57:579-585.

Lamari Darkrim F, Malbrunot P, Tartaglia GP. 2002. Review of hydrogen storage by adsorption in carbon nanotubes. International Journal of Hydrogen Energy 27:193-202.

Lamed RF, Lobos JH, Su TM. 1988. Effects of stirring and hydrogen on fermentation products of *Clostridium thermocellum*. Applied and Environmental Microbiology 54:1216-1221.

Larsen L, Nielsen P, Ahring BK. 1997. *Thermoanaerobacter mathranii* sp. nov., an ethanol-producing, extremely thermophilic anaerobic bacterium from a hot spring in Iceland. Archives of Microbiology 168:114-119.

Lawford HG, Rousseau JD. 1999. The effect of glucose on high-level xylose fermentations by recombinant Zymomonas in batch and fed-batch fermentations. Applied Biochemistry and Biotechnology 77-79:235–249.

Lawford HG, Rousseau JD. 2002. Performance testing of *Zymomonas mobilis* metabolically engineered for cofermentation of glucose, xylose, and arabinose. Applied Biochemistry and Biotechnology 98-100:429–448.

Lay JJ, Fan K-S, Hwang JI, Chang JI, Hsu P-C. 2005. Factors affecting hydrogen production from food wastes by Clostridium-rich composts. Journal of Environmental Engineering 131:595-602.

Lay J-J, Lee Y-J, Noike T. 1999. Feasibility of biological hydrogen production from organic fraction of municipal solid waste. Water Research 33:2579-2586.

Lay J-J. 2000. Modeling and optimization of anaerobic digested sludge converting starch to hydrogen. Biotechnology and Bioengineering 68:269-278.

Lay JJ. 2001. Biohydrogen generation by mesophilic anaerobic fermentation of microcrystalline cellulose. Biotechnology and Bioengineering 74:281-287.

Lay J-J, Fan K-S, Chang J, Ku C-H. 2003. Influence of chemical nature of organic wastes on their conversion to hydrogen by heat-shock digested sludge. International Journal of Hydrogen Energy 28:1361-1367.

Lee CK, Ordal ZJ. 1967. Regulatory effect of pyruvate on the glucose metabolism of *Clostridium thermosaccharolyticum*. Journal of Bacteriology 94:530-536.

Lee J. 1997. Biological conversion of lignocellulosic biomass to ethanol. Journal of Biotechnology 56:1–24.

Lee K-S, Lin P-J, Chang J-S. 2006a. Temperature effects on biohydrogen production in a granular sludge bed induced by activated carbon carriers. International Journal of Hydrogen Energy 31:1648–1657.

Lee K-S, Lo Y-C, Lin P-J, Chang J-S. 2006b. Improving biohydrogen production in a carrier-induced granular sludge bed by altering physical configuration and agitation pattern of the bioreactor. International Journal of Hydrogen Energy 31:1648-1657.

Lee K-S, Lo Y-S, Lo Y-C, Lin P-J, Chang J-S. 2003. H_2 production with anaerobic sludge using activated-carbon supported packed-bed bioreactors. Biotechnology Letters 25:133-138.

Lee K-S, Lo Y-S, Lo Y-C, Lin P-J, Chang J-S. 2004a. Operation strategies for biohydrogen production with a high-rate anaerobic granular sludge bed bioreactor. Enzyme and Microbial Technology 35:605-612.

Lee K-S, Wu J-F, Lo Y-S, Lo Y-C, Lin P-J, Chang J-S. 2004b. Anaerobic hydrogen production with an efficient carrier-induced granular sludge bed bioreactor. Biotechnology and Bioengineering 87: 648-657.

Lee MJ, Zinder SH. 1988. Hydrogen partial pressures in a thermophilic acetate-oxidising methanogenic coculture. Applied and Environmental Microbiology 54:1457-1461.

Lee YJ, Miyahara T, Noike T. 2001. Effect of iron concentration on hydrogen fermentation. Bioresource Technology 80:227-231.

Lettinga G. 1995. Anaerobic digestion and wastewater treatment systems. Antonie van Leeuwenhoek 67:3-28.

Levin DB, Islam R, Cicek N, Spaarling R. 2006. Hydrogen production by *Clostridium thermocellum* 27405 from cellulosic biomass substrates. International Journal of Hydrogen Energy 31:1496-1503.

Levin DB, Pitt L, Love M. 2004. Biohydrogen production: prospects and limitations to practical application. International Journal of Hydrogen Energy 29:173-185.

Levin DB. 2004. Re: Biohydrogen production: prospects and limitations to practical application. International Journal of Hydrogen Energy 29:1425-1426.

Li S-L, Kuo S-C, Lin J-S, Lee ZK, Wang YH, Cheng S-S. 2006. Performance evaluation for anaerobic hydrogen fermentation process treating kitchen waste. The 2006 Asian bio-Hydrogen symposium, November 19, 2006, Feng Chia University, Taiwan. 108-120 p.

Li C, Fang HHP. 2007a. Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. Critical Reviews in Environmental Science and Technology 37:1–39.

Li C, Fang HHP. 2007b. Inhibition of heavy metals on fermentative hydrogen production by granular sludge. Chemosphere 67:668-673.

Li YF, Ren NQ, Chen Y, Zheng GX. 2007. Ecological mechanism of fermentative hydrogen production by bacteria. International Journal of Hydrogen Energy 32:755–760.

Liang T-M, Cheng S-S, Wu K-L. 2002. Behavioral study on hydrogen fermentation reactor installed with silicone rubber membrane. International Journal of Hydrogen Energy 27:1157-1165.

Lide DR. 2007. CRC Handbook of chemistry and physics. 88th edition, 2007-2008. CRC Press/Taylor & Francis Group: Boca Raton, FL. 2640 p.

Lin CY, Jo CH. 2003. Hydrogen production from sucrose using an anaerobic sequencing batch reactor process. Journal of Chemical Technology and Biotechnology 78, 678-684.

Lin C-N, Wu S-Y, Chang J-S. 2006a. Fermentative hydrogen production with a draft tube fluidized bed reactor containing silicone-gel-immobilized anaerobic sludge. International Journal of Hydrogen Energy 31:2200-2210.

Lin C-N, Wu S-Y, Lee K-S, Lin P-J, Lin C-Y, Chang J-S: 2007a. Integration of fermentative hydrogen process and fuel cell for on-line electricity generation. International Journal of Hydrogen Energy 32:802-808.

Lin C-Y, Chang R-C. 1999. Hydrogen production during the anaerobic acidogenic conversion of glucose. Journal of Chemical Technology and Biotechnology 74:498-500.

Lin C-Y, Chang RC. 2004. Fermentative hydrogen production at ambient temperature. International Journal of Hydrogen Energy 29:715-720.

Lin C-Y, Chen H-P. 2006. Sulfate effect on fermentative hydrogen production using anaerobic mixed microflora. International Journal of Hydrogen Energy 31:953-960.

Lin C-Y, Cheng C-H. 2006. Fermentative hydrogen production from xylose using anaerobic mixed microflora. International Journal of Hydrogen Energy 31:832–840.

Lin C-Y, Hung C-H, Chen C-H, Chung W-T, Cheng L-H. 2006b. Effects of initial cultivation pH on fermentative hydrogen production from xylose using natural mixed cultures. Process Biochemistry 41:1383-1390.

Lin C-Y, Lay CH. 2004a. Carbon/nitrogen-ratio effect on fermentative hydrogen production by mixed microflora. International Journal of Hydrogen Energy 29:41-45.

Lin C-Y, Lay CH. 2004b. Effects of carbonate and phosphate concentrations on hydrogen production using anaerobic sewage sludge microflora. International Journal of Hydrogen Energy 29:275-281.

Lin C-Y, Lay CH. 2005. A nutrient formulation for fermentative hydrogen production using anaerobic sewage sludge microflora. International Journal of Hydrogen Energy 30:285-292.

Lin CY, Lee CY, Tseng IC, Shiao IZ. 2006c. Biohydrogen production from sucrose using base-enriched anaerobic mixed microflora. Process Biochemistry 41:915-919.

Lin C-Y, Shei S-H. 2008. Heavy metal effects on fermentative hydrogen production using natural mixed microflora. International Journal of Hydrogen Energy 33:587–593.

Lin P-Y, Whang L-M, Wu Y-R, Ren W-J, Hsiao C-J, Li S-L, Chang J-S. 2007b. Biological hydrogen production of the genus *Clostridium*: Metabolic study and mathematical model simulation. International Journal of Hydrogen Energy 32:1728-1735.

Lin Y, Tanaka S. 2006. Ethanol fermentation from biomass resources: current state and prospects. Applied Microbiology and Biotechnology 69:627-642.

Liu D, Liu D, Zeng RJ, Angelidaki I. 2006. Hydrogen and methane production from household solid waste in the two-stage fermentation process. Water Research 40:2230-2236.

Liu G, Shen J .2004. Effects of culture and medium conditions on hydrogen production from starch using anaerobic bacteria. Journal of Bioscience and Bioengineering 98:251–256.

Liu H, Cheng S, Logan BE. 2005a. Production of electricity from acetate or butyrate using a single-chamber microbial fuel cell. Environmental Science and Technology 39:658–662.

Liu H, Grot S, Logan BE. 2005b. Electrochemically assisted microbial production of hydrogen from a fermentation end product. Environmental Science and Technology 39:4317-4320.

Liu H, Fang HHP. 2002. Hydrogen production from wastewater by acidogenic granular sludge. Water Science and Technology 47(1):153-158.

Liu H, Ramnarayanan R, Logan BE. 2004a. Production of electricity during wastewater treatment using a single chamber microbial fuel cell. Environmental Science and Technology 38:2281-2285.

Liu SY, Rainey FA, Morgan HW, Mayer F, Wiegel J. 1996. *Thermoanaerobacterium aotearoense* sp. nov., a slightly acidophilic, anaerobic thermophile isolated from various hot springs in New Zealand, and emendation of the genus *Thermoanaerobacterium*. International Journal of Systematic Bacteriology 46:388–396.

Liu W-Z, Wang A-J, Ren N-Q, Zhao X-Y, Liu L-H, Yu Z-G, Lee D-J. 2008. Electrochemically assisted biohydrogen production from acetate. Energy & Fuels 22:159-163.

Liu Y, Xu H-L, Yang S-F, Tay J-H. 2003. Mechanisms and models for anaerobic granulation in upflow anaerobic sludge blanket reactors. Water Research 37:661-673.

Liu Y, Yang SF, Tay JH, Liu QS, Qin L, Li Y. 2004b. Cell hydrophobicity is a triggering force of biogranulation. Enzyme and Microbial Technology 34:371–379.

Liu YQ, Liu Y, Tay JH. 2004c. The effects of extracellular polymeric substances on the formation and stability of biogranules. Applied Microbiology and Biotechnology 65:143–148.

Logan BE, Hamelers B, Rozendal R, Schröder U, Keller J, Freguia S, Aelterman P, Verstraete W, Rabaey K. 2006. Microbial fuel cells: Methodology and technology. Environmental Science and Technology 40: 5181-5192.

Logan BE, Oh S-E, Kim AS, van Ginkel S. 2002. Biological hydrogen production measured in batch anaerobic respirometers. Environmental Science and Technology 36:2530-2535.

Logan BE, Regan JM. 2006. Electricity-producing bacterial communities in microbial fuel cells. Trends in Microbiology 14:512-518.

Logan BE. 2004. Biologically extracting energy from wastewater – Biohydrogen production and microbial fuel cells. Environmental Science and Technology 38:160A–167A.

Logan BE, Grot S. 2006. Bio-electrochemically assisted microbial reactor that generates hydrogen gas and methods of generating hydrogen gas. United States Patent 20060011491.

van Loosdrecht MCM, van, Lyklema J, Norde W, Schraa G, Zehnder AJB. 1987. The role of bacterial cell wall hydrophobicity in adhesion. Applied and Environmental Microbiology 53:1893-1897.

van Loosdrecht MCM, Zehnder AJB. 1990. Energetics of bacterial adhesion. Experientia 46:817-822.

Lopes Pinto FA, Troshina O, Lindblad P. 2002. A brief look at three decades of research on cyanobacterial hydrogen evolution. International Journal of Hydrogen Energy 27:1209-1215.

Lovins A. 2005. Twenty hydrogen myths. Rocky Mountain Institute. Report #E03-05. Internet document, accessed 7.1.2008, available at: http://www.rmi.org/images/other/Energy/E03-05_20HydrogenMyths.pdf.

Lovitt RW, Longin R, Zeikus JG. 1984. Ethanol production by thermophilic bacteria: Physiological comparison of solvent effect on parent and alcohol-tolerant strains of *Clostridium thermohydrosulfuricum*. Applied and Environmental Microbiology 48:171–177.

Lovitt RW, Shen G-J, Zeikus JG. 1988. Ethanol production by thermophilic bacteria: Biochemical basis for ethanol and hydrogen tolerance in *Clostridium thermohydrosulfuricum*. Journal of Bacteriology 170:2809-2815.

Lu W, Wen J, Chen Y, Sun B, Jia X, Liu M, Caiyin Q. 2007. Synergistic effect of *Candida maltosa* HY-35 and *Enterobacter aerogenes* W-23 on hydrogen production. International Journal of Hydrogen Energy 32:1059–1066.

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G, Förster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, König A, Liss T, Lüßmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann A, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer K-H. 2004. ARB: a software environment for sequence data. Nucleic Acids Research 32:1363-1371.

Lynd LR, Baskaran S, Casten S. 2001. Salt accumulation resulting from base added for pH control, and not ethanol, limits growth of *Thermoanaerobacterium thermosaccharolyticum* HG-8 at elevated feed xylose concentrations in continuous culture. Biotechnology Progress 17:118-125.

Lynd LR. 1989. Production of ethanol from lignocellulosic materials using thermophilic bacteria. Critical evaluation of potential and review. Advanced Biochemical Engineering/Biotechnology 38:1-52.

Ma K, Zhou ZH, Adams MWW. 1994. Hydrogen production from pyruvate by enzymes purified from the hyperthermophilic archaeon, *Pyrococcus furiosus*: A key role for NADPH. FEMS Microbiology Letters 122:245-250.

Madigan MT, Martenko JM, Parker J. 2000. Brock biology of microorganisms 9th edition. Prentice Hall, Upper Saddle River, New Jersey, USA. 991 p.

Maijzat A, Yoshida M, Watanabe M, Nakamura M, Matsumoto J. 1997. Hydrogen gas production from glucose and its microbial kinetics in anaerobic systems. Water Science and Technology 36(6-7) 279-286.

Mandal B, Nath K, Das D. 2006. Improvement of biohydrogen production under decreased partial pressure of H_2 by *Enterobacter cloacae*. Biotechnology Letters 28(11):831–835.

Maness P-C, Weaver PF. 2002. Hydrogen production from a carbon-monoxide oxidation pathway in *Rubrivivax gelatinosus*. International Journal of Hydrogen Energy 27:1407-1411.

Marbán G, Valdés-Solís T. 2007. Towards the hydrogen economy? International Journal of Hydrogen Energy 32: 1625-1637.

Marin P, Alkalay D, Guerrero L, Chamy R, Sciappacasse MC. 1999. Design and start-up of an anaerobic fluidized-bed bioreactor. Water Science and Technology 40(8):63-70.

Matsumoto M, Nishimura Y. 2007. Hydrogen production by fermentation using acetic acid and lactic acid. Journal of Bioscience and Bioengineering 103:236–241.

McGovern PE, Zhang J, Tang J, Zhang Z, Hall GR, Moreau RA, Nunez A, Butrym ED, Richards MP, Wang C-S, Cheng G, Zhao Z, Wang C. 2004. Fermented beverages of pre- and proto-historic China. PNAS 01: 17593–17598.

McLean GF, Niet T, Prince-Richard S, Djilali N. 2002. An assessment of alkaline fuel cell technology. International Journal of Hydrogen Energy 27:507-526.

van der Meer T, Kinnunen PH.-M, Kaksonen AH, Puhakka JA. 2007. Effect of fluidized-bed carrier material on biological ferric sulphate generation. Minerals Engineering 20:782–792.

Melin ES, Järvinen KT, Puhakka JA. 1998. Effects of temperature on the chlorophenol degradation kinetics in fluidized-bed reactors with different biomass carriers. Water Research 32:81-90.

Melis A. 2002. Green alga hydrogen production: progress, challenges and prospects. International Journal of Hydrogen Energy 27:1217-1228.

Merida WM, Maness P-C, Brown RC, Levin DB. 2004. Enhanced hydrogen production from indirectly heated, gasified biomass, and removal of carbon gas emissions using a novel biological gas reformer. International Journal of Hydrogen Energy 29:283–290.

Miller TL, Wolin MJ. 1973. Formation of hydrogen and formate by *Ruminococcus albus*. Journal of Bacteriology 116:836-846.

Milne TA, Elam CC, Evans RJ. 2001. Hydrogen from biomass – State of the art and research challenges. Annual Report, International Energy Agency, Agreement on the Production and Utilization of Hydrogen; Task 16, Hydrogen from Carbon-Containing Materials. Internet document, accessed 16.11.2007, available at: http://www1.eere.energy.gov/hydrogenandfuelcells/pdfs/ hydrogen_biomass.pdf. 82p.

Min B, Kim JR, Oh SE, Regan JM, Logan BE. 2005. Electricity generation from swine wastewater using microbial fuel cells. Water Research 39:4961-4968.

Min B, Logan BE. 2004 Continuous electricity generation from domestic wastewater and organic substrate in a flat plate microbial fuel cell. Environmental Science and Technology. 38:5809-5814.

Mishra J, Khurana S, Kumar N, Ghosh AK, Das D. 2004. Molecular cloning, characterization, and overexpression [Fe]-hydrogenase isolated from a high rate of hydrogen producing *Enterobacter cloacae* IIT-BT 08. Biochemical and Biophysical Research Communications 324:679–685.

Mistry FR, Cooney CL. 1989. Production of ethanol by *Clostridium thermosaccharolyticum*: I. Effect of cell recycle and environmental parameters. Biotechnology Bioengineering 34:1295–1304.

Miyake J, Mao X-Y, Kawamura S. 1984. Photoproduction of hydrogen from glucose by a co-culture of a photosynthetic bacterium and *Clostridium butyricum*. Journal of Fermentation Technology 62:531-535.

Mizuno O, Dinsdale R, Hawkes FR, Hawkes DL, Noike T. 2000a. Enhancement of hydrogen production from glucose by nitrogen gas sparging. Bioresource Technology 73:59-65.

Mizuno O, Ohara T, Shinya M, Noike T. 2000b. Characteristics of hydrogen production from bean curd manufacturing waste by anaerobic microflora. Water Science and Technology 42(3-4):345-350.

Moniruzzaman M, Dien BS, Skory CD, Chen ZD, Hespell BB, Ho NWY, Dale BE, Bothast RJ. 1997. Fermentation of corn fibre sugars by an engineered xylose utilizing *Saccharomyces* yeast strain. World Journal of Microbiology and Biotechnology 13:341–346

Morimoto K, Kimura T, Sakka K, Ohmiya K. 2005. Overexpression of a hydrogenase gene in *Clostridium paraputrificum* to enhance hydrogen gas production. FEMS Microbiology Letters 246:229–234.

Morimoto M, Atsuko M, Atif AAY, Bgan MA, Fakhru'l-Razi A, Iyuke SE, Bakir AM. 2004. Biological production of hydrogen from glucose by natural anaerobic microflora. International Journal of Hydrogen Energy 29:709-713.

Mu Y, Yu HQ. 2006. Biological hydrogen production in a UASB reactor with granules. I: Physicochemical characteristics of hydrogen-producing granules. Biotechnology and Bioengineering 94:980:987.

Mu Y, Zheng X-J, Yu H-Q, Zhu R-F. 2006. Biological hydrogen production by anaerobic sludge at various temperatures. International Journal of Hydrogen Energy 31:780–785.

Mu Y, Yu H-Q, Wang G. 2007. Evaluation of three methods for enriching H_2-producing cultures from anaerobic sludge. Enzyme and Microbial Technology 40:947–953.

Nakamura M, Kanube H, Matsumoto J. 1993. Fundamental studies on hydrogen production in the acid-forming phase and its bacteria in anaerobic treatment processes – the effects of solids retention time. Water Science and Technology 28(7):81-88.

Nakashimada Y, Rachman MA, Kakizono T, Nishio N. 2002. Hydrogen production of *Enterobacter aerogenes* altered by extracellular and intracellular redox states. International Journal of Hydrogen Energy 27:1399-1405.

Nandi R, Sengupta S. 1998. Microbial production of hydrogen: an overview. Critical Reviews in Microbiology 24:61-84.

Nath K, Das D. 2003. Hydrogen from biomass. Current Science 85:265-271.

Nath K, Das D. 2004. Improvement of fermentative hydrogen production: various approaches. Applied Microbiology and Biotechnology 65:520-529.

National Hydrogen Association. 2007a. Key hydrogen messages. Internet document, accessed 18.12.2007, available at:www.hydrogenassociation.org.

National Hydrogen Association. 2007b. History of hydrogen. Internet document, accessed 18.12.2007, available at: http://www.hydrogenassociation.org/general/factSheet_history.pdf.

National Hydrogen Association. 2007c. Hydrogen production from nuclear energy. Internet document, accessed 18.12.2007, available at: http://www.hydrogenassociation.org/general/factSheet_productionNuclear.pdf

National Hydrogen Association. 2007d. Renewable hydrogen production using electrolysis. Internet document, accessed 18.12.2007, available at: http://www.hydrogenassociation.org/general/factSheet_productionRenewables.pdf

Ng TK, Ben-Bassat A, Zeikus JG. 1981. Ethanol production by thermophilic bacteria: fermentation of cellulosic substrates by cocultures of *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*. Applied and Environmental Microbiology 41:1337-1343.

Nguyen TAD, Kim J-P, Kim M-S, Oh Y-K, Sim SJ. 2008. Optimization of hydrogen production by hyperthermophilic eubacteria, *Thermotoga maritima* and *Thermotoga neapolitana* in batch fermentation. International Journal of Hydrogen Energy 33:1483-1488.

Ni M, Leung DYC, Leung MKH, Sumathy K. 2006a. An overview of hydrogen production from biomass. Fuel Processing Technology 87:461-472.

Ni M, Leung DYC, Leung MKH. 2007. A review on reforming bio-ethanol for hydrogen production. International Journal of Hydrogen Energy 32 3238-3247.

Ni M, Leung KHC, SumathyK, Leung DYC. 2006b. Potential of renewable hydrogen production for energy supply in Hong Kong. International Journal of Hydrogen Energy 31: 1401-1412.

van Niel EW, Claassen PAM, Stams AJM. 2003. Substrate and product inhibition of hydrogen production by the extreme thermophile, *Caldicellulosiruptor saccharolyticus*. Biotechnology and Bioengineering 81:255-262.

van Niel EWJ, Budde MAW, Haas GG, van der Wal FJ, Claassen PAM, Stams AJM. 2002. Distinctive properties of high hydrogen producing extreme thermophiles, *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii*. International Journal of Hydrogen Energy 27:1391-1398.

van Niel EWJ, Claassen PAM, Stams AJM. 2003. Substrate and product inhibition of hydrogen production by the extreme thermophile, *Caldicellulosiruptor saccharolyticus*. Biotechnology and Bioengineering 81:255-262.

Nielsen AT, Amandusson H, Bjorklund R, Dannetun H, Ejlertsson J, Ekedahl L-G, Lundstrom I., Svensson BH. 2001. Hydrogen production from organic waste. International Journal of Hydrogen Energy 26:547-550.

Nigam JN, Ireland RS, Margaritis A, Lachance MA. 1985. Isolation and screening of yeasts that ferment D-xylose directly to ethanol. Applied and Environmental Microbiology 50:1486-1489.

Nikhil, Visa A, Yli-Harja O, Lin C-Y, Puhakka JA. 2008. Application of the Clustering Hybrid Regression approach to model xylose-based fermentative hydrogen production. Energy & Fuels 22:128–133.

Niven RK. 2005. Ethanol in gasoline: environmental impacts and sustainability review article. Renewable and Sustainable Energy Reviews 9:535–555.

Noike T, Mizuno O. 2000. Hydrogen fermentation of organic municipal wastes. Water Science and Technology 42(12):155-162.

Noike T, Takabatake H, Mizuno O, Ohba M. 2002. Inhibition of hydrogen fermentation of organic wastes by lactic acid bacteria. International Journal of Hydrogen Energy 27:1367-1371.

Ntaikou I, Gavala HN, Kornaros M, Lyberatos G. 2008.Hydrogen production from sugars and sweet sorghum biomass using *Ruminococcus albus*. International Journal of Hydrogen Energy 33:1153-1163.

Odom JM, Wall JD. 1983. Photoproduction of H_2 from cellulose by an anaerobic bacterial coculture. Applied and Environmental Microbiology 45:1300-1305.

Ogino H, Miura T, Ishimi Km Seki M, Yoshida H. 2005. Hydrogen production from glucose by anaerobes. Biotechnology Progress 21:1786-1788.

Oh S-E, van Ginkel S, Logan BE. 2003. The relative effectiveness of pH control and heat treatment for enhancing biohydrogen gas production. Environmental Science and Technology 37:5186-5190.

Oh S-E, Iyer P, Bruns MA, Logan BE. 2004a. Biological hydrogen production using a membrane bioreactor. Biotechnology Bioengineering 87:119-127.

Oh S-E, Logan BE. 2005. Hydrogen and electricity production from a food processing wastewater using fermentation and microbial fuel cell technologies. Water Research 39:4673–4682.

Oh Y-K, Kim H-J, Park S, Kim M-S, Ryu DDY. 2008a. Metabolic-flux analysis of hydrogen production pathway in *Citrobacter amalonaticus* Y19. International Journal of Hydrogen Energy 33:1471-1482.

Oh Y-K, Kim SH, Kim M-S, Park S. 2004b. Thermophilic biohydrogen production from glucose with trickling biofilter. Biotechnology and Bioengineering 88:690-698.

Oh Y-K, Park S, Seol E-H, Kim SH, Kim M-S, Hwang J-W, Ryu DDY. 2008b. Carbon and energy balances of glucose fermentation with hydrogen-producing bacterium *Citrobacter amalonaticus* Y19. Journal of Microbiology and Biotechnology 18:532-538

Oh Y-K, Seol E-H, Kim JR, Park S. 2004c. Photoproduction of hydrogen from acetate by a chemolithotrophic bacterium *Rhodopseudomonas Palustris* P4. International Journal of Hydrogen Energy 29:1115-1121.

Ohta K, Alterthum F, Ingram LO. 1990. Effects of environmental conditions on xylose fermentation by recombinant *Escherichia coli*. Applied and Environmental Microbiology 56:463-465.

Ohta K, Beall DS, Mejia JP, Shanmugam KT, Ingram LO. 1991a. Genetic improvement of *Escherichia coli* for ethanol production: Chromosomal integration of *Zymomonas mobilis* genes encoding pyruvate decarboxylase and alcohol dehydrogenase II. Applied and Environmental Microbiology 57:893-900

Ohta K, Beall DS, Mejia JP, Shanmugam KT, Ingram LO. 1991b. Metabolic engineering of *Klebsiella oxytoca* M5A1 for ethanol production from xylose and glucose. Applied and Environmental Microbiology 57:2810-2815.

Okamoto M, Miyahara T, Mizuno O, Noike T. 2000. Biological hydrogen production of materials characteristic of the organic fraction of municipal solid wastes. Water Science and Technology 41(3):25-32.

Olsson L, Hahn-Hägerdal B. 1996. Fermentation of lignocellulosic hydrolysates for ethanol production. Enzyme and Microbial Technology 18:312-331.

van Ooteghem SA, Beer SK, Yue PC. 2001. hydrogen production by the thermophilic bacterium *Thermotoga neapolitana*. Proceedins of the DOE Hydrogen Program Review, NREL/CP-570-30535. 11p. Internet document, accessed 13.11.2007, available at: http://www.eere.energy.gov/hydrogenandfuelcells/pdfs/30535h.pdf.

van Ootgehem SA, Jones A, van der Lelie D, Dong B, Mahajan D. 2004. H_2 production and carbon utilization by *Thermotoga neapolitana* under anaerobic and microaerobic growth conditions. Biotechnology Letters 26:1223-1232.

Ordal EJ, Halvorson HO. 1939. A comparison of hydrogen production from sugars and formic acid by normal and variant strains of *Escherichia coli*. Journal of Bacteriology 138:199-220.

Orlygsson J, Baldursson SRB. 2007. Phylogenetic and physiological studies of four hydrogen producing thermoanaerobes from Icelandic geothermal areas. Icelandic Agricultural Sciences 20:93-105.

O-Thong S, Prasertsan P, Intrasungkha N, Dhamwichukorn S, Birkeland N-K. 2007. Improvement of biohydrogen production and treatment efficiency on palm oil mill effluent with nutrient supplementation at thermophilic condition using an anaerobic sequencing batch reactor. Enzyme and Microbial Technology 41:583-590.

O-Thong S, Prasertsan P, Karakashev D, Angelidaki I. 2008. Thermophilic fermentative hydrogen production by the newly isolated *Thermoanaerobacterium thermosaccharolyticum* PSU-2. International Journal of Hydrogen Energy 33:1204-1214.

Page RDM. 1996. TreeView: An application to display Phylogenetic trees on personal computers. Computer Applications in Biosciences 12:357-358.

Palazzi E, Fabiano B, Perego P. 2000. Process development of continuous hydrogen production by *Enterobacter aerogenes* in a packed column reactor. Bioprocess Engineering 22:205-213.

Palo DR, Daigle RA, Holladay JD. 2007. Methanol steam reforming for hydrogen production. Chemical Reviews 107:3992-4021.

Pandey A, Soccol CR, Mitchell DM. 2000. New developments in solid state fermentation: I-bioprocesses and products. Process Biochemistry 35:1153–1169.

Pandey A. 2003. Solid-state fermentation. Biochemical Engineering Journal 13:81–84.

Park W, Hyun S, Oh S-E, Logan BE, Kim IS. 2005. Removal of headspace CO_2 increases biological hydrogen production. Environmental Science and Technology 39:4416-4420.

Patel GB. 1984. Ethanol production during D-xylose, L-arabinose, and D-ribose fermentation by *Baeteroides polypragmatus*. Applied Mierobiology and Biotechnology 20:111-117.

Patel GB, MacKenzie CR, Agnew BJ. Fermentation of xylose and hemicellulose hydrolysates by an ethanol-adapted culture of *Bacteroides polypragmatus*. Archives of Microbiology 146:68-73.

Pavlostathis SG, Giraldo-Gomez E. 1991. Kinetics of anaerobic treatment: A critical review. In Critical Reviews in Environmental Control 21:411-490.

Peguin S, Soucaille P. 1995. Modulation of carbon and electron flow in *Clostridium acetobutylicum* by iron limitation and methyl viologen addition. Applied and Environmental Microbiology 66:403-405.

Phillips S, Aden A, Jechura J, Dayton D, Eggema T. 2007. Thermochemical ethanol via indirect gasification and mixed alcohol synthesis of lignocellulosic biomass. National Renewable Energy Laboratory of U.S. Department of Energy. Technical Report NREL/TP-510-41168. Internet document, accessed 15.3.2008, available at: http://www.nrel.gov/docs/fy07osti/41168.pdf.

Pinkerton FE, Meisner GP, Meyer MS, Balogh MP, Kundrat MD. 2005.Hydrogen desorption exceeding ten weight percent from the new quaternary hydride $Li_3BN_2H_8$. Journal of Physical Chemistry B 109:6-8.

Plombin C. 2003. Biogas as vehicle fuel. A European overview. Trendsetter Report No 2003:3. Internet document, accessed 1.3.2008, available at: http://213.131.156.10/xpo/bilagor/20040115134708.pdf.

Podesta JJ, Estrella CN, Esteso MA. 1996. Evaluation of the absorption on mild steel of hydrogen evolved in glucose fermentation by pure cultures of *Clostridium acetobutylicum* and *Enterobacter*. Sensors and Actuators B 32:27-31.

Qureshi N, Annous BA, Ezeji TC, Karcher P,Maddox IS. 2005.Biofilm reactors for industrial bioconversion processes: employing potential of enhanced reaction rates. Microbial Cell Factories 2005: 4-24.

Rabaey K, Lissens G, Siciliano SD, Verstraete, W. 2003. A microbial fuel cell capable of converting glucose to electricity at high rate and efficiency. Biotechnology Letters 25:1531-1535.

Rabaey K, Ossieur W, Verhaege M, Verstraete W. 2005. Continuous microbial fuel cells convert carbohydrates to electricity. Water Science and Technology 52(1-2):515-23.

Rabaey K, Verstraete W. 2005. Microbial fuel cells: novel biotechnology for energy generation. Trends in Biotechnology 23:291-298.

Rabaey K, Clauwaert P, Aelterman P, Verstraete W. 2007. Tubular microbial fuel cells for efficient electricity generation. Environmental Science and Technology 39: 8077-8082.

Rachman MA, Furutani Y, Nakashimada Y, Kakizono T, Nishio N. 1997. Enhanced hydrogen production in altered mixed acid fermentation of glucose by *Enterobacter aerogenes*. Journal of Fermentation and Bioengineering 83: 358-363.

Rachman MA, Nakashimada Y, Kakizono T, Nishio N. 1998. Hydrogen production with high yield and high evolution rate by self-flocculated cells of *Enterobacter aerogenes* in a packed-bed reactor. Applied Microbiology and Biotechnology 49:450-454.

Rainey FA, Donnison AM, Janssen PH, Saul D, Rodrigo A, Bergquist PL, Daniel RM, Stackebrandt E, Morgan HW. 1994. Description of *Caldicellulosiruptor saccharolyticus* gen. nov., sp. nov.: an obligately anaerobic, extremely thermophilic, cellulolytic bacterium. FEMS Microbiology Letters 120:263-266.

Rajeshwari KV, Balakrishnan M, Kansal A, Kusum Lata, Kishore VVN. 2000. State-of-the-art of anaerobic digestion technology for industrial wastewater treatment. Renewable and Sustainable Energy Reviews 4:135-156.

Ramachandran R, Menon RK. 1998. An overview of industrial uses of hydrogen. International Journal of Hydrogen Energy 23:593-598.

Ren NQ, Wang B, Huang J-C. 1997. Ethanol-type fermentation from carbohydrate in high rate acidogenic reactor. Biotechnology and Bioengineering 54:428–433.

Ren NQ, Chua H, Chan SY, Tsang YF, Wang YJ, Sin N. 2007. Assessing optimal fermentation type for bio-hydrogen in continuous-flow acidogenic reactors. Bioresource Technology 98:1774–1780.

Ren NQ, Gong ML. 2006. Acclimation strategy of a biohydrogen producing population in a continuous-flow reactor with carbohydrate fermentation. Engineering Life Science 6:403-409.

Ren NQ, Li J, Li B, Wang Y, Liu S. 2006. Biohydrogen production from molasses by anaerobic fermentation with a pilot-scale bioreactor system. International Journal of Hydrogen Energy 31:2147–2157.

Riis T, Hagen EF, Vie PJS, Ulleberg Ø. 2006a. Hydrogen production R&D: Priorities and gaps. In: Hydrogen production and storage: R&D priorities and gaps. International Energy Agency (IEA), Hydrogen Implementing Agreement, IEA, Paris, France. 38 p. Internet document, accessed 18.12.2007, available at: http://www.iea.org/Textbase/papers/2006/hydrogen.pdf.

Riis T, Sandrock G, Ulleberg Ø, Vie PJS. 2006b. Hydrogen storage R&D: Priorities and gaps. In: Hydrogen production and storage: R&D priorities and gaps. International Energy Agency (IEA), Hydrogen Implementing Agreement, IEA, Paris, France. 38 p. Internet document, accessed 18.12.2007, available at: http://www.iea.org/Textbase/papers/2006/hydrogen.pdf.

Rind D. 2002. The Sun's role in climate variations. Science 296:673-677.

Rittmann BE. 1982. Comparative performance of biofilm reactor types. Biotechnology and Bioengineering 24:1341-1370.

Rittmann BE. 2006. Microbial ecology to manage processes in environmental biotechnology. TRENDS in Biotechnology 24:261-266.

Roehr M. 2001. Introduction. In: The biotechnology of ethanol – classical and future applications. Ed: Roehr M. Wiley-WCH, Weinheim, Germany. 1-3 p.

Rogner H-H, Zhou D, Bradley R, Crabbé P, Edenhofer O, Hare B, Kuijpers L, Yamaguchi M. 2007: Introduction. In Climate Change 2007: Mitigation. Contribution of Working Group III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Edited by: Metz B, Davidson OR, Bosch PR, Dave R, Meyer LA. Internet document, accessed 1.3.2008, available at: http://www.ipcc.ch/pdf/assessment-report/ar4/wg3/ar4-wg3-chapter1.pdf.

Rosillo-Calle F, Cortez LAB. 1998. Towards proalcool II: A revire of the Brazilian Bioethanol programme. Biomass and Bioenergy 14:115-124.

van Ruijven B, van Vuuren DP, de Vries B. 2007. The potential role of hydrogen in energy systems with and without climate policy. International Journal of Hydrogen Energy 32:1655-1672.

Saint-Amans S, Girbal L, Andrade J, Ahrens K, Soucaille P. 2001. Regulation of carbon and electron flow in *Clostridium butyricum* VPI 3266 grown on glucose-glycerol mixtures. Journal of Bacteriology 183:1748–1754.

Sakai S, Nakashimada Y, Yoshimoto H, Watanabe S, Okada H, Nishio N. 2004. Ethanol production from H_2 and CO_2 by a newly isolated thermophilic bacterium, *Moorella* sp. HUC22-1. Biotechnology Letters 26:1607–1612.

Sakintuna B, Lamari-Darkrim F, Hirscher M. 2007. Metal hydride materials for solid hydrogen storage:A review. International Journal of Hydrogen Energy 32:1121-1140.

Santoro N, Konisky J. 1987. Characterization of bromoethanesulfonate resistant mutants of *Methanococcus voltae*: evidence of a coenzyme M. transport system. Journal of Bacteriology 169:660-665.

Schäfer T, Schönheit P. 1992. Maltose fermentation to acetate, CO_2 and H_2 in the anaerobic hyperthermophilic archaeon *Pyrococcus furiosus*: evidence for the operation of a novel sugar fermentation pathway. Archives of Microbiology 158:188-202.

Schlapbach L, Züttel A. 2001. Hydrogen – storage materials for mobile applications. Nature 414:353-358.

Schmidt JE, Ahring BK. 1996. Granular sludge formation in upflow anaerobic sludge blanket (UASB) reactors. Biotechnology and Bioengineering 49:229-246.

Schönheit P, Schäfer T. 1995. Metabolism of hyperthermophiles. World Journal of Microbiology & Biotechnology 11:26-57.

Schröder C, Selig M, Schönheit P. 1994. Glucose fermentation to acetate, CO_2 and H_2 in the anaerobic hyperthermophilic eubacterium *Thermotoga maritima*: involvement of the Embden–Meyerhof pathway. Archives of Microbiology 161:460–470.

Segers L, Verstraete W. 1983. Conversion of organic acids to H_2 by *Rhodospirillaceae* grown with glutamate or dinitrogen as nitrogen source. Biotechnology and Bioengineering 25:2843-2853.

Selig M, Xavier KB, Santos H, Schönheit P. 1997. Comparative analysis of Embden–Meyerhof and Entner–Doudoroff glycolytic pathways in hyperthermophilic archaea and the bacterium *Thermotoga*. Archives of Microbiology 167:217–232.

Senn T, Pieper HJ. 2001. Classical methods. In: The biotechnology of ethanol – classical and future applications. Ed: Roehr M. Wiley-WCH, Weinheim, Germany. 7-86 p.

Sequeira CAC, Brito PSD, Mota AF, Carvalho JL, Rodrigues LFFTTG, Santos DMF; Barrio DB, Justo DM. 2007. Fermentation, gasification and pyrolysis of carbonaceous residues towards usage in fuel cells. Energy Conversion and Management 48:2203–2220.

Sherif SA, Zeytinoglu N, Veziroglu TN. 1997. Liquid hydrogen: Potential, problems, and a proposed research program. International Journal of Hydrogen Energy 22: 683-688.

Shieh WK, Keenan JD. 1986. Fluidized bed biofilm reactors for wastewater treatment. Advances in Biochemical Engineering/Biotechnology 33:132-169.

Shin H-K, Kim S-H, Paik B-C. 2003. Characteristics of hydrogen production from food waste and waste activated sludge. Journal of Water and Environment Technology 1:177-187.

Shin H-S, Youn J-H, Kim S-H. 2004. Hydrogen production from food waste in anaerobic mesophilic and thermophilic acidogenesis. International Journal of Hydrogen Energy 29:1355-1363.

Shin HS, Youn JH. 2005. Conversion of food waste into hydrogen by thermophilic acidogenesis. Biodegradation 16:33-44.

Shin H-S, Kim S-H, Han S-K, Kim H-W, Oh S-E. 2005. Current technical development in continuous H_2 and CH_4 production from organic waste. Proceedings of The 2005 Biohydrogen Technology Symposium and the 3rd International Workshop on Innovative Anaerobic Technology, 9 -11th December 2005, Taichung, Taiwan, 28-36 p.

Shin J-H, Yoon J-H, Ahn EK, Kim M-S, Sim SJ, Park TH. 2007. Fermentative hydrogen production by the newly isolated *Enterobacter asburiae* SNU-1. International Journal of Hydrogen Energy 3:192-199.

Sjolander NO. 1937. Studies on anaerobic bacteria XII. The fermentation products of *Clostridium thermosaccharolyticum*. Journal of Bacteriology 34:419-428.

Slininger PJ, Bothast RJ, Okos MR, Ladisch MR. 1985. Comparative evaluation of ethanol production by xylose fermenting yeasts presented high xylose concentrations. Biotechnology. Letters. 7:431-436.

Smith MR. 1983. Reversal of 2-bromoethanesulfonate inhibition of methanogenesis in *Methanosarcina* sp. Journal of Bacteriology 156:516-523.

Solanki SK, Usoskin IG, Kromer B, Schüssler M, Beer J. 2004. Unusual activity of the Sun during recent decades compared to the previous 11,000 years. Nature 1084-1087.

Solomon BO, Zheng A-P, Biebl H, Schlieker H, Posten C, Deckwer W-D. 1995. Comparison of the energetic efficiencies of hydrogen and oxychemicals formation in *Klebsiella pneumoniae* and *Clostridium butyricum* during anerobic growth on glycerol. Journal of Biochemistry 39:107-117.

Sommer P, Georgieva T, Ahring BK. 2004. Potential for using thermophilic anerobic bacteria for bioethanol production from hemicellulose. Biochemical Society Transactions 32:283-289.

Sonne-Hansen J, Mathrani IM, Ahring BK. 1993. Xylanolytic anaerobic thermophiles from Icelandic hot-springs. Applied Microbiology and Biotechnology 38:537-541.

Soutschek, E., Winter, J., Schindler, F., Kandler, O. 1994. *Acetomicrobium flavidum*, gen. nov., sp. nov., a thermophilic, anaerobic bacterium from sewage sludge, forming acetate, CO_2 and H_2 from glucose. Systematic and Applied Microbiology 5:377-390.

Sparling R, Daniels L. 1987. The specificity of growth inhibition of methanogenic bacteria by bromoethanesulfonate. Canadian Journal of Microbiology 33:1132-1136.

Sparling R, Risbey D, Poggi-Varaldo HM. 1997. Hydrogen production from inhibited anaerobic composters. International Journal of Hydrogen Energy 22:563-566.

Speece RE. 1983. Anaeribic biotechnology for industrial waste water treatment. Environmental Science and Technology 17:416A-427A.

Sprenger GA. 1996. Carbohydrate metabolism in *Zymomonas mobilis*: a catabolic highway with some scenic routes. FEMS Microbiology Letters 145:301-307.

Stams AJM. 1994. Metabolic interactions between anaerobic bacteria in methanogenic environments. Antonie van Leeuwenhoek 66:271-294.

Steinkraus KH. 2004. Industrialisation of indigenous fermented foods. 2nd edition. CRC press. 600 p.

Sudha Rani K, Seenayya G. 1999. High ethanol tolerance of new isolates of *Clostridium thermocellum* strains SS21 and SS22.World Journal of Microbiology and Biotechnology 15:173-178.

Sudha Rani K, Swamy MV, Seenayya G. 1997. Increased ethanol production by metabolic modulation of cellulose fermentation in *Clostridium thermocellum*. Biotechnology Letters 19: 819–823.

Sudha Rani K, Swamy MV, Sunitha D, Haritha D, Seenayya G. 1996. Improved ethanol tolerance and production in strains of *Clostridium theumocellum*. World Journal of Microbiology Biotechnofogy 12:57-60.

Suihko M-L, Enari T-M. 1981. The production of ethanol from D-glucose and D-xylose by different *Fusarium* strains. Biotechnology Letters 3:723-728.

Sung S, Raskin L, Duangmanee T, Padmasiri S, Simmons JJ. 2002. Hydrogen production by anaerobic microbial communities exposed to repeated heat treatments. Proceedings of the 2002 U.S. DOE Hydrogen Program Review. NREL/CP-610-32405. Internet document, accessed 18.2.2008, available at: http://www1.eere.energy.gov/hydrogenandfuelcells/pdfs/32405a13.pdf

Sutton DD, Starr MP. 1959. Anaerobic dissimilation of glucose by *Erwinia amylovora*. Journal of Bacteriology 78:427-431.

Svensmark H. 1998. Influence of cosmic rays on Earth's climate. Physical Review Letters 81:5027-5030.

Svensmark H, Friis-Christensen E. 2007. Reply to Lockwood and Fröhlich - The persistent role of the Sun in climate forcing. Danish National Space Center Scientific Report 3/2007. Internet document, accessed on 11.3.2008, available at: http://www.spacecenter.dk/publications/scientific-report-series/Scient_No._3.pdf/view.

Swings J, De Ley J. 1977. The biology of *Zymomonas*. Bacteriological Reviews 41:1-46.

Taguchi F, Chang JD, Mizukami N, Saito-Taki T, Hasegawa K, Morimoto M.1993. Isolation of a hydrogen-producing bacterium, *Clostridium beijerinckii* strain AM21B, from termites. Canadian Journal of Microbiology 39:726-730.

Taguchi F, Mizukami N, Saito-Taki T, Hasegawa K. 1995a. Hydrogen production from continuous fermentation of xylose during growth of *Clostridium* sp. strain No. 2. Canadian Journal of Microbiology 41:536-540.

Taguchi F, Mizukami N, Yamada K, Hasegawa K, Saito-Taki T. 1995b. Direct conversion of cellulosic materials to hydrogen by *Clostridium* sp. strain no. 2. Enzyme and Microbial Technology 17:147-150.

Tamagnini P, Axelsson R, Lindberg P, Oxelfelt F, Wünschiers R, Lindblad P. 2002. Hydrogenases and hydrogen metabolism of cyanobacteria. Microbiology and Molecular Biology Reviews 66:1-20.

Tanisho S, Ishiwata Y. 1994. Continuous hydrogen production from molasses by the bacterium *Enterobacter aerogenes.* International Journal of Hydrogen Energy 19:807-812.

Tanisho S, Ishiwata Y. 1995. Continuous hydrogen production from molasses by fermentation using urethane foam as a support of flocks. International Journal of Hydrogen Energy 20:541-545.

Tanisho S, Kuromoto M, Kadokura N. 1998. Effect of CO_2 removal on hydrogen production by fermentation. International Journal of Hydrogen Energy 23:559-563.

Tanner RS, Miller LM, Yang D. 1993. *Clostridium ljungdahlii* sp. nov., an acetogenic species in clostridial ribosomal-RNA homology group-I. International Journal of Systematic Bacteriology 43:232-236.

Tao Y, Chen Y, Wu Y, He Y, Zhou Z. 2007. High hydrogen yield from a two-step process of dark- and photo-fermentation of sucrose. International Journal of Hydrogen Energy 32:200-206.

Taylor CD, Wolfe RS. 1974. Structure and methylation of coenzyme M ($HSCH_2CH_2SO_3$). The Journal of Biological Chemistry 249:4879-4885.

Temudo MF, Kleerebezem R, van Loosdrecht M. 2007. Influence of the pH on (open) mixed culture fermentation of glucose: A chemostat study. Biotechnology and Bioengineering 98:69-79.

Teplyakov VV, Gassanova LG, Sostina EG, Slepova EV, Modigell M, Netrusov AI. 2002. Lab-scale bioreactor integrated with active membrane system for hydrogen production: experience and prospects. International Journal of Hydrogen Energy 27:1149-1155.

Thauer RK, Jungermann K, Decker K. 1977. Energy conservation in chemotrphic anaerobic bacteria. Bacteriological Reviews 41:100-180.

Toivola A, Yarrow D, van den Bosch E, van Dijken JP, Scheffers WA. 1984. Alcoholic fermentation of D-xylose by yeasts. Applied and Environmental Microbiology 47:1221-1223.

Tolan J S, Finn RK. 1987. Fermentation of D-xylose and L-arabinose to ethanol by *Erwinia chrysanthemi*. Applied and Environmental Microbiology 53:2033-2038.

Tolvanen KES, Koskinen PEP, Ylikoski A, Ollikka P, Hemmilä I, Puhakka JA, Karp M. 2008. Quantification of *Clostridium butyricum* with real-time PCR using lanthanide labeled probes and time-resolved fluorometry. International Journal of Hydrogen Energy 33:542–549.

Tromp TK, Shia R-L, Allen M, Eiler JM, Yung YL. 2003. Potential environmental impact of a hydrogen economy on the stratosphere. Science 300:1740-1742.

Tsygankov AA, Fedorov AS, Talipova IV, Laurinavichene TV, Miyake J, Gogotov IN, Rao KK, Hall DO. 1998a. Application of immobilized phototrophic bacteria for simutaneous waste water treatment and hydrogen photoproduction. Applied Biochemistry and Microbiology 34:1-5. (In Russian)

Tsygankov AA, Fedorov AS, Talipova IV, Laurinavichene TV, Miyake J, Gogotov IN. 1998b. Use of immobilized phototrophic microorganisms for waste water treatment and simultaneous production of hydrogen. Applied Biochemistry and Microbiology 34:362-366. (In Russian)

Turcot J, Bisaillon A, Hallenbeck PC. 2008. Hydrogen production by continuous cultures of *Escherchia coli* under different nutrient regimes. International Journal of Hydrogen Energy 33:1465-1470.

Turner JA. 2004. Sustainable hydrogen production. Science 305:972-974.

Ueno Y, Fukui H, Goto M. 2007. Operation of a two-stage fermentation process producing hydrogen and methane from organic waste. Environmental Science and Technology 41:1413-1419.

Ueno Y, Haruta S, Ishii M, Igarashi Y. 2001a. Microbial community in anaerobic hydrogen-producing microflora enriched from sludge compost. Applied Microbiology and Biotechnology 57:555-562.

Ueno Y, Haruta S, Ishii M, Igarashi Y. 2001b. Characterization of a microorganism isolated from the effluent of hydrogen fermentation by microflora. Journal of Bioscience and Bioengineering 92:397-400.

Ueno Y, Kawai T, Sato S, Otsuka S, Morimoto M. 1995. Biological production of hydrogen from cellulose by natural anaerobic microflora. Journal of Fermentation and Bioengineering 79(4):395-397.

Ueno Y, Otsuka S, Morimoto M. 1996. Hydrogen production from industrial wastewater by anaerobic microflora in chemostat culture. Journal of Fermentation and Bioengineering 82:194-197.

Ueno Y, Sasaki D, Fukui H, Haruta S, Ishii M, Igarashi Y. 2006. Changes in bacterial community during fermentative hydrogen and acid production from organic waste by thermophilic anaerobic microflora. Journal of Applied Microbiology 101:331-343.

United Nations. 1997. Kyoto protocol to the United Nations framework convention on climate change. Internet document, accessed 4.3.2008, available at: http://unfccc.int/resource/docs/convkp/kpeng.pdf.

United Nations. 2007. Kyoto protocol status of ratification. Internet document, accessed 4.3.2008, available at: http://unfccc.int/files/kyoto_protocol/background/status_of_ratification/application/pdf/kp_ratification.pdf.

United Nations. 2008. Kyoto protocol. Internet document, accessed 4.3.2008, available at: http://unfccc.int/kyoto_protocol/items/2830.php.

US Government. 2005. Energy policy act of 2005. Public law 109–58—AUG. 8, 2005. Internet document, accessed 1.3.2008, available at: http://www.doi.gov/iepa/EnergyPolicyActof2005.pdf.

US Energy Agency. 2002. National hydrogen energy roadmap. Internet document, accessed 18.12.2007, available at: http://www.hydrogen.energy.gov/roadmaps.html.

US DOE. 2002. National hydrogen energy roadmap. November 2002. United States Department of Energy, Energy Efficiency and Renewable Energy. Internet document, accessed 7.1.2008, available on-line: http://www1.eere.energy.gov/hydrogenandfuelcells/pdfs/national_h2_roadmap.pdf

US DOE. 2006. Handbook for handling, storing, and dispensing E85. U.S. Department of Energy, Energy Efficiency and Renewable Energy. July 2006. 31 p. Internet document, accessed 5.1.2008, available at:http://www.eere.energy.gov/afdc/pdfs/40243.pdf

US DOE. 2007a. Clean cities fact sheet. Flexible fuel vehicles: Powered by a renewable American fuel. March 2007. 2 p. Internet document, accessed 5.1.2008, available at:http://www.eere.energy.gov/afdc/pdfs/41131.pdf.

US DOE. 2007b. Properties of fuels. US Department of Energy: Energy efficiency and renewable energy. Internet document, accessed 19.12.2007, available at: http://www.eere.energy.gov/afdc/altfuel/fuel_properties.html.

US Fuel Cell Council 2004a. Fuel Cells: Residential Applications. Internet document, accessed 8.1.2008, available at: http://www.usfcc.com/about/Fuel_Cells_for_Residential.pdf.

US Fuel Cell Council 2004b. Fuel Cells: Stationary Power - Industrial Applications. Internet document, accessed 8.1.2008, available at: http://www.usfcc.com/about/Fuel_Cells_for_Industrial_Applications.pdf.

US Fuel Cell Council 2004c. Fuel Cells: Transportation Applications. Internet document, accessed 8.1.2008, available at: http://www.usfcc.com/about/Fuel_Cells_in_Transportation.pdf.

Valdez-Vazquez I, Rios-Leal E, Esparza-Garcia F, Cecchi F, Poggi-Varaldo HM. 2005. Semi-continuous solid substrate anaerobic reactors for H_2 production from organic wastes: Mesophilic versus thermophilic regime. International Journal of Hydrogen Energy 30:1383-1391.

Valdez-Vazquez I, Rios-Leal E, Munoz-Paez KM, Carmona-Matinez A, Poggi-Varaldo HM. 2006. Effect of inhibition treatment, type of inocula, and incubation temperature on batch H_2 production from organic solid waste. Biotechnology and Bioengineering 95:343-349.

Vardar-Schara G, Maeda T, Wood TK. 2008. Metabolically engineered bacteria for producing hydrogen via fermentation. Microbial Biotechnology 1:107–125.

Vaughan-Martini A, Martini A. 1995. Facts, myths and legends on the prime industrial microorganism. Journal of Industrial Microbiology 14:514-522.

Venkata Mohan S, Lalit Babu V, Sarma PN. 2007. Anaerobic biohydrogen production from dairy wastewater treatment insequencing batch reactor (AnSBR): Effect of organic loading rate. Enzyme and Microbial Technology 41:506–515.

Venkata Mohan S, Lalit Babu V, Sarma PN. 2008. Effect of various pretreatment methods on anaerobic mixed microflora to enhance biohydrogen production utilizing dairy wastewater as substrate. Bioresource Technology 99:59-67.

Verenium 2008. Jennings demonstration project. Internet document, accessed 29.7.2008, available at: http://www.verenium.com/pdf/Jennings_factsh.pdf.

Verhees C, Kengen SWM, Tuininga JE, Schut GJ, Adams MWW, De Vos WM, van der Oost J. 2003. The unique features of glycolytic pathways in Archaea. Biochemical Journal 375:231–246.

Verne J. 1879. Mysterious island. Internet document, accessed 20.3.2008, available at: http://www.literature-web.net/verne/mysteriousisland.

Vignais PM, Billoud B. 2007. Occurrence, classification, and biological function of hydrogenases: An overview. Chemical Reviews 107:4206-4272.

Vignais PM, Mgnin J-P, Willisin JC. 2006. Increasing biohydrogen production by metabolic engineering. International Journal of Hydrogen Energy 31:1478–1483.

Vijayaraghavan K, Ahmad D, Ibrahim MKB. 2006a. Biohydrogen generation from jackfruit peel using anaerobic contact filter. International Journal of Hydrogen Energy 31:569-579.

Vijayaraghavan K, Ahmad D, Ibrahim MKB, Herman HNB. 2006b. Isolation of hydrogen generating microflora from cowdung for seeding anaerobic digester. International Journal of Hydrogen Energy 31:708– 720.

Vijayaraghavan K, Ahmad D, Soning C. 2007. Bio-hydrogen generation from mixed fruit peel waste using anaerobic contact filter. International Journal of Hydrogen Energy 32:4754-4760.

Vijayaraghavan K, Ahmad D. 2006. Biohydrogen generation from palm oil mill effluent using anaerobic contact filter. International Journal of Hydrogen Energy 31:1284-1291.

de Vrije T, Claassen PAM. 2003. Dark hydrogen fermentations. In: Bio-methane & Bio-hydrogen: Status and perspectives of biological methane and hydrogen production. Ed: Reith JH, Wiffels RH, Barten H. Dutch Biological hydrogen Foundation, The Netherlands. 103-123 p. Available on the Internet: http://www.biohydrogen.nl/publicfiles/16_20804_2_Bio_methane_and_Bio_hydrogen_2003.pdf (Accessed 28.12.2007).

de Vrije T, de Haas GG, Tan GB, Keijsers ERP, Claassen PAM. 2002. Pretreatment of *Miscanthus* for hydrogen production by *Thermotoga elfii*. International Journal of Hydrogen Energy 27:1381-1390.

de Vrije T, Mars AE, Budde MAW, Lai MH, Dijkema C, de Waard P, Claassen PAM. 2007. Glycolytic pathway and hydrogen yield studies of the extreme thermophile *Caldicellulosiruptor saccharolyticus*. Applied Microbiology and Biotechnology 74:1358–1367.

Wang CC, Chang CW, Chu CP, Lee DJ, Chang B-V, Liao CS. 2003a. Producing hydrogen from wastewater sludge by *Clostridium bifermentas*. Journal of Biotechnology 102:83-91.

Wang CC, Chang CW, Chu CP, Lee DJ, Chang B-V, Liao CS, Tay JH. 2003b. Using filtrate of waste biosolids to effectively produce bio-hydrogen by anaerobic fermentation. Water Research 37:2789-2793.

Wang C-H, Lin P-J, Chang J-S. 2006. Fermentative conversion of sucrose and pineapple waste into hydrogen gas in phosphate-buffered culture seeded with municipal sewage sludge. Process Biochemistry 41:1353-1358.

Wang H-W, Chang J-S. 2008. Continuous biohydrogen production from starch with granulated mixed bacterial microflora. Energy&Fuels 22:93-97.

Wang A, Ren N, Shi Y, Lee D-J. 2008. Bioaugmented hydrogen production from microcrystalline cellulose using co-culture—*Clostridium acetobutylicum* X_9 and *Ethanoligenens harbinense* B_{49}. International Journal of Hydrogen Energy DOI: 10.1016/j.ijhydene.2007.10.017.

Weissman JC, Benemann JR. 1977. Hydrogen production by nitrogen-starved cultures of *Anabaena cylindrica*. Applied and Environmental Microbiology 33:123-131.

Wiegel J, Ljungdahl LG, Rawson JR. 1979. Isolation from soil and properties of the extreme thermophile *Clostridium thermohydrosulfuricum*. Journal of Bacteriology 139: 800-810.

Wiegel J. 1980. Formation of ethanol by bacterial. A pledge for the use of extreme thermophilic anaerobic bacteria in industrial ethanol fermentation processes. Experientia 36:1434-1446.

Wiegel J, Ljungdahl LG. 1981. *Thermoanaerobacter ethanolicus* gen. nov., spec. nov., a new, extreme thermophilic, anaerobic bacterium. Archives of Microbiology 128:343-348.

Woodward J, Orr M, Cordray K, Greenbaum E. 2000a. Efficient production of hydrogen from glucose-6-phosphate. Proceedings of the 2000 DOE Hydrogen Program review, May 9-11, 2000, San Ramon, U.S. Internet document, accessed 20.12.2007, available at: http://www1.eere.energy.gov/hydrogenandfuelcells/pdfs/28890w.pdf.

Woodward J, Orr M, Cordray K, Greenbaum E. 2000b. Enzymatic production of biohydrogen. Nature 405:1014-1015.

Woodward J, Orr M. 1998. Enzymatic conversion of sucrose to hydrogen. Biotechnology and Progress 14:897-902.

Wouter Wisselink H, Toirkens MJ, del Rosario Franco Berriel M, Winkler AA, van Dijken JP, Pronk JT, van Maris AJA. 2007. Engineering of *Saccharomyces cerevisiae* for efficient anaerobic alcoholic fermentation of L-arabinose. Applied and Environmental Microbiology 73:4881-4891.

Wu J-H, Lin C-Y. 2004. Biohydrogen production by mesophilic fermentation of food wastewater. Water Science and Technology 49(5-6):223-228.

Wu S-Y, Hung C-H, Lin C-N, Chen H-W, Lee A-S, Chang J-S. 2006a. Fermentative hydrogen production and bacterial community structure in high-rate anaerobic bioreactors containing silicone-immobilized and self-flocculated sludge. Biotechnology and Bioengineering 93:934-946.

Wu S-Y, Hung C-H, Lin C-Y, Lin P-J, Lee K-S, Lin C-N, Chang F-Y, Chang J-S. 2008a. HRT-dependent hydrogen production and bacterial community structure of mixed anaerobic microflora in suspended, granular and immobilized sludge systems using glucose as the carbon substrate. International Journal of Hydrogen Energy 33:1542-1549.

Wu SY, Lee KS, Lin CN, Shiu BR, Huang YS, Lin PJ, Chang JS. 2005a. Effect of cell immobilization approaches on high-rate hydrogen production in CSTRs. Proceedings of The 2005 Biohydrogen Technology Symposium and the 3rd International Workshop on Innovative Anaerobic Technology, 9 -11th December 2005, Taichung, Taiwan, 81-87 p.

Wu S-Y, Lin C-N, Chang J-S, Chang J-S. 2005b. Biohydrogen production with anaerobic sludge immobilized by ethylene-vinyl acetate copolymer. International Journal of Hydrogen Energy 30:1375-1381.

Wu S-Y, Lin C-N, Chang J-S, Lee K-S, Lin P-J. 2002. Microbial hydrogen production with immobilized sewage sludge. Biotechnology Progress 18:921-926.

Wu S-Y, Lin C-N, Chang J-S. 2003. Hydrogen production with immobilized sewage sludge in three-phase fluidized-bed bioreactors. Biotechnology Progress 19:828-832.

Wu S-Y, Lin C-Y, Lee K-S, Hung C-H, Chang J-S, Lin P-J, Chang F-Y. 2008b. Dark fermentative hydrogen production from xylose in different bioreactors using sewage sludge microflora. Energy&Fuels, 22:113–119.

Wu K-J, Chang C-F, Chang J-S. 2007a. Simultaneous production of biohydrogen and bioethanol with fluidized-bed and packed-bed bioreactors containing immobilized anaerobic sludge. Process Biochemistry 42:1165–1171.

Wu K-J, Lo Y-C, Chen S-D, Chang J-S. 2007b. Fermentative production of biofuels with entrapped anaerobic sludge using sequential HRT shifting operation in continuous cultures. Journal of the Chinese Institute of Chemical Engineers 38:205–213.

Wyman CE. 1999. Biomass ethanol: Technical Progress, opportunities, and commercial challenges. Annual Reviews of Energy and Environment 1999. 24:189–226.

Xing D, Ren N, Wang A, Li Q, Feng Y, Ma F. 2008. Continuous hydrogen production of auto-aggregative *Ethanoligenens harbinense* YUAN-3 under non-sterile condition. International Journal of Hydrogen Energy 33:1489-1495.

Yang H, Shao P, Lu T, Shen J, Wang D, Xu Z, Yuan X. 2006. Continuous bio-hydrogen production from citric acid wastewater via facultative anaerobic bacteria. International Journal of Hydrogen Energy 31:1306-1313.

Yang H, Shen J. 2006. Effect of ferrous iron concentration on anaerobic bio-hydrogen production from soluble starch. International Journal of Hydrogen Energy 31:2137–2146.

Yang P, Zhang R, McGarvey JA, Benemann J. 2007. Biohydrogen production from cheese processing wastewater by anaerobic fermentation using mixed microbial communities. International Journal of Hydrogen Energy 32:4761 – 4771.

Yokoyama H, Moriya N, Ohmori H, Waki M. Ogino A, Tanaka Y. 2007a. Community analysis of hydrogen-producing extreme thermophilic anaerobic microflora enriched from cow manure with five substrates. Applied Microbiology and Biotechnology 77:213–222.

Yokoyama H, Waki M, Moriya N, Yasuda T, Tanaka Y, Haga K. 2007b. Effect of fermentation temperature on hydrogen production from cow waste slurry by using anaerobic microflora within the slurry. Applied Microbiology and Biotechnology 74:474–483.

Yokoi H, Maki R, Hirose J, Hayashi S. 2002. Microbial production of hydrogen from starch-manufacturing wastes. Biomass and Bioenergy 22:389-395.

Yokoi H, Mori M, Hirose J, Hayashi S, Takasaki Y. 1998a. H_2 production from starch by a mixed culture of *Clostridium butyricum* and *Rhodobacter* sp. M.19. Biotechnology Letters 20:895-899.

Yokoi H, Tokushige T, Hirose J, Hayashi S, Takasaki Y. 1998b. H_2 production from starch by a mixed culture of *Clostridium butyricum* and *Enterobacter aerogenes*. Biotechnology Letters 20: 143–147.

Yokoi H, Ohkawara T, Hirose J, Hayashi S, Takasaki Y. 1995. Characteristics of H_2 production by aciduric *Enterobacter aerogenes* strain HO-39. Journal of Fermentation and Bioengineering 80:571-574.

Yokoi H, Saitsu A, Uchida H, Hirose J, Hayashi S, Takasaki Y. 2001. Microbial hydrogen production from sweet potato starch residue. Journal of Bioscience and Bioengineering 91:58-63.

Yokoi H, Tokushige T, Hirose J, Hayashi S, Takasaki Y. 1997. H_2 production by immobilized cells of aciduric *Enterobacter aerogenes* Strain HO-39. Journal of Fermentation and Bioengineering 83:481-484.

Yomano LP, York SW, Ingram LO. 1998. Isolation and characterization of ethanol-tolerant mutants of *Escherichia coli* KO11 for fuel ethanol production. Journal of Industrial Microbiology and Biotechnology 20:132–13.8

Yoshida A, Nishimura T, Kawaguchi H, Inui M, Yukawa H. 2005. Enhanced hydrogen production from formic acid by formate hydrogen lyase-overexpressing *Escherichia coli* strains. Applied and Environmental Microbiology 71:6762–6768.

Yoshida A, Nishimura T, Kawaguchi H, Inui M, Yukawa H. 2006. Enhanced hydrogen production from glucose using ldh- and frd-inactivated *Escherichia coli* strains. Applied Microbiology and Biotechnology 73:67–72.

Yoshida A, Nishimura T, Kawaguchi H, Inui M, Yukawa H. 2007. Efficient induction of formate hydrogen lyase of aerobically grown *Escherichia coli* in a three-step biohydrogen production process. Applied Microbiology and Biotechnology 74:754–760.

Yu H, Zhu Z, Hu W, Zhang H. 2002b. Hydrogen production from rice winery wastewater in an upflow anaerobic reactor by using mixed anaerobic cultures. International Journal of Hydrogen Energy 27:1359-1365.

Yu H-Q, Fang HHP, Gu G-W. 2002a. Comparative performance of mesophilic and thermophilic acidogenic upflow reactors. Process Biochemistry 38:447-454.

Yu H-Q, Mu Y. 2006.Biological hydrogen production in a UASB reactor with granules. II: Reactor performance in 3-year operation, Biotechnology and Bioengineering 94:988-995.

Zaldivar J, Ingram LO. 1999. Effect of organic acids on the growth and fermentation of ethanologenic *Escherichia coli* LY01. Biotechnology and Bioengneering 66:203-210.

Zaldivar J, Martinez A, Ingram LO. 1999. Effect of selected aldehydes on the growth and fermentation of ethanologenic *Escherichia coli*. Biotechnology and Bioengneering 65:24-33.

Zaldivar J, Martinez A, Ingram LO. 2000. Effect of alcohol compounds found in hemicellulose hydrolysate on the growth and fermentation of ethanologenic *Escherichia coli*. Biotechnology and Bioengneering 68:524-530.

Zaldivar J, Nielsen J, Olsson L. 2001. Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. Applied and Microbiology and Biotechnology 56:17-34.

Zeikus JG. 1980. Chemical and fuel production by anaerobic bacteria. Annual Reviews Microbiology 34:423-464.

Zhang H, Bruns MA, Logan BE. 2006a. Biological hydrogen production by *Clostridium acetobutylicum* in an unsaturated flow reactor. Water Research 40: 728–734.

Zhang JJ, Li XY, Oh SE, Logan BE. 2004. Physical and hydrodynamic properties of flocs produced during biological hydrogen production. Biotechnology and Bioengineering 88:854–860.

Zhang M, Chou Y, Picataggio S, Finkelstein M. 1998. *Zymomonas mobilis* strain for xylose utilization and arabinose fermentation. U.S. Patent 5,843,760.

Zhang M-L, Fan Y-T, Xing Y, Pan C-M, Zhang G-S, Lay J-J. 2007a. Enhanced biohydrogen production from cornstalk wastes with acidification pretreatment by mixed anaerobic cultures. Biomass and Bioenergy 31:250–254.

Zhang M, Eddy C, Deanda K, Finkelstein M, Picataggio, S.1995. Metabolic engineering of a pentose metabolism pathway in ethanologenic *Zymomonas mobilis*. Science 267:240–243.

Zhang T, Liu H, Fang HHP. 2003. Biohydrogen production from starch in wastewater under thermophilic condition. Journal of Environmental Management 69:149-156.

Zhang Y, Liu G, Shen J. 2005. Hydrogen production in batch culture of mixed bacteria with sucrose under different iron concentration. International Journal of Hydrogen Energy 30: 855-860.

Zhang Y, Shen J. 2006. Effect of temperature and iron concentration on the growth and hydrogen production of mixed bacteria. International Journal of Hydrogen Energy 31:441-446.

Zhang Z-P, Show K-Y, Tay J-H, Liang DT, Lee D-J, Jiang W-J. 2006b. Effect of hydraulic retention time on biohydrogen production and anaerobic microbial community. Process Biochemistry 41: 2118–2123.

Zhang Z-P, Show K-Y, Tay J-H, Liang DT, Lee D-J. 2008a. Biohydrogen production with anaerobic fluidized bed reactors—A comparison of biofilm-based and granule-based systems. International Journal of Hydrogen Energy 33:1559-1564.

Zhang Z-P, Show K-Y, Tay J-H, Liang DT, Lee D-J. 2008b. Enhanced continuous biohydrogen production by immobilized anaerobic microflora. Energy&Fuels 22:87–92.

Zhang Z-P, Tay J-H, Show K-Y, Yan R, Liang DT, Lee D-J, Jiang W-J. 2007b. Biohydrogen production in a granular activated carbon anaerobic fluidized bed reactor. International Journal of Hydrogen Energy 32:185-191.

Zhang Z-P, Tay J-H, Show K-Y, Yan R, Liang DT, Lee D-J, Jiang W-J. 2007c. Rapid formation of hydrogen-producing granules in an anaerobic continuous stirred tank reactor induced by acid incubation. Biotechnology and Bioengineering 96:1040-1050.

Zheng XJ, Yu HQ. 2005. Inhibitory effects of butyrate on biological hydrogen production with mixed anaerobic cultures. Journal of Environmental Management 74:65-70.

Zhou W, Imai T, Ukita M, Sekine M, Higuchi T. 2006.Triggering forces for anaerobic granulation in UASB reactors. Process Biochemistry 41:36–43.

Zhu H, Suzuki T, Tsygankov AA, Asada Y, Miyake J. 1999. Hydrogen production from tofu wastewater by *Rhodobacter sphaeroides* immobilized in agar gels. International Journal of Hydrogen Energy 24:305-310.

Zhu Y, Yang S-T. 2004. Effect of pH on metabolic pathway shift in fermentation of xylose by *Clostridium tyrobutyricum*. Journal of Biotechnology 110:143–157.

Zhu Y, Liu X, Yang S-T. 2005. Construction and characterization of pta gene-deleted mutant of *Clostridium tyrobutyricum* for enhanced butyric acid fermentation. Biotechnology and Bioengineering 90:154-166.

Zinder SH. 1990. Conversion of acetic acid to methane by thermophiles. FEMS Microbiology Reviews 75:125-138.

Zoutberg GR, Willemsberg R, Smit G, Texeira de Mattos MJ, Neijssel OM. 1989. Aggregate-formation by *Clostridium butyricum*. Applied Microbiology and Biotechnology 32:17–21.

Zürrer H, Bachofen R. 1979. Hydrogen production by the photosynthetic bacterium *Rhodospirillum rubrum*. Applied and Environmental Microbiology 37:789-793.