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Biohydrogen, Bioelectricity and Bioalcohols from Cellulosic Materials

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ABSTRACT

The demand for renewable energy is increasing due to increasing energy demand and global warming associated with increasing use of fossil fuels. Renewable energy can be derived from biological production of energy carriers from cellulosic biomass. These biochemical processes include biomass fermentation to hydrogen, methane and alcohols, and bioelectricity production in microbial fuel cells (MFCs). The objective of this study was to investigate the production of different energy carriers (hydrogen, methane, ethanol, butanol, bioelectricity) through biochemical processes. Hydrogen production potential of a hot spring enrichment culture from different sugars was determined, and hydrogen was produced continuously from xylose. Cellulolytic and hydrogenic cultures were enriched on cellulose, cellulosic pulp materials, and on silage at different process conditions. The enrichment cultures were further characterized. The effect of acid pretreatment on hydrogen production from pulp materials was studied and compared to direct pulp fermentation to hydrogen. Electricity and alcohol(s) were simultaneously produced from xylose in MFCs and the exoelectrogenic and alcohologenic enrichment cultures were characterized. In the end, the energy yields obtained from different biochemical processes were determined and compared.

Hydrogen production potential from various hexose and pentose sugars was investigated with a hot spring enrichment culture. Lignocellulosic and cellulosic materials contain hexose and pentose sugars and thus, their efficient utilization for hydrogen production is important. The culture favored pentoses over hexoses for hydrogen fermentation with the highest yield of 0.71 mol H_2/mol xylose. Hydrogen was further produced continuously from xylose in a completely stirred tank reactor at 37°C and 45°C. Highest hydrogen yield and production rate at 45°C were 1.97 mol H_2/mol xylose and 7.3 mmol H_2/L/h, respectively, and were considerably higher than at 37°C. *Clostridium acetobutylicum* and *Citrobacter freundii* were the only bacteria detected at 45°C.

Cellulolytic and hydrogenic cultures were enriched on cellulose from compost and rumen fluid materials at elevated temperatures. Elevated temperatures are associated with increased chemical and enzymatic reaction rates and hydrogen yields. Furthermore, elevated temperatures may inhibit hydrogen consuming bacteria and enhance biomass hydrolysis. The need and effects of heat treatments on hydrogen production potentials were determined. Hydrogen consumers remained absent even in cultures that were not heat-treated, while heat treatment enhanced hydrogen production at certain conditions.

The highest hydrogen and ethanol yields of 0.4 mol H_2/mol hexose (1.9 mol H_2/mol $hexose_{degraded}$) and 0.2 mol EtOH/mol hexose (1.0 mol EtOH/mol $hexose_{degraded}$), respectively, were obtained with rumen fluid culture without heat treatment at 60°C and associated with 21 % cellulose hydrolysis. The rumen fluid enrichment culture contained mainly Clostridial species, from which a cellulolytic hydrogen-producer *Clostridium stercorarium* dominated. With compost enrichment culture, the highest hydrogen yields were obtained after heat treatment at 80°C for 20 min, although hydrogen was also produced without heat treating the culture. At 52°C, 1.4 mol H_2/mol hexose (2.4 mol H_2/mol $hexose_{degraded}$) and 0.4 mol EtOH/mol hexose (0.8 mol EtOH/mol $hexose_{degraded}$) were produced with 57 % cellulose degradation, while hydrogen production was negligible at temperatures above 52°C. Compost enrichment culture consisted of bacteria belonging to genera *Thermoanaerobacterium* and

Clostridium, from which *Clostridium cellulosi* and *C. stercorarium* dominated. With both enrichment cultures, hydrogen yields were controlled by cellulose degradation efficiencies.

Hydrogen and methane were produced from dry and wet pulp materials at different pH values. Compost enrichment culture did not produce methane at pH 9, whilst at pH 6 methane was produced from all tested substrates but dry conifer pulp. These pH values could be successfully used to enrich cellulolytic hydrogen-producing cultures. Fermentation of dry pulps at pH 6 resulted in 160 mL H_2/g TS. The highest hydrogen and methane yields were 560 mL H_2/g TS from wet birch pulp at pH 6 and 4800 mL CH_4/g TS from wet conifer pulp at pH 7, respectively. Inhibition of methanogens with BESA (2-Bromoethanesulfonic acid) resulted in decreased hydrogen yields, which may have resulted from the inhibitory effects of BESA on some Clostridial species. Cellulolytic and hydrogenic cultures enriched on pulp materials belonged mainly to phyla *Bacteroidetes*, *Firmicutes* and *Proteobacteria*.

Direct pulp fermentation to hydrogen was compared to hydrogen fermentation from acid hydrolyzed pulps. Wet and dry pulps were hydrolyzed with concentrated sulfuric acid at 37°C. The optimal times for hydrolysis and the following sugars yields were 33-37 % after 90 min with wet pulps and 70-84 % after 180 min with dry pulps, respectively. Fermentation of dry conifer pulp hydrolysate resulted in 63 mL H_2/g TS. In conclusion, higher hydrogen yields were obtained from direct pulp fermentation to hydrogen (120 mL H_2/g TS). However, hydrogen production from acid hydrolyzed pulp took 10 days, while direct fermentation was completed in 28 days.

Indigenous grass silage bacteria were enriched for hydrogen production at different silage concentrations. Lowest silage concentration of 25 g/L resulted in the highest hydrogen yield of 163 mL H_2/g TS, while increasing silage concentrations up to 200 g/L decreased the hydrogen yields but increased the cumulative hydrogen production. Silage fermentation to hydrogen was associated with bacteria related to *Ruminobacillus xylanolyticum*, *Acetanaerobacterium elongatum* and *Clostridium populeti*.

Compost and anaerobic digester samples were enriched on xylose in MFCs resulting in simultaneous production of electricity and ethanol/butanol. Alcohol production was dependent on xylose concentration. Low xylose concentration of 1.0 g/L resulted in electron recoveries of 13-24 % and 40-65 % as electricity and ethanol, respectively. With higher xylose concentration of 4.0 g/L, electrons were directed mainly to butanol (33 %) and 4 % of the electrons were recovered as electricity. *Ruminobacillus xylanolyticum* was mainly responsible for xylose degradation in MFCs, while denitrifying bacteria, *Comamonas denitrificans* and *Paracoccus pantotrophus*, produced electricity from soluble metabolites.

In this study, hydrogen, methane, alcohols and electricity were produced at laboratory scale in batch systems. The highest overall energy yields of 167 kJ/g TS and 113-130 kJ/g TS were obtained from direct pulp fermentation to both hydrogen and methane and from simultaneous production of electricity and butanol in MFCs, respectively. Cellulose fermentation resulted in the simultaneous production of hydrogen and ethanol with the highest overall energy yield of 4.9 kJ/g TS with compost enrichment culture. The highest energy yield as hydrogen, 5.3-6.0 kJ/g TS, was obtained from wet pulps.

In summary, bacterial cultures producing different energy carrier(s) can be enriched from the same environmental sample by controlling the enrichment conditions. For example, compost sample was enriched for the production of (i) hydrogen and ethanol from cellulose at elevated

temperatures by heat-treating the sample, (ii) hydrogen and/or methane from pulp materials at 37°C by changing the pH values, and (iii) electricity and alcohol(s) at 37°C in MFCs by changing xylose concentrations. It was shown that different operational conditions enrich for different microbial communities that are responsible for changes in fermentation patterns. In this study, cultures carrying out simultaneous cellulose hydrolysis and hydrogen fermentation were enriched from different sources at different operational conditions. These cultures were successfully utilized for cellulose to hydrogen fermentation in batch systems. Based on these results further research should be conducted on continuous hydrogen production from cellulosic materials.

TIIVISTELMÄ

Uusiutuvien energianlähteiden tarve kasvaa, koska energian tarve maailmassa lisääntyy ja koska fossiilisten polttoaineiden käyttö aiheuttaa ilmaston lämpenemistä. Uusiutuvia energianlähteitä voidaan tuottaa biologisesti selluloosapitoisesta biomassasta, muun muassa tuottamalla vetyä, metaania tai alkoholeja fermentaatiolla tai tuottamalla sähköä biologisissa polttokennoissa (MFC). Tässä työssä tutkittiin eri energian kantajien (vety, metaani, etanoli, butanoli, biosähkö) tuottamista biokemiallisten prosessien avulla. Kuumasta lähteestä rikastetun viljelmän vedyntuottopotentiaali erilaisista sokereista määritettiin panospulloissa, jonka jälkeen tutkittiin jatkuvatoimista vedyn tuotto ksyloosista. Selluloosaa hajottavia ja vetyä tuottavia viljelmiä rikastettiin erilaisissa olosuhteissa käyttäen raaka-aineina selluloosaa, paperimassaa sekä säilörehua. Rikastusviljelmien bakteeriyhteisöt karakterisoitiin. Paperimassaa esikäsiteltiin happokäsittelyllä ja vedyntuottopotentiaali tuotetuista hydrolysaateista määritettiin ja sitä verrattiin suoraan vedyn tuottoon paperimassasta. Biologisissa polttokennoissa tuotettiin samanaikaisesti sähköä ja alkoholeja käyttäen ksyloosia raaka-aineena ja saadut rikastusviljelmät karakterisoitiin. Lopuksi erilaisten energiantuottoprosessien energiasaantoja vertailtiin toisiinsa.

Työssä määritettiin kuumasta lähteestä rikastetun viljelmän vedyntuottopotentiaalit erilaisista pentoosi- ja heksoosisokereista. Selluloosapitoinen materiaali sisältää suuret määrät pentoosi- ja heksoosisokereita, joten vedyn tuotto näistä sokereista on tärkeää. Suuremmat vetysaannot saatiin pentooseista kuin heksooseista ja suurin saanto oli 0.71 mol H_2/mol ksyloosi. Vetyä tuotettiin myös jatkuvatoimisesti ksyloosista sekä 37°C:ssa että 45°C:ssa. Huomattavasti suuremmat vetysaannot ja vedyntuottonopeudet, 1.97 mol H_2/mol ksyloosi ja 7.2 mmol H_2/L/h, saatiin 45°C:ssa. *Clostridium acetobutylicum* ja *Citrobacter freundii* olivat vallitsevat bakteerit 45°C:ssa.

Selluloosaa hajottavat ja vetyä tuottavat mikrobiviljelmät rikastettiin komposti- ja lehmän pötsinäytteistä korkeissa lämpötiloissa käyttäen selluloosaa raaka-aineena. Korkeiden lämpötilojen on osoitettu kasvattavan kemiallisten ja entsymaattisten reaktioiden reaktionopeuksia sekä vetysaantoja. Korkeat lämpötilat voivat myös inhiboida vetyä kuluttavia mikro-organismeja sekä edistää biomassa hajoamista. Viljelmien lämpökäsittelyn vaikutus vedyntuottopotentiaaleihin määritettiin. Vetyä kuluttavia mikro-organismeja ei havaittu edes viljelmissä, joita ei lämpökäsitelty. Lisäksi viljelmien lämpökäsittely kasvatti vetysaantoja tietyissä olosuhteissa.

Suurimmat vety- ja etanolisaannot, 0.4 mol H_2/mol heksoosi (1.9 mol H_2/mol $\text{heksoosi}_{\text{hajotettu}}$) ja 0.2 mol EtOH/mol heksoosi (1.0 mol EtOH/mol $\text{heksoosi}_{\text{hajotettu}}$), tuotettiin pötsinäytteellä 60°C:ssa, jolloin 21 % selluloosasta hajotettiin. Rikastusviljelmä sisälsi pääasiassa *Clostridium*-suvun bakteereja, joista selluloosaa hajottava ja vetyä tuottava *Clostridium stercorarium* hallitsi. Kompostinäytteellä suurimmat vetysaannot tuotettiin lämpökäsitellyllä (80°C, 20 min) rikastusviljelmällä, vaikkakin vetyä tuotettiin myös lämpökäsittelemättömällä rikastusviljelmällä. Lämpötilassa 52°C tuotettiin 1.4 mol H_2/mol heksoosi (2.4 mol H_2/mol $\text{heksoosi}_{\text{hajotettu}}$) ja 0.4 mol EtOH/mol heksoosi (0.8 mol EtOH/mol $\text{heksoosi}_{\text{hajotettu}}$), jolloin selluloosasta hajosi 57 %. Rikastusviljelmä ei tuottanut vetyä yli 52°C lämpötiloissa. Rikastusviljelmä koostui *Thermoanaerobacteria* ja *Clostridium*-sukujen lajeista, joista *Clostridium cellulosi* ja *C. stercorarium* hallitsivat. Molemmilla rikastusviljelmillä selluloosan hajotustehokkuudet rajoittivat vedyn tuottoa.

Vetyä ja metaania tuotettiin kuivista ja kosteista paperimassoista eri pH-arvoilla. Kompostista rikastettu viljelmä ei tuottanut metaania pH 9:ssä, eikä pH 6:ssa kuivasta havupuusta tehdystä paperimassasta. Näillä pH-arvoilla rikastettiin viljelmät, jotka tuottivat vain vetyä paperimassoista. Vedyntuottosaanto kuivista paperimassoista oli 160 mL H_2/g TS. Suurin vetysaanto, 560 mL H_2/g TS, tuotettiin kosteasta koivusta valmistetusta paperimassasta, ja suurin metaanisaanto, 4800 mL CH_4/g TS, kosteasta havupuusta tehdystä paperimassasta. Metanogeenejä inhiboitiin bromoetaanisulfonihapolla (BESA), mikä pienensi vetysaantoa. Selluloosaa hajottavat ja vetyä tuottavat rikastusviljelmät sisälsivät bakteereja jaksoista *Bacteroidetes*, *Firmicutes* ja *Proteobacteria*.

Suoraa vedyn tuottoa paperimassoista verrattiin vedyn tuottoon happokäsitellyistä paperimassoista. Kosteita ja kuivia paperimassoja esikäsiteltiin konsentroidulla rikkihapolla 37°C:ssa. Optimiaika esikäsittelylle määritettiin ja saadut sokerisaannot analysoitiin, ja ne olivat 90 min ja 33-37 % kuivilla ja 180 min ja 70-84 % kosteilla paperimassoilla. Vetysaanto happokäsitellystä kuivasta havupuusta valmistetusta paperimassasta oli 63 mL H_2/g TS. Suuremmat vetysaannot saatiin, kun vetyä tuotettiin suoraan paperimassasta (120 mL H_2/g TS). Vedyn tuottoon kuluva aika erosi kuitenkin suuresti. Vedyn tuotto happokäsitellystä paperimassasta vei 10 päivää, kun taas suora vedyn tuotto paperimassasta kesti 28 päivää.

Säilörehun bakteereja rikastettiin vedyn tuottoon erilaisissa säilörehun konsentraatioissa. Suurin vetysaanto, 163 mL H_2/g TS, tuotettiin alhaisimmalla säilörehukonsentraatiolla (25 g/L). Suuremmat säilörehu-konsentraatiot (50-200 g/L) pienensivät vetysaantoja, mutta suurensivat kumulatiivista vedyn tuottoa. Säilörehusta rikastettu vetyä tuottava viljelmä sisälsi muun muassa bakteerit *Ruminobacillus xylanolyticum*, *Acetanaerobacterium elongatum* ja *Clostridium populeti*.

Sähkön ja alkoholien tuottajia rikastettiin biologisissa polttokennoissa sekä komposti- että mädättämönäytteistä käyttäen ksyloosia raaka-aineena. Alkoholien tuotto riippui ksyloosin konsentraatiosta. Elektroneista 13-24 % hyödynnettiin sähkön tuottoon ja 40-65 % etanolin tuottoon matalammalla ksyloosin konsentraatiolla (1.0 g/L). Suuremmalla ksyloosin konsentraatiolla (4.0 g/L) 33 % elektroneista hyödynnettiin butanolin tuottoon ja vain 4 % sähkön tuottoon. *Ruminobacillus xylanolyticum* hajotti pääasiassa ksyloosia MFC:issä, kun taas denitrifikaatiobakteerit, *Comamonas denitrificans* ja *Paracoccus pantotrophus*, tuottivat sähköä ksyloosista saaduista aineenvaihduntatuotteista.

Tässä työssä vetyä, metaania, alkoholeja ja sähköä tuotettiin laboratoriomittakaavassa panoskokeilla. Suurimmat energiasaannot saavutettiin, kun paperimassasta tuotettiin sekä vetyä että metaania (167 kJ/g TS) ja kun biologisissa polttokennoissa tuotettiin samanaikaisesti sähköä ja butanolia (113-130 kJ/g TS). Puhtaasta selluloosasta tuotettiin sekä vetyä että etanolia, joista suurin energiasaanto (4.9 kJ/g TS) tuotettiin kompostirikastusviljelmällä. Suurin energiasaanto vedyn muodossa tuotettiin kosteista paperimassoista (5.3-6.0 kJ/g TS).

Yhteenvetona voidaan todeta, että rikastusviljelmiä, jotka tuottavat eri energiankantajia, voidaan rikastaa samasta näytteestä säätämällä rikastusolosuhteita. Esimerkiksi kompostinäytteestä rikastettiin viljelmiä, jotka tuottivat (1) vetyä ja etanolia puhtaasta selluloosasta korkeissa lämpötiloissa lämpökäsittelyn jälkeen, (2) vetyä ja/tai metaania paperimassoista 37°C:ssa eri pH-arvoilla, ja (3) sähköä ja alkoholeja biologisissa polttokennoissa 37°C:ssa eri ksyloosin konsentraatioilla. Tässä työssä osoitettiin, että erilaiset olosuhteet rikastavat erilaisia mikrobiyhteisöjä, jotka vastaavasti tuottavat erilaisia

energiankantajia. Tässä työssä rikastettiin viljelmiä, jotka panosolosuhteissa tuottivat vetyä selluloosapitoisista materiaaleista. Tämän työn tuloksien perusteella tulisi suunnitella kokeita, joissa vetyä tuotetaan jatkuvatoimisesti selluloosapitoisista materiaaleista.

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This thesis in based on the work carried out mainly at the Department of Chemistry and Bioengineering, Tampere University of Technology, Tampere, Finland, and partially at the Department of Chemical Engineering, Feng Chia University, Taichung, Taiwan.

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LIST OF ORIGINAL PUBLICATIONS

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

I Mäkinen AE, Nissilä ME, Puhakka JA. 2012. Dark fermentative hydrogen production from xylose by a hot spring enrichment culture. International Journal of Hydrogen Energy 37: 12234-12240.

II Nissilä ME, Tähti HP, Rintala JA, Puhakka JA. 2011. Effect of heat treatment on hydrogen production potential and microbial community of thermophilic compost enrichment cultures. Bioresource Technology 102: 4501-4506.

III Nissilä ME, Tähti HP, Rintala JA, Puhakka JA. 2011. Thermophilic hydrogen production from cellulose with rumen fluid enrichment cultures: Effects of different heat treatments. International Journal of Hydrogen Energy 36: 1482-1490.

IV Nissilä ME, Li YC, Wu SY, Lin CY, Puhakka JA. 2012. Hydrogenic and methanogenic fermentation of birch and conifer pulps. Applied Energy 100: 58-65.

V Li YC, Nissilä ME, Wu SY, Lin CY, Puhakka JA. 2012. Silage as source of bacteria and electrons for dark fermentative hydrogen production. International Journal of Hydrogen Energy 37: 15518-15524.

VI Nissilä ME, Sulonen MLK, Puhakka JA. Simultaneous production of electricity and alcohols from xylose in microbial fuel cells. Submitted for publication.

THE AUTHOR'S CONTRIBUTION

Paper I: Marika Nissilä and Annukka Mäkinen planned the experiments, performed the experimental work, interpreted the results and wrote the paper. Annukka Mäkinen is the corresponding author.

Paper II: Marika Nissilä performed the experimental work, wrote the paper, interpreted the results and is the corresponding author. The experiments were planned by Marika Nissilä and Hanne Tähti.

Paper III: Marika Nissilä performed the experimental work, wrote the paper, interpreted the results and is the corresponding author. The experiments were planned by Marika Nissilä and Hanne Tähti.

Paper IV: Marika Nissilä and Ya-Chieh Li planned and performed the experimental work. Marika Nissilä wrote the paper, interpreted the results and is the corresponding author.

Paper V: Marika Nissilä and Ya-Chieh Li planned and performed the experimental work, interpreted the results and wrote the paper. Prof. Chu-Yi Wu is the corresponding author.

Paper VI: Mira Sulonen and Anni Ylä-Outinen performed the experimental work. Marika Nissilä planned the experiments, participated in the experimental work, interpreted the results, wrote the paper and is the corresponding author.

The experimental work was carried out under the supervision of Prof. J.A. Puhakka (Papers I-VI), Prof. J.A. Rintala (Papers II-III) and Prof. Chu-Yi Wu (Papers IV-V).

ABBREVIATIONS

ABE	Acetone-butanol-ethanol fermentation
AFEX	Ammonia fiber expansion
ASBR	Anaerobic sequencing batch reactor
ATP	Adenosine triphosphate
BES	Bioelectrochemical system
BESA	2-Bromoethanesulfonic acid
c	Cohesion domain
CBH	Fusarian head blight contaminated barley hull
CBM	Cellulose binding motif
CBP	Consolidated bioprocessing
CE	Coulombic efficiency
CEM	Cation exchange membrane
COD	Chemical oxygen demand
CoTMPP	Cobalt tetramethoxyphenyl-phorphyrin
CSTR	Completely stirred tank reactor
DGGE	Denaturing gradient gel electrophoresis
DM	Dry matter
DPG	Dried distillers grain
EJ	Exajoule (10^{18} J)
en	endoglucanase
ex	exoglucanase
Fd	Ferredoxin
Fd(oxd)	Oxidized ferredoxin
Fd(red)	Reduced ferredoxin
Fhl	Formate:hydrogen lyase
g	β-glucosidase
GC-FID	Gas chromatograph-flame ionization detector
GC-TCD	Gas chromatograph-thermal conductivity detector
H_2ase	Hydrogenase
HCF	Hexacyanoferrate
HMF	5-hydroxymethylfurfural
HPLC	High performance liquid chromatography
HPR	Hydrogen production rate
HRT	Hydraulic retention time
HSW	Household solid waste
HY	Hydrogen yield
I	Current
MEC	Microbial electrolysis cell
MFC	Microbial fuel cell
MWTP	Municipal wastewater treatment sludge
Nfor	NADH:Fd oxidoreductase
NO_x	Mono-nitrogen oxides (NO, NO_2)
OFMSW	Organic fraction of municipal solid waste
P	Power
PCR	Polymerase chain reaction
PD	Power density
Pfor	Puryvate:ferredoxin oxidoreductase

pH_2	Partial pressure of hydrogen
PJ	Petajoule (10^{15} J)
POME	Palm oil mill effluent
Pt	Platinum
qPCR	Quantitative PCR
R	Resistance
SSF	Simultaneous saccharification and fermentation
TS	Total solids
U	Voltage
UASB	Upflow anaerobic sludge blanket reactor
VFA	Volatile fatty acid
VS	Volatile solids
VSS	Volatile suspended solids

1. INTRODUCTION

In 2008, the total global energy consumption was in the range of 515-530 EJ (exajoules, 10^{18} J) (IEA 2010, U.S. EIA 2011) and was produced from oil, coal, gas, renewable energy, and nuclear energy (Figure 1) (IPPC 2011). Most of the energy is produced from fossil fuels that results in the production of CO_2 emissions that are associated with climate change (IPPC 2011). In addition, fossil fuels are diminishing. For example, it is estimated that oil reserves are consumed by 2050 (Saxena et al. 2009). Furthermore, energy requirements are increasing due to population growth that is estimated to increase to 8.5 billion in 2035 (IEA 2010). The world energy consumption is expected to increase to 700-810 EJ by 2035 (IEA 2010, U.S. EIA 2011). The problems related to fossil fuels can be reduced and the world energy production can be increased by increasing the share of renewable energy. Renewable energy sources (Figure 1) include hydro, wind, solar, geothermal, biomass and marine energy (IEA 2010). These processes may produce directly electricity and/or heat or some of the processes can be harnessed for the production of different energy carriers, such as hydrogen or ethanol.

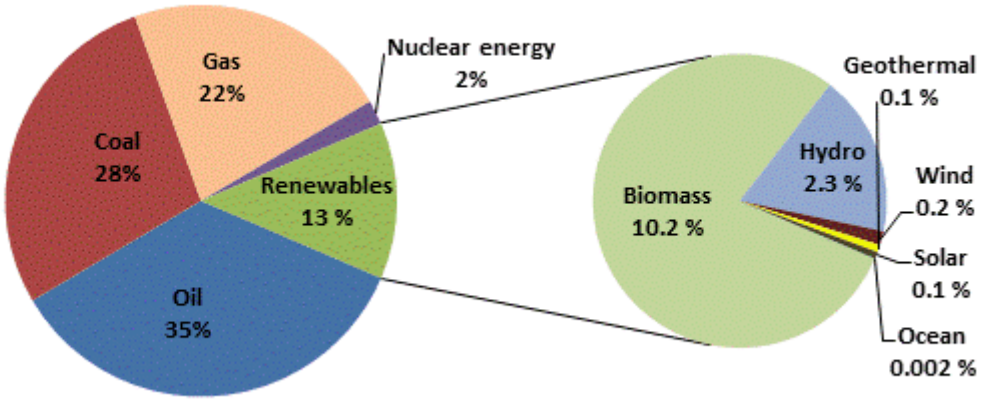


Figure 1. The distribution of energy sources and the shares of renewable energy (adapted from IPPC 2011).

Production of energy carriers from biomass is favored since biomass is available locally, its conversion into biological energy carriers is feasible without high capital investments, and using biomass applications may reduce greenhouse gas emissions and create new jobs (Hoogwijk et al. 2003). Furthermore, although biomass utilization for energy production releases CO_2 it does not increase the greenhouse gases, since biomass binds CO_2 from the atmosphere during growth (Chandra et al. 2012). In 2008, 10 % of the annual global energy (i.e., 50.3 EJ) was produced from biomass (IPPC 2011). Almost 70 % of this energy originated from wood and the rest consisted of agricultural biomass, charcoal, recovered wood, wood industry residues, municipal solid waste and landfill gases, forest residues, and black liquor (IPPC 2011). Biomass can be converted to energy through thermochemical processes, such as combustion (heat/electricity), gasification (syngas), pyrolysis or liquefaction (bio-oils), through physicochemical processes that produce biodiesel by oil extraction, or through biochemical processes, including anaerobic digestion (CH_4) or fermentation to ethanol, butanol or hydrogen (for a review, see Srirangan et al. 2012).

Methane has been produced at full scale through anaerobic digestion for decades. Biologically produced methane can be combusted for heat and/or electricity, or it can be upgraded to vehicle fuels or fed to the gas grid (Antoni et al. 2007, Appeals et al. 2011). In addition, anaerobic digestion results in residual digestate that is nutrient rich and can be used as fertilizer in agriculture (Tambone et al. 2009). Presently, ethanol is fermented from corn (the United Sates) or from sugarcane (Brazil) (Srirangan et al. 2012) but research on lignocellulose fermentation to ethanol is increasing. Ethanol can be used directly as vehicle fuel or it can be blended with gasoline, typically with concentration of 10 % ethanol (Demirbas et al. 2009). Butanol is considered better vehicle fuel than ethanol due to its higher heating value, lower volatility, fewer ignition problems, better viscosity, easier distribution, and better safety (Jin et al. 2011). Butanol is produced at large scale through chemical processes (Srirangan et al. 2012). Biological butanol fermentation with clostridial species has attained increased attention recently (Ezeji et al. 2007a).

Hydrogen is at the moment produced by reforming, pyrolysis, biomass gasification, or electrolysis (for a review, see Holladay et al. 2009). In 2004, 48 % of the hydrogen was produced from natural gas, 30 % from heavy oils and naphtha, 18 % from coal and 4 % from water through electrolysis (Logan 2004). Biological hydrogen production through dark fermentation has been studied extensively in the last decade. Hydrogen is considered a good energy carrier due to its high energy content (122 MJ/kg) (Busby 2005). Hydrogen can be used for electricity production in fuel cells or combusted with air with the production of water and small amounts of NO_x (Dincer 2002, Zhu et al. 2008). In addition to methane, ethanol, butanol and hydrogen fermentation, biochemical processes for energy include electricity production with microbial fuel cells (MFCs), where bacteria oxidize organic substrates at the anode and generate electricity (for a review, see Logan et al. 2006).

This thesis focuses on the production of different energy carriers through biochemical processes, including fermentation of hydrogen, methane, ethanol and butanol, and electricity production in MFCs. The summary part of the thesis presents literature review on (i) pretreatment and hydrolysis of cellulosic materials, (ii) fermentation of cellulose with anaerobic, cellulolytic bacteria, (iii) principles of dark fermentative hydrogen production, (iv) ethanol and butanol fermentation, (v) conversion of organic materials to electricity, and (vi) the options for sequential production of different energy carriers. In the experimental part of this thesis (i) cellulolytic, hydrogenic cultures were enriched from different sources at different operational conditions, (ii) parameters affecting direct hydrogen fermentation from cellulose were studied, (iii) effects of acid pretreatment on hydrogen production potential was determined, (iv) hydrogen production from different sugars was examined, and continuous hydrogen production from xylose was determined, and (v) simultaneous production of electricity and alcohol(s) in MFCs was studied. The main aim of this thesis was to enrich and manage microbial communities for the production of various energy carriers, characterize them, and compare the different energy carrier production processes.

2. CELLULOSIC MATERIALS AND THEIR TREATMENT FOR THE PRODUCTION OF DIFFERENT ENERGY CARRIERS

2.1 Renewable, cellulosic materials

Cellulosic materials are composed of cellulose and hemicellulose. Lignocellulose contains also lignin that binds to cellulose and hemicellulose limiting their hydrolysis (Lee 1997, Kumar et al. 2008). Cellulose molecules are bound together by hemicellulose that consists of pentoses, hexoses and sugar acids (Hendriks and Zeeman 2009). Cellulose is a linear polysaccharide composed of thousands of glucose molecules connected by β-glycosidic bonds (Carere et al. 2008). Cellulose can have either a crystalline or an amorphous structure. In the crystalline structure cellulose molecules are tightly packed together with hydrogen bonds (for reviews, see Schwarz 2001, Levin et al. 2009), while the amorphous structure contains large gaps and irregularities and is hydrolyzed much faster (Kumar et al. 2008, Brodeur et al. 2011).

The annual, worldwide production of lignocellulosic material is about 220 billion tons (dry weight) (Chandra et al. 2012) consisting of agricultural, forestry and food processing residues, energy crops, municipal solid waste, aquatic plants and algae (Appels et al. 2011, Cheng et al. 2011a). In Finland, around 9.5 million tons (dry weight) of renewable materials are produced annually including manure, municipal and industrial wastewaters, sewage and septic tank sludge, energy crops as well as by-products and wastes from plant production (Lehtomäki et al. 2007, Tähti and Rintala 2010). The annual biomass potential in Finland, Europe and in the world is estimated to be 87.8 PJ (petajoules, 10^{15} J) (Tähti and Rintala 2010), 24.6 EJ (de Wit and Faaij 2010), and 104 EJ (Demirbas et al. 2009), respectively. The estimates of annual renewable energy potentials are presented in Table 1. The selection of cellulosic material for the production of different energy carriers depends on material´s cost, availability, carbohydrate content and biodegradability (Kapdan and Kargi 2006). Depending on the composition of the cellulosic and/or lignocellulosic material (reviewed by Hamelinck et al. 2005, Mosier et al. 2005, Chandra et al. 2007, Saratale et al. 2008) it may require pretreatment or hydrolysis before utilization for biological energy production (Figure 2).

Table 1. The estimated annual theoretical energy potential of different renewable materials produced in Finland, Europe or globally (Hoogwijk et al. 2003, Nikolau et al. 2003, Ericsson and Nilsson 2006, Demirbas et al. 2009, Tähti and Rintala 2010).

Material	In Finland (PJ)	In Europe (EJ)	Globally (EJ)
Manure	12.6	nr	9-25[b]
Biomass from agriculture	64.1	16.1-21.1[a]	54.6 / 8-1100[b]
Forestry residues	2.5	1.6-2.2[a]	41.6 / 10-16[b]
Compostable waste	0.9	nr	1-3[b]
Sewage sludge	1.4	0.1	nr
Food industry wastes	0.7	nr	nr

[a] including 15 EU countries (2006), two candidates, Belarus and Ukraine, [b] estimated energy potentials in year 2050, nr: not reported

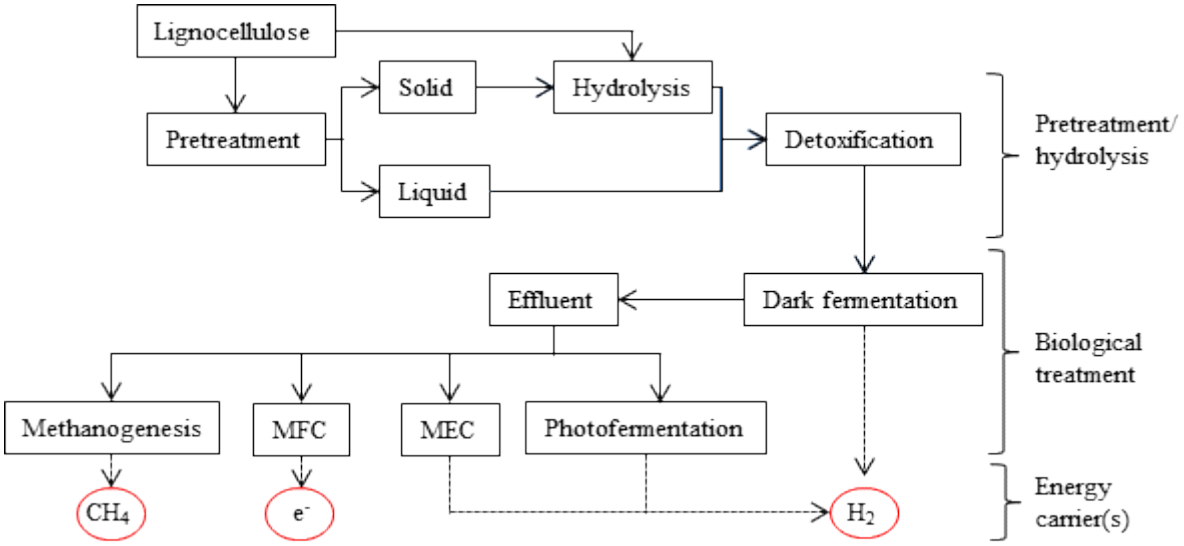


Figure 2. Lignocellulosic and cellulosic substrates may require pretreatment and/or hydrolysis prior to utilization for the production of different energy carriers. In this diagram dark fermentative hydrogen production is considered as the main pathway after which the effluent can be utilized for the production of additional energy carriers. (Modified from Ren et al. 2009)

2.2 Pretreatment methods

Pretreatment, i.e. prehydrolysis, breaks the lignin seal of the lignocellulosic material and modifies the size, structure and chemical composition of the substrate (Mosier et al. 2005). Pretreatment hydrolyzes some of the hemicellulose, decreases cellulose crystallinity and increases cellulose surface area (Ren et al. 2009). Pretreated substrate can be further hydrolyzed to obtain high sugar yields. Selection of pretreatment method depends on the type of raw material, operating conditions and the desired energy carrier (Kumar et al. 2008, Hendriks and Zeeman 2009). Pretreatment is usually done with physical procedures (mechanical comminution), such as milling or grinding (Table 2). An example of mechanical comminution is ball milling, where lignocellulose is degraded with mechanical shear stress and impaction (Lin et al. 2010a). Physical pretreatments are, however, considered too costly for large scale applications (Brodeur et al. 2011).

2.3 Cellulose hydrolysis

Depending on the substrate composition, it can be first pretreated to remove lignin or it can be directly hydrolyzed to sugars. Hydrolysis can be done with physicochemical, chemical or biological methods, such as acid, alkaline, liquid hot water or enzymatic treatments (Table 2). Hydrolysis method (and proceeding pretreatment) should fulfill the following requisites: (i) increase sugar yield, (ii) avoid degradation or loss of sugars, (iii) minimize the formation of inhibitory by-products, (iv) be cost-effective, and (v) recover lignin that can be further converted to co-products (for reviews, see Chandra et al. 2007 and Brodeur et al. 2011). The advantages and disadvantages of different pretreatment and hydrolysis methods are listed in Table 2.

Physicochemical hydrolysis processes include steam explosion, ammonia fiber expansion (AFEX), and liquid hot water. Steam explosion is conducted at high temperature and pressure for a short amount of time followed by rapid release of the applied pressure (Lee et al. 1999,

Brodeur et al. 2011). AFEX resembles steam explosion process. It exposes the substrate to liquid anhydrous ammonia by using high pressures and moderate temperatures followed by rapid pressure release (Kim et al. 2008). In liquid hot water pretreatment, substrate is degraded by using water at high temperature and pressure that maintains the water in a liquid state (Kim et al. 2008).

Acid, alkaline and solvent extraction are examples of chemical hydrolysis methods. Acid pretreatments can be done with diluted or concentrated acids. Diluted acid hydrolysis often occurs with low acid concentrations at increased temperature (Panagiotopoulos et al. 2009, Chang et al. 2011a) or at increased temperature and pressure (Phowan et al. 2010, Lakaniemi et al. 2011). Concentrated acid hydrolysis, on the other hand, proceeds at milder conditions (ambient temperature and normal pressure) with high acid concentrations, usually over 40 % (Chu et al. 2011, Li et al. 2011). Alkaline hydrolysis also proceeds at milder conditions with low alkaline concentrations but longer treatment times (Pakarinen et al. 2009). Solvent extraction is carried out with solvents, such as ionic liquids, at normal pressure and increased temperature. The biomass is separated from ionic liquids by mixing it with water (Samayam and Schall 2010). Biological hydrolysis can be done with cellulolytic enzymes, such as cellulase, alpha-amylase or glucoamylase (Panagiotopoulos et al. 2009, Lakshmidevi and Muthukumar 2010), or by utilizing living microorganisms, such as fungi or bacteria, for the substrate hydrolysis. Microbial hydrolysis is discussed more in the next Chapter 3.1.

Pretreatment and hydrolysis may lead to production of inhibiting compounds, such as furfural, 5-hydroxymethylfurfural (HMF) and carboxylic acids, which inhibit subsequent biological processes. In this regard, detoxification is required and can be done with chemical, physical or biological methods (for a review, see Palmqvist and Hahn-Hägerdal 2000). Inhibiting compounds have been removed, e.g., with charcoal, cation exchange resin, activated carbon, overliming (Lee et al. 1999, Sainio et al. 2011), or with yeast (Zhang et al. 2010a). Detoxification should be low-cost, easily integrated into the process and selectively remove inhibitors (for a review, see Palmqvist and Hahn-Hägerdal 2000).

Table 2. Advantages and disadvantages of different pretreatment and hydrolysis methods.

Pretreatment/ hydrolysis method	Advantages	Disadvantages	Reference(s)
Physical			
Mechanical comminution	+ Increases surface area of cellulose + Decreases cellulose crystallinity and the degree of polymerization + Proceeds at ambient conditions + Does not produce any inhibitors	- High energy requirements - Does not remove lignin	Inoue et al. 2008, Lin et al. 2010a, Yeh et al. 2010, for reviews, see Sun and Cheng 2002, Chandra et al. 2007, Hendriks and Zeeman 2009
Physicochemical			
Steam explosion	+ Removes hemicellulose and transforms lignin + Increases surface area of cellulose + Minimizes sugar degradation + Does not excessively dilute sugars + Low energy input, cost effective + No addition of external catalyst	- Incomplete degradation of lignin-carbohydrate matrix - Partial hemicellulose degradation - May release chemical inhibitors, such as furfural and HMF	Sun and Cheng 2002, Okuda et al. 2008, for reviews, see Negro et al. 2003, Mosier et al. 2005, Agbor et al. 2011, Brodeur et al. 2011
AFEX	+ Increases surface area of cellulose + Low formation of inhibitors + Recovery of solid materials	- Ammonia recycling is required - Increased lignin content reduces process efficiency - Ammonia is expensive	For a review, see Brodeur et al. 2011
Liquid hot water	+ Removes and separates pure hemicellulose + Increases surface area of cellulose + Decreases degree of polymerization and lignin content + Does not produce any inhibitors + No need for washing or neutralization + No addition of external catalyst	- High energy demands - Large water requirements	Hamelinck et al. 2005, Okuda et al. 2008, Kumar et al. 2011, for reviews, see Mosier et al. 2005, Agbor et al. 2011, Brodeur et al. 2011
Chemical			
Acid	+ Hydrolysis lignin and hemicellulose + Increases surface area of cellulose + High sugar yield + Fast and easy to perform + Diluted acid: low acid concentration minimizes corrosion + Concentrated acid: mild process conditions, acid can be recovered with anion exchanger	- Production of inhibitors, such as furfural, HMF and acetate - Requires detoxification and neutralization of acids (concentrated acids should be recovered) - Expensive construction materials needed due to corrosion - Use of high concentrations of acid increases environmental concerns and catalyst costs	Chu et al. 2011, Han et al. 2012, for reviews, see von Sivers and Zacchi 1994, Sun and Cheng 2002, Hamelinck et al. 2005, Mosier et al. 2005, Pattra et al. 2008, Dwivedi et al. 2009, Chang et al. 2011a

Table 2. Continued

Pretreatment/ hydrolysis method	Advantages	Disadvantages	Reference(s)
Alkaline	+ Removes all lignin and some of the hemicellulose + Increases surface area of cellulose + Decreases cellulose crystallinity and degree of polymerization + Proceeds at ambient conditions + Low formation of inhibitors	- Increasing lignin content decreases effectiveness - Conversion of some alkali to irrecoverable salts - Lignin structure is altered	For reviews, see Hamelinck et al. 2005, Mosier et al. 2005, Dwivedi et al. 2009, Agbor et al. 2011
Solvent extraction	+ Hydrolysis lignin and hemicellulose + Proceeds at ambient conditions + Many solvents, e.g. ionic liquids, can be recovered + Does not produce any inhibitors	- High costs - Solvent should be recovered and recycled	For a review, see Brodeur et al. 2011
Biological	+ Proceeds at ambient environmental conditions + Low energy requirements and low maintenance costs + High yields of reduced sugars + Few side reactions + Does not produce any inhibitors + No problems caused by high pressure or corrosion	- Delignification is difficult and often the rate-limiting step - Rate of hydrolysis is usually low	For reviews, see Lee 1997, Sun and Cheng 2002, Hamelinck et al. 2005, Saratale et al. 2008
Fungal	+ Efficient biodegradation of lignin + High cell growth rate + No need for chemicals	- Long treatment time - Careful control of growth conditions is required - Requires a large amount of space for treatment	Shi et al. 2009, for reviews, see Lee 1997, Chandra et al. 2007
Bacterial	+ Easy to operate + Anaerobic, thermophilic bacteria: high growth rates, high metabolic rates on cellulose, increased enzyme stability	- Slow production rate of enzymes - Poor efficiency - Consumption of hydrolyzed products by non-cellulolytic bacteria may lead to low sugar yields	Lo et al. 2011 , for reviews, see Lee 1997, Saratale et al. 2008
Enzymatic	+ Enzymes are biodegradable and environmentally friendly + Recovery and recycle of enzymes possible + No need for special equipment	- End product (cellobiose and glucose) inhibition - Lignin has to be degraded by pretreatment - Production of enzymes is expensive - Instability of enzymes in, e.g., organic solvents - Crystalline cellulose is degraded slowly	Adsul et al. 2009, for reviews, see Schwarz 2001, von Sivers and Zacchi 1994, Hamelinck et al. 2005, Chandra et al. 2007, Dwivedi et al. 2009

AFEX: ammonia fiber expansion, HMF: 5-hydroxymethylfurfural

3. ANAEROBIC CELLULOSE FERMENTATION

Lignin can be degraded biologically only with some aerobic fungal species, e.g., with white rot fungi (Lee 1997, Kumar et al. 2008). Most cellulose is degraded in aerobic conditions, while only 5-10 % of cellulose is degraded in anaerobic environments (Carere et al. 2008). Anaerobic microorganisms degrade cellulose either with a multi-enzyme complex called cellulosome or with multiple enzymes that are simultaneously active and interact with each other (for a review, see Schwarz 2001). Anaerobic cellulase production has a high specific activity but the enzyme synthesis rate is slow (Saratale et al. 2008) and thus, cellulose degradation may take days (O´Sullivan et al. 2006). Many anaerobic, cellulolytic bacteria have been isolated, while the cellulose degradation mechanisms have been identified only with some of the strains.

3.1 Cellulase enzymes and the cellulosome

There are three major groups of cellulases: endoglucanases, exoglucanases, and β-glucosidases (Figure 3). The cellulose chain is randomly cut to smaller pieces by endoglucanases, exoglucanases cut cellobiose units from the ends of cellulose molecules, and β-glucosidases degrade cellobiose and cellodextrins into glucose monomers (for reviews, see Lee 1997, Kumar et al. 2008). The efficiency of hydrolysis is dependent on the amount of individual enzymes and often cellulose hydrolysis is limited by the fact that microorganisms do not excrete appropriate levels of all three enzymes. In general, β-glucosidases do not accumulate quickly enough, which increases the cellobiose concentration that may cause feedback inhibition for endo- and exoglucanases (Lee 1997, Kumar et al. 2008). Furthermore, the efficiency of hydrolysis depends on the crystallinity and particle size of cellulose, the association of cellulose with hemicellulose and lignin, and the ratio of the three cellulase enzymes (Lee 1997, Fields et al. 2000, Schwarz 2001, Yuan et al. 2011a).

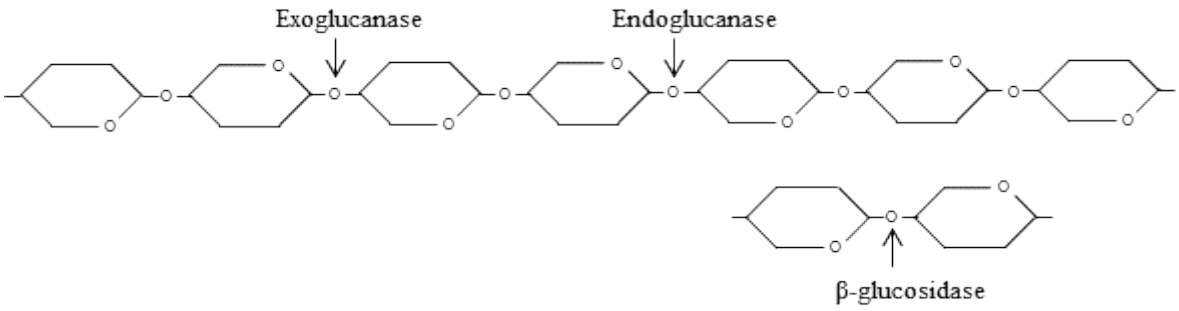


Figure 3. The structure of cellulose (up) and cellobiose (down) and the sites where the cellulase enzymes act (modified from Kumar et al. 2008).

Cellulases can be excreted as single enzymes or they can form multi-enzyme -complexes, called cellulosomes (Figure 4). Cellulosome is an extracellular complex that contains all three types of cellulases and a large non-enzymatic protein, called scaffoldin (Carere et al. 2008, Levin et al. 2009). Scaffolding binds the cellulosome to the bacterial cell wall and thus, promotes enzyme activity near the bacterial cell decreasing diffusion losses of hydrolytic products (Schwarz 2001, Kumar et al. 2008, Levin et al. 2009). Cellulosome can degrade both amorphous and crystalline cellulose and varies between bacterial species (Lynd et al. 2002, Carere et al. 2008).

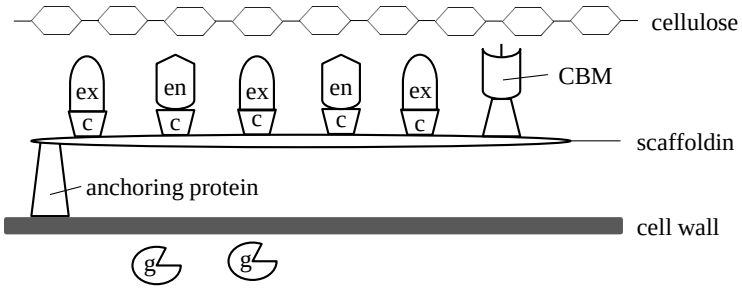


Figure 4. Schematic presentation of cellulosome. Anchoring protein links the scaffoldin protein to the cell wall. Cellulose binding motif (CBM) binds the cellulosome to cellulose. Cohesion domains (c) attach the exoglucanases (ex) and endoglucanases (en) to the scaffoldin. β-glucosidases (g) are located inside the bacterial cell. (Adapted from Lynd et al. 2002, Walter et al. 2007)

3.2 Anaerobic, cellulolytic bacteria

Several anaerobic, cellulolytic bacteria have been characterized (Table 3). Although all these bacteria have been shown do degrade cellulose their cellulase production rates and thus, cellulose degradation rates may not be sufficient enough for significant cellulose utilization (Lynd et al. 2002). Most *Clostridia*, such as *Clostridium thermocellum*, excrete cellulosomes that bind the cell to the substrate (Schwarz 2001, Levin et al. 2006). Other species produce free cellulase enzymes. Hydrogen and/or ethanol fermentation from cellulose has been reported with many bacteria (Table 3). In addition, butanol fermentation has been observed in a few cellulolytic species. Direct electricity production from cellulose was reported by Rezaei et al. (2009) with a pure culture of *Enterobacter cloacae*.

Bacterial production of energy carriers (H_2, ethanol, butanol and/or electricity) from cellulose is often performed with mixed cultures that include both cellulolytic bacteria and bacteria responsible for energy carrier(s) production. Cellulose hydrolysis and following energy carrier(s) production can be divided into two separate steps (Lo et al. 2008, Lo et al. 2011) or they can be conducted in the same reactor, in a process called consolidated bioprocessing (CBP) or simultaneous saccharification and fermentation (SSF). Advantages of CBP include reduced reactor volume and fermentation time, lower costs and energy inputs, lower risk of contamination with external microorganisms, and higher conversion efficiencies (Lynd et al. 2005, Lin and Tanaka 2006, Carere et al. 2008). However, the optimal conditions for cellulolytic bacteria often differ from the optimal growth of energy carrier producers (Lin and Tanaka 2006, Kumar et al. 2008), which may complicate the choice of process conditions. In two-stage process, the cellulose hydrolysis and energy carrier production can be optimized separately. However, the costs and complexity of the process are increased (Saratale et al. 2008).

Table 3. Anaerobic, cellylolytic bacteria, their isolation sources, cellulose hydrolysis types (cellulosome or free cellulases), optimal growth temperature and pH. Cellulose fermentation to different energy carriers (hydrogen, ethanol or butanol) is also indicated. (Modified from Schwarz 2001 and Lynd et al. 2002)

						Energy carriers			
Genus	**Species**	**Source**	**FC/CM**	**T (ºC)**	**pH**	**H_2**	**EtOH**	**ButOH**	**Reference(s)**
Clostridium	*thermocellum*	Hot spring	CM, FC	50-55	nr	+	+	(+)	Weimer and Zeikus 1977, Stainthorpe and Williams 1988, Lynd et al. 1989, Levin et al. 2006
	cellulolyticum	Decaying grass	CM	32-35	nr	+	+	-	Petitdemange et al. 1984, Pagés et al. 1997
	cellulovorans	Wood chips	CM	37	7.0	+	-	-	Sleat et al. 1984, Doi and Tamaru 2001
	papyrosolvens	Freshwater sediment	CM	25-30	nr	+	+	-	Madden et al. 1982, Cavedon et al. 1990, Pohlschröder et al. 1995
	phytofermentans	Forest soil	FC	37	8.0-8.5	+	+	-	Warnick et al. 2002, Zhang et al. 2010b,c
	termitidis	Gut of a termite	nr	37	7.5	+	+	-	Hethener et al. 1992
	herbivorans	Pig intestine	nr	39-42	6.8-7.2	(+)	(+)	-	Varel et al. 1995
	cellulosi	Cow manure	nr	55-60	7.3-7.5	+	+	-	Yanling et al. 1991
	josui	Compost	CM	45	7.0	+	+	-	Kakiuchi et al. 1998, Sukhumavasi et al. 1988
	aldrichii	Wood digester	nr	35	7	+	-	-	Yang et al. 1990
	cellulofermentans	Soil sample	nr	37-40	7.0-7.2	+	+	-	Yanling et al. 1991
	celerescens	Cow manure	nr	35	7.0	+	+	-	Palop et al. 1989
	longisprorum	Bison rumen	nr	35-42	nr	+	+	-	Varel 1989
	alkalicellum	Soda lake	nr	35-40	9.0	+	+	-	Zhilina et al. 2005
	cellobiosparum	Soil	nr	38	6.0-6.5	+	+	-	Hungate 1944
	hungatei	Soil	nr	30-40	7.2	+	+	-	Monserrate et al. 2001
	stercorarium	Compost	FC	65	7.3	+	+	-	Madden 1983
	clariflavum	Sludge	nr	55-60	7.5	+	+	-	Shiratori et al. 2009
	thermopapyrolyticum	Riverside mud	nr	59	nr	+	+	+	Méndez et al. 1991
Acetivibrio	*cellulolyticus*	Sewage sludge	CM	35	7.0	+	(+)	-	Patel et al. 1980, Ding et al. 1999
	cellulosolvens	Fermentor	CM	35-37	6.5-7.5	+	+	-	Khan et al. 1984
Bacteroides	*cellulosolvens*	Sewage sludge	CM	42	7.0	+	+	-	Murray et al. 1984, Lamed et al. 1991
Ruminococcus	*albus*	Bovine rumen	CM	meso	nr	+	+	-	Halliwell and Bryant 1963, Miller and Wolin 1973, Ohara et al. 2000
	flavefaciens	Bovine rumen	CM	meso	nr	-	-	-	Halliwell and Bryant 1963
	succinogenes	Rumen	nr	meso	nr	-	-	-	Fields et al. 2000

Table 3. Continued

Genus	Species	Source	FC/CM	T (ºC)	pH	Energy carriers			Reference(s)
						H_2	EtOH	ButOH	
Caldicellulosiruptor	*saccharolyticus*	Hot spring	FC	70	7.0	+	(+)	-	Rainey et al. 1994, Willquist et al. 2011, Te'o et al. 1995
	lactoaceticus	Hot spring	FC	68	7.0	+	(+)	-	Mladenovska et al. 1995
	krisjanssonii	Hot spring	FC	78	7.0	+	(+)	-	Bredholt et al. 1999
Fervidobacterium	*islandicum*	Hot spring	nr	65	7.2	+	+	-	Huber et al. 1990
Fibrobacter [a]	*succinogenes*	Bovine rumen	CM	meso	nr	-	-	-	Halliwell and Bryant 1963, Montgomery et al. 1988, Fields et al. 2000
Spirochaeta	*thermophila*	Hot spring	nr	66-68	7.5	+	-	-	Aksenova et al. 1992
Enterobacter	*cloacae*	Paper recycling plant wastewater	nr	nr	nr	-	-	-	Rezaei et al. 2009
Thermotoga	*neapolitana*	Marine sediment	nr	80	7	+	-	-	Jannasch et al. 1988, Nguyen et al. 2008
	maritime	Marine sediment	nr	80	6.5	+	-	-	Huber et al. 1986, Nguyen et al. 2008

[a] Former *Bacteroides succinogenes*

FC: free cellulases, CM: cellulosome, EtOH: ethanol, ButOH: butanol, nr: not reported, meso: mesophilic, +: ferments cellulose to energy carrier, (+): ferments cellulose to small concentrations of energy carrier, -: does not ferment cellulose to energy carrier or it has not been reported

4. DARK FERMENTATIVE HYDROGEN PRODUCTION

Hydrogen can be produced biologically through direct or indirect photolysis, photofermentation, dark fermentation, or with microbial electrolysis cells (MEC). In direct and indirect photolysis light energy is used to split water into hydrogen and oxygen by green algae or cyanobacteria, respectively. However, the photosynthetic conversion efficiencies are low and generated oxygen inhibits hydrogenase- and nitrogenase-enzymes responsible for H_2 production. In photofermentation, photosynthetic bacteria utilize light energy and small organic acids to produce hydrogen. Various waste materials are amenable to photo-fermentation but the light conversion efficiencies remain low. (For reviews, see Nath and Das 2004, Das and Veziroglu 2008, Holladay et al. 2009, Lee et al. 2010, Hallenbeck et al. 2012) MECs are an emerging technology where bacteria oxidize organic substrates in the anode and the formed protons and electrons combine at the cathode to form H_2 gas. Hydrogen yields are high and the formed H_2 gas is pure, however the process requires an addition of small amount of electricity to work (for a review, see Lee et al. 2010).

In nature, methane is produced through anaerobic digestion (Figure 5). Acidogenic bacteria degrade the substrate into volatile fatty acids (VFAs), alcohols, H_2 and CO_2 that are further converted to methane by methanogens. If the methanogenic reactions are inhibited, hydrogen can be produced through dark fermentation. The advantages of dark fermentative hydrogen production are that it does not require light energy, it has wide substrate versatility (including cellulosic waste streams) and high hydrogen production rates, and it can be operated in non-sterile conditions and in simple reactors (Valdez-Vazquez et al. 2005a, Wang and Wan 2009, Hallenbeck et al. 2012).

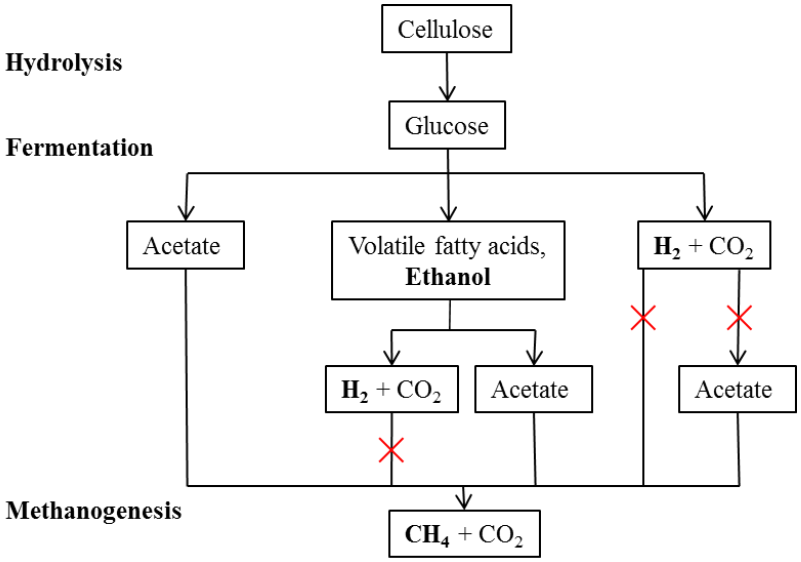


Figure 5. Biological conversion of cellulose that in nature produces methane. When the marked (X) reactions are inhibited, hydrogen can be produced through dark fermentation.

4.1 Hydrogen producing pathways

In dark fermentation, sugars are first fermented into puryvate that is further converted into biomass, ATP and by-products, such as volatile fatty acids and alcohols. Hydrogen and VFAs

are produced at exponential growth phase, while at stationary phase reactions shift to solvent (ethanol, butanol, acetone) production that is also associated with decrease in pH below 4.5 (Yu et al. 2002, Hallenbeck 2005). Acetate and butyrate are the main fermentation by-products associated with hydrogen production (Li and Fang 2007). The highest theoretical hydrogen yields with acetate or butyrate as the sole soluble metabolite are 4 mol H_2/mol hexose and 3.33 mol H_2/mol pentose or 2 mol H_2/mol hexose and1.67 mol H_2/mol pentose, respectively (Table 4). Dark fermentative hydrogen production proceeds through Enteric- or Clostridial (Embden-Meyerhof)-type pathways (Hallenbeck et al. 2009, Valdez-Vazquez and Poggi-Varaldo 2009) that produce at maximum 2 or 4 mol H_2/mol glucose, respectively. Enteric-type pathway is used by facultative anaerobes, such as *Escherichia coli*, whereas strict anaerobes, e.g., *Clostridium butyricum*, produce hydrogen through Clostridial-pathway (Lee et al. 2010).

Table 4. Reactions affecting dark fermentative hydrogen production.

End product	Reaction	Reference
H_2 production		
Acetate	$C_6H_{12}O_6 + 2\ H_2O \rightarrow 2\ CH_3COOH + 2\ CO_2 + \mathbf{4\ H_2}$	Hallenbeck 2005
Butyrate	$C_6H_{12}O_6 + 2\ H_2O \rightarrow CH_3CH_2COOH + 2\ CO_2 + \mathbf{2\ H_2}$	Hallenbeck 2005
Acetate/butyrate	$C_6H_{12}O_6 \rightarrow 0.5\ CH_3COOH + 0.75\ CH_3CH_2COOH + 2\ CO_2 + \mathbf{2\ H_2}$	Barros and Silva 2012
Acetate/ethanol	$C_6H_{12}O_6 + H_2O \rightarrow CH_3COOH + CH_3CH_2OH + 2\ CO_2 + \mathbf{2\ H_2}$	Hwang et al. 2004
Isopropanol	$C_6H_{12}O_6 + 4\ H_2O \rightarrow CH_3CH(OH)CH_3 + 3\ CO_2 + \mathbf{3\ H_2}$	Mitchell 1998
Butyrate to H_2[a]	$CH_3CH_2COOH + 2\ H_2O \rightarrow 2\ CH_3COOH + \mathbf{H_2}$	Valdez-Vazquez and
Propionate to H_2[a]	$CH_3CH_2COOH + 3\ H2O \rightarrow CH_3COOH + CO_2 + \mathbf{3\ H_2}$	Poggi-Varaldo 2009
Lactate and acetate to H_2	$CH_3CHOHCOOH + 0.5\ CH_3COOH \rightarrow 0.83\ CH_3CH_2COOH + 0.75\ CO_2 + 0.5\ \mathbf{H_2} + 0.83\ H_2O$	Juang et al. 2011
H_2 utilization		
Propionate to valerate	$CH_3CH_2COOH + \mathbf{6\ H_2} + 2\ CO_2 \rightarrow C_5H_{10}O_2 + 2\ H_2O$	Kim et al. 2010
Sulfate reduction	$\mathbf{4\ H_2} + SO_4^{2-} + H^+ \rightarrow HS^- + 4\ H_2O$	Conrad and Wetter 1990
Methanogenesis	$\mathbf{4\ H_2} + CO_2 \rightarrow CH_4 + 2\ H_2O$	Conrad and Wetter 1990
Acetogenesis	$\mathbf{4\ H_2} + 2\ CO_2 \rightarrow CH_3COOH + 2\ H_2O$	Müller 2003
Other reactions		
Acetate	$C_6H_{12}O_6 \rightarrow 3\ CH_3COOH$	Müller 2003
Ethanol	$C_6H_{12}O_6 \rightarrow 2\ CH_3CH_2OH + 2\ CO_2$	Guo et al. 2010a
Lactate	$C_6H_{12}O_6 \rightarrow 2\ CH_3CHOHCOOH + 2\ CO_2$	Guo et al. 2010a

[a] anaerobic oxidation (syntrophy)

In Enteric-pathway (Figure 6) the formate:hydrogen lyase (Fhl) is induced and hydrogen is produced from formate (Hallenbeck 2009) yielding at the highest 2 mol H_2/mol glucose. This reaction takes place only at acidic conditions with high concentrations of formate. Enteric-pathway is also called mixed acid fermentation since it produces many fermentation products, including lactate, acetate, ethanol, formate, H_2 and CO_2 (Hallenbeck 2005). In Clostridial-pathway (Figure 6) puryvate is oxidized to acetyl-CoA and CO_2 by puryvate:ferredoxin oxidoreductase (Pfor) and at the same time electrons are transferred to ferredoxin (Fd) (Kraemer and Bagley 2007). Fd transfers electrons to hydrogenase resulting in the production of 2 mol H_2/mol glucose (Hallenbeck 2005). At low partial pressure of hydrogen ($pH_2 < 10^{-3}$ atm), the NADH produced during glycolysis is reoxidized by electron transfer to Fd by NADH:Fd oxidoreductase (Nfor) resulting in additional production of 2 mol H_2/mol glucose (Kraemer and Bagley 2007). At high H_2 partial pressures, electrons are utilized for ethanol and butanol production instead of hydrogen (Valdez-Vazquez and Poggi-Varaldo 2009).

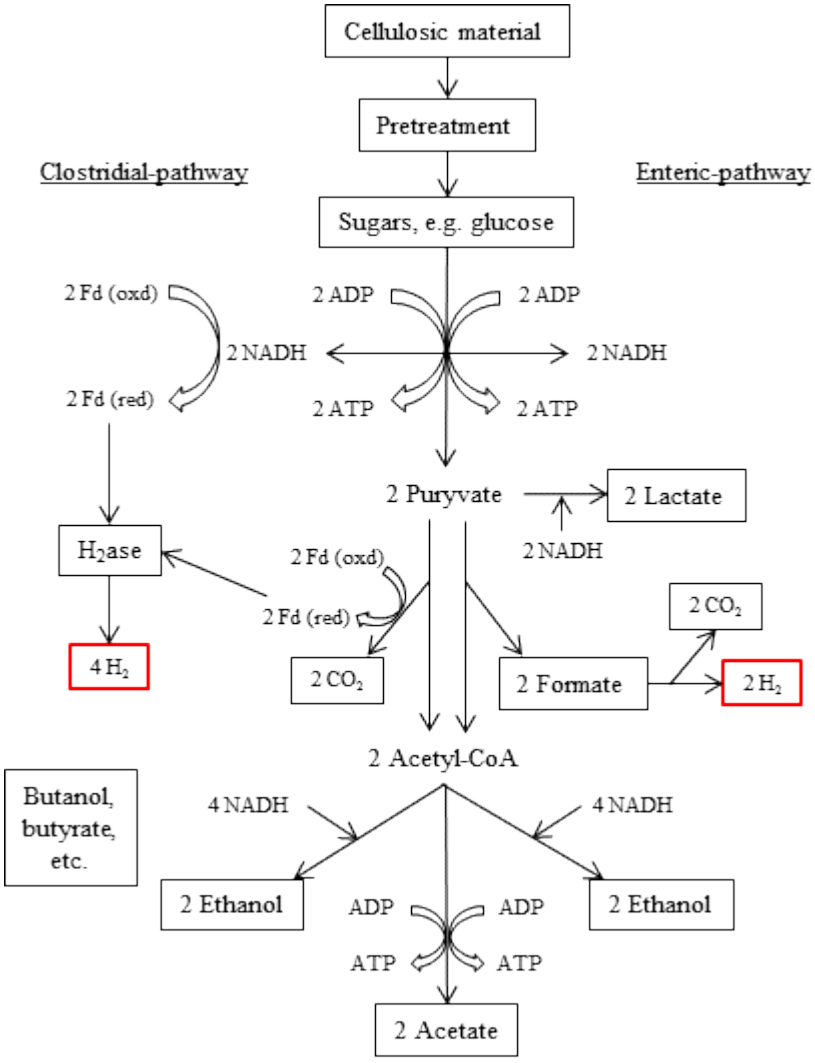


Figure 6. Hydrogen production pathways. Clostridial-type pathway is presented on the left and Enteric-type pathway on the right (modified from Hallenbeck 2005, 2009, Hallenbeck et al. 2012). Fd(oxd): oxidized ferredoxin, Fd(red): reduced ferredoxin, H_2ase: hydrogenase.

4.2 Hydrogen consumers and pretreatment of H_2 producing cultures

Hydrogen fermentation can be done either with pure or mixed cultures. Pure cultures used for H_2 production include, e.g., *C. butyricum* (Wang and Jin 2009), *C. thermocellum* (Levin et al. 2006), *Caldicellulosiruptor saccharolyticus* (van Niel et al. 2002), and *C. acetobutylicum* (Zhang et al. 2006). However, mixed cultures are preferred since they contain cellulolytic bacteria and can utilize complex feedstocks, they can be used in non-aceptic conditions, and they are more robust to changes (Hallenbeck and Ghosh 2009). Hydrogen producing mixed cultures have been obtained, for example from anaerobic digester sludge (Lee et al. 2009, O-Thong et al. 2009), compost (van Ginkel et al. 2001, Calli et al. 2008a), cow dung (Lin and Hung 2008), soil (Luo et al. 2008, Ravindran et al. 2010), hot spring (Karadag et al. 2009), sediment (Kawagoshi et al. 2005), and rotted wood crumbs (Wang et al. 2011a). The disadvantage of mixed cultures is that they often contain hydrogen-consuming microorganisms, such as methanogens, homoacetogens or sulfate-reducers, and/or bacteria directing electrons to other by-products than hydrogen (Table 4). For example, formate production consumes hydrogen (Li and Fang 2007), while lactate production directs the metabolic pathways away from hydrogen production (Hallenbeck et al. 2009). Furthermore,

lactic acid bacteria excrete proteins called bacteriocins that have bactericidal activity against Gram-positive bacteria, such as *Clostridia* and thus, may inhibit hydrogen production (Noike et al. 2002, Valdez-Vazquez and Poggi-Varaldo 2009).

To ensure high hydrogen yields, H_2-consuming microorganisms have to be inhibited. Heat treatment kills methanogens and non-sporulating eubacteria, inhibits the growth of lactic acid bacteria, and enriches for spore-forming bacteria, such as *Clostridia* (Noike et al. 2002, Valdez-Vazquez et al. 2005a). However, heat treatment does not always prevent acetogenesis (Oh et al. 2003, Mu et al. 2007) or growth of spore-forming lactic acid bacteria (Karadag et al. 2009). In addition, heat treatment may also inhibit some non-spore forming cellulolytic and hydrogenic bacteria (Kawagoshi et al. 2005, Zhu and Beland 2006, Lin and Hung 2008) decreasing hydrogen yields. Acid and alkaline treatments also enrich for spore-forming hydrogen producers (Lee et al. 2009, Kim et al. 2010). 2-Bromoethanesulfonic acid (BESA) is a specific inhibitor of methanogens (Valdez-Vazquez et al. 2005b), linoleic acid inhibits hydrogenotrophic methanogens (Chaganti et al. 2012), and chloroform suppresses hydrogen consuming microorganisms (Chang et al. 2011b). In organic load-shock treatment, high substrate concentration results in accumulation of VFAs and decrease in pH (O-Thong et al. 2009). Low pH inhibits the growth of methanogens, since their optimal pH is in the range of 6.8-7.2 (Chandra et al. 2012). In addition, growth of acetogens was reported unfavorable at pH values below 5.5 (Calli et al. 2008a).

During continuous H_2 production heat-treatment or addition of chemicals is not economical, since feeds are non-sterile and treatment of microbial communities should be repeated regularly. In continuous mode, hydrogen producers can be inhibited by changing the operational parameters, for example, by increasing temperature (Shin and Youn 2005, Yokoyama et al. 2007a), decreasing pH (Valdez-Valquez et al. 2005a, Calli et al. 2008b), or by reducing hydraulic retention time (HRT).

4.3 Parameters affecting cellulose degradation and hydrogen production

Biohydrogen production from cellulosic materials is often limited by the hydrolytic activity of cellulolytic microorganisms (Guo et al. 2010a). Furthermore, hydrogen fermentation often suffers from low H_2 yields (Hallenbeck et al. 2012) associated with excessive soluble metabolite production or presence of H_2 consuming microorganisms. Cellulose hydrolysis and hydrogen fermentation can be increased by optimizing process parameters (Table 5). However, optimal process conditions for hydrogen fermentation and cellulose hydrolysis often differ. For example, efficient cellulose hydrolysis has been reported near neutral pH (Hu et al. 2004, Lo et al. 2008), while hydrogen yields from sugars are often the highest at lower pH values ranging from 5.0 to 5.5 (van Ginkel and Sung 2001, Calli et al. 2008b, Karadag and Puhakka 2010a).

Table 5. Main parameters affecting simultaneous cellulose hydrolysis and hydrogen fermentation.

Parameter	Affects	Reference(s)

Temperature	- Solubility of gases and effect of pH_2
	- Chemical and enzymatic reaction rates, stability of enzymes, cellulase adsorption and hydrolysis efficiency
	- Viscosity and surface tension that affect the energy required for mixing
	- H_2 production rate and yield, lag time of H_2 production
	- Metabolic pathways
	- Microbial community composition and cell densities
	- High temperature (> 50ºC) results in
	  - Simultaneous biomass hydrolysis and treatment of pathogens
	  - Increased H_2 yields and absence of most H_2-consuming bacteria
	  - Increased energy demand
		Van-Wyk 1997, Noike et al. 2002, van Niel et al. 2002, Levin et al. 2004, Valdez-Vazquez et al. 2005a, Yokoyama et al. 2007a, Lin et al. 2008, Lo et al. 2008, Karadag et al. 2009, Karadag and Puhakka 2010b, for reviews, see Lee 1997, Schwarz 2001, Hallenbeck 2005, Hawkes et al. 2007, Ren et al. 2009

pH	- Production and release of cellulases, hydrolysis efficiency - H_2 production rate and yield, lag time of H_2 production - Metabolic pathways - Microbial community composition - Changes nutritional substance supply and toxicity of harmful substances	Chyi and Levine 1992, Fang and Liu 2002, Hu et al. 2005, Fang et al. 2006, Li et al. 2007, Wang et al. 2007, Liu et al. 2008, Lin and Hung 2008, Calli et al. 2008b, Wang and Zhao 2009, Karadag and Puhakka 2010a, for reviews, see Kapdan and Kargi 2006, Ren et al. 2009
Alkalinity	- Low alkalinity leads to decrease in pH - H_2 content and production rate, lag time of H_2 production	Lin and Lay 2004, Li et al. 2007
Redox potential	- Rate and efficiency of cellulose utilization - H_2 production rate and yield - Use of reducing agents increases production costs	For reviews, see Lynd et al. 2002, Kapdan and Kargi 2006
H_2 partial pressure (pH_2)	- Metabolic pathways - H_2 production rate and yield (temperature dependent) - Redox potential of H^+/H_2 and electron flow from ferredoxin (Fd) to H_2	Levin et al. 2004, Li et al. 2007, Valdez-Vazquez and Poggi-Varaldo 2009

Carbon source	- Microbial community composition
	- Crystallinity and available surface area affect
	  - Hydrolysis rate
	  - H_2 production yield
	  - Metabolic pathways
	- Substrate concentration affects
	  - Cellulase production and hydrolysis efficiency
	  - H_2 production rate and yield
	  - Metabolic pathways
	  - High substrate concentration may cause substrate inhibition on H_2 production
		Hu et al. 2005, Kim et al. 2006a, Li et al. 2007, Liu et al. 2008a, Luo et al. 2008, Karadag et al. 2009, Ravindran et al. 2010, Ren et al. 2010, Antonopoulou et al. 2011, Yuan et al. 2011a, for reviews, see Lynd et al. 2002, Kumar et al. 2008, Saratale et al. 2008, Guo et al. 2010a

Hydraulic retention time (HRT)	- Substrate conversion: larger cellulose particles require longer HRT - Metabolic pathways - H_2 production rate and yield - Biomass content and H_2 consuming microorganisms - Oxidation-reduction potential - Low HRT may lead to wash out of granular bacterial biomass - High HRT may lead to product inhibition due to accumulation of VFAs	Chyi and Levine 1992, Ueno et al. 1996, Yu et al. 2002, Han and Shin 2004, Fan et al. 2006a, Vijayaraghavan and Ahmad 2006

Table 5. Continued

Parameter	Affects	Reference(s)
Nutrients		
Nitrogen	- Cellulase production - The most essential nutrient required for bacterial growth - Excess nitrogen may cause ammonia inhibition - C/N ratio affects stability of H_2 production	Singh et al. 1991, Menon et al. 1994, Yokoyama et al. 2007b, Kim et al. 2010, for a review, see Lynd et al. 2002
Phosphorus	- Cellulase production	For a review, see Lynd et al. 2002
Sulfur	- Cellulase production - Activity of hydrogenases and ferredoxins that contain sulfur	For reviews, see Lee et al. 2001, Lynd et al. 2002, Hawkes et al. 2007, Wang and Wan 2008
Iron	- Activity of hydrogenases - H_2 production rate and yield - Metabolic pathways - Optimal concentration is temperature dependent	Zhang and Shen 2006, Karadag et al. 2009, Karadag and Puhakka 2010c, Lee et al. 2001, for a review, see Wang and Wan 2008
Nickel	- H_2 yield - Metabolic pathways - Microbial community composition	Karadag and Puhakka 2010c
Magnesium	- Needed in the activation of 10 enzymes of the glycolysis process - Absence may inhibit anabolism and H_2 production	Liu et al. 2008b
Vitamins	- Cellulase production	For a review, see Lynd et al. 2002

4.4 Hydrogen production from cellulosic materials

Dark fermentative hydrogen production from sugars, such as glucose or xylose, has been extensively studied. These studies have provided valuable information on the fundamental effects of different parameters on hydrogen production potentials and microbial communities as well as on the metabolic pathways involved in dark fermentation. However, if hydrogen is produced at large-scale, the process has to be capable of utilizing cellulosic materials for hydrogen fermentation with or without pretreatment. Research on the amenability of cellulosic and lignocellulosic materials to direct hydrogen fermentation in batch mode has been active recently (Table 6). Furthermore, continuous or semi-continuous hydrogen production from these substrates has also been successful (Table 6).

Table 6. Simultaneous cellulose hydrolysis and hydrogen production from renewable materials in batch, semi-continuous or continuous mode.

Substrate	Culture	Reactor	T (ºC)	pH	HRT (h)	HY (mol H_2/mol hexose)	HPR (L H_2/L/d)	Reference
Batch								
Cellulose	Sludge compost	Flask	60	nr	-	2.4	nr	Ueno et al. 1995
Cellulose	Digested sludge	Flask	37	7.0	-	0.39	nr	Lay 2001
Cellulose	H_2-producing sludge	Flask	55	6.5	-	0.76	nr	Liu et al. 2003
Cellulose	Cow dung microflora	Flask	55	7.5	-	0.50	nr	Lin and Hung 2008
Cellulose	Soil sample	Flask	35	7.0	-	0.22	nr	Lo et al. 2008
Cellulose	Cow dung compost	Flask	37	6.8	-	2.09	0.79	Ren et al. 2010
Cattle wastewater	Sewage sludge	Flask	45	5.5	-	2.55	nr	Tang et al. 2008
Cassava stillage	Digested sludge	Flask	60	6.0	-	67.8 mL H_2/g VS	nr	Luo et al. 2010
Cow waste slurry	Cow waste slurry	Flask	60	7.0	-	29.3 mL H_2/g VS	nr	Yokoyama et al. 2007a
Cow dung	Cow dung	Flask	60	6.6	-	5.05 mL H_2/g TS	nr	Yokoyama et al. 2007b
Cow manure and waste milk	Cow manure	Flask	55	6.4	-	59.5 mL H_2/g VS	nr	Lateef et al. 2012
Delignified wood fibers	*C. thermocellum*	Flask	nr	7.08	-	2.32	nr	Levin et al. 2006
DDG Barley hull CBH	*C. thermocellum*	Flask	60	7.2	-	0.23 0.21 0.22	0.13 0.05 0.14	Magnusson et al. 2008
Fodder maize Chicory fructans Perennial ryegrass	Digester sludge	Flask	35	5.2	-	62.4 mL H_2/g TS 218 mL H_2/g TS 75.6 mL H_2/g TS	4.5 3.2 6.0	Kyazze et al. 2008
Organic HSW	Enriched H_2 bacteria	Flask	70	7.3	-	169.5 mL H_2/g VS	nr	Liu et al. 2008a
OFMSW	Digested sludge Enriched H_2 bacteria	Flask	37	nr	-	140 mL H_2/g VS 180 mL H_2/g VS	nr nr	Lay et al. 1998
POME	Anaerobic lagoon	Flask	45	6.0	-	0.23	76.0	Yossan et al. 2012
Rice slurry	Digested sludge	Flask	37	4.5	-	2.59	nr	Fang et al. 2006
Sweet potato	Sewage sludge	Flask	37	6.7	-	1.24	7.46	Lay et al. 2012
Waste biosolids Filtrate from biosolids	Waste biosolids	Flask	35	nr	-	0.11 0.95-1.43	nr	Wang et al. 2003a
Wheat stalk	Digested sludge	Flask	35	nr	-	31.6 mL H_2/g TS	nr	Yuan et al. 2011a

Table 6. Continued

Substrate	Culture	Reactor	T (ºC)	pH	HRT (h)	HY (mol H_2/mol hexose)	HPR (L H_2/L/d)	Reference
Semi-continuous								
Cellulose	Sludge compost	Fermenter	60	6.4	72	2.0	0.72	Ueno et al. 2001
Food waste	Enriched bacteria	Flask	55	5.5	120	1.8	1.74	Shin et al. 2004
Food waste	Digested sludge	Tank	35	5.3	24	0.87	nr	Kim and Shin 2008
Food waste	Digested sludge	ASBR	35	5.3	36	0.69	nr	Kim et al. 2010
OFMSW	Digested sludge	Flask	55	6.4	nr	3.2	nr	Valdez-Vazquez et al. 2005a
POME	Digested sludge	ASBR	60	5.5	24	2.24	6.1	O-Thong et al. 2007
Sugar factory wastewater	Sludge compost	Chemostat	60	6.8	12	2.52	4.77	Ueno et al. 1996
Continuous								
Cellulose	Rotted wood crumbs	Flask	60	7.0	32	1.82	0.24	Wang et al. 2011b
Brewery waste	Compost	CSTR	37	5.5	18	0.34	3.1	Fan et al. 2006b
Corn starch	Digested sludge	CSTR	35	5.3	12	0.92	4.29	Arooj et al. 2008
Food waste	Digested sludge	CSTR	55	5.5	120	2.2	1.0	Shin and Youn 2005
Olive pulp	Digested sludge	CSTR	55	5.6	29	38.5 mL H_2/g TS	nr	Gavala et al. 2005
Organic HSW	Enriched bacteria	CSTR	70	nr	72	78.8 mL H_2/g VS	nr	Liu et al. 2008a
Rice winery wastewater	MWTP	UASB	55	5.5	2	2.14	3.81	Yu et al. 2002
Sugar beet juice	Digested sludge	CSTR	32	5.2	nr	0.9	nr	Hussy et al. 2005
Sweet sorghum	Sweet sorghum	CSTR	35	5.3	12	0.86	3.48	Antonopoulou et al. 2008
Wheat starch	Digested sludge	CSTR	35	5.2	18	1.28	nr	Hussy et al. 2003

HY: hydrogen yield, HPR: hydrogen production rate, nr: not reported, TS: total solids, VS: volatile solids

CBH: fusarium head blight contaminated barley hull, DDG: dried distillers grain, HSW: household solid waste, OFMSW: organic fraction of municipal solid waste, POME: palm oil mill effluent, MWTP: municipal wastewater treatment sludge

ASBR: anaerobic sequencing batch reactor, CSTR: completely stirred tank reactor, UASB: upflow anaerobic sludge blanket

Hydrogen yields from cellulosic materials are often lower than those obtained from sugars (for a review, see Kapdan and Kargi 2006), because the complex structure of cellulose may result in low hydrolysis efficiencies. Even lower hydrogen yields have been reported with lignocellulosic materials (Kyazze et al. 2008, Magnusson et al. 2008, Yuan et al. 2011a) due to the presence of lignin that interferes with cellulose hydrolysis. Hydrogen yields from lignocellulosic materials can be increased by pretreating the substrates with, e.g., diluted acid treatment or enzymes (Table 7). For example, Han et al. (2012) reported that diluted acid pretreatment of soybean straw resulted in 11-fold increase in cumulative H_2 yields when compared to raw soybean straw. However, pretreatment may lead to production of inhibitors (furfural, HMF) that decrease hydrogen production (Cao et al. 2010). Adding a pretreatment step before hydrogen fermentation also complicates the process and increases the production and maintenance costs.

Dark fermentative hydrogen production has been extensively studied at laboratory scale. Furthermore, few studies on hydrogen fermentation have been performed at pilot-scale (Ren et al. 2006, Kim et al. 2010, Lin et al. 2011). A 400 L pilot-scale fermentor has been operated at Feng Chia University (Taiwan) with sucrose as substrate. The first reported experiments obtained hydrogen production rates and yields of 5.2–15.6 L H_2/L/d and 1.0–2.3 mol H_2/mol sucrose, respectively (Lin et al. 2010b,c). Optimization of agitation rate, HRT and substrate concentration increased the hydrogen production rate and yield to 28.4 L H_2/L/d and 3.8 mol H_2/mol sucrose, respectively (Lin et al. 2011). Furthermore, the microbial community composition of the reactor has been monitored both quantitatively and qualitatively with quantitative PCR (qPCR) (Cheng et al. 2011b).

More complex substrates have also been studied at pilot-scale. Ren et al. (2006) used a 1480 L bioreactor for over 200 days with molasses as substrate. They reported a hydrogen production rate of 5.6 L H_2/L/d corresponding to 26.1 mol H_2/kg $COD_{removed}$. Organic loading rate of over 68 kg COD/m^3/d decreased hydrogen production due to VFA inhibition (Ren et al. 2006). Kim et al. (2010) produced hydrogen from food waste in a 150 L anaerobic sequencing batch reactor (ASBR). Alkaline shock increased the hydrogen yield considerably, after which the hydrogen yield stabilized to 0.69 mol H_2/mol hexose. More pilot-scale studies are needed with actual wastewaters and wastes and the processes need further optimization before hydrogen fermentation can be practiced at full scale.

Table 7. Hydrogen production from hydrolysates.

Substrate	Pretreatment method	Sugar yield	Culture	T (ºC)	pH	HY (mol H_2/mol hexose)	Reference
Beer lees waste	Diluted acid	nr	Cow dung compost	36	6.5	68.6 mL H_2/g VS	Fan et al. 2006b
Barley grains Corn grains	Enzymatic	97.0 g/L, 56 %[a] 108 g/L, 74 %[a]	*Caldicellulosiruptor saccharolyticus*	70	7.0	nr	Panagiotoupoulos et al. 2009
Carboxymethyl cellulose	Bacterial, soil sample	5.53 g/L 55.3 %[b]	*Clostridium pasteurianum*	35	7.0	0.20	Lo et al. 2008
Cassava pulp	Diluted acid	nr	*Clostridium butyricum*, *Enterobacter aerogenes*	36	5.5	2.76	Phowan et al. 2010
CMC	Bacterial, *Cellulomonas uda*	2.88 g/L 14.4 %[b]	*Clostridium butyricum*	37	7.5	1.58	Lo et al. 2011
Cornstalk wastes	Enzymatic	29.0 %	Enrichment culture	36	6.5	122 mL H_2/g VS	Guo et al. 2010b
Cornstalk wastes	Alkaline	nr	Rotted wood crump	60	7.0	155 mL H_2/g VS	Cao et al. 2012
Corn stover	Diluted acid	12.2 g/L	*Thermoanaerobacterium thermosaccharolyticum*	60	7.0	2.24	Cao et al. 2009
Corn stover	Diluted acid	nr	*Clostridium thermocellum*	55	6.8	1.67	Lalaurette et ala. 2009
Corn stover	Neutral steam explosion Acid steam explosion	27.3 g/L 51.5 g/L	Digested sludge	35	5.5	2.84 3.0	Datar et al. 2007
Cotton cellulose	Diluted acid	73.9 %[b]	Mixed culture	37	8.2	0.99	Chu et al. 2011
Fruits and vegetables waste	Alkaline	nr	Wastewater sludge	35	5-6	17.8 mL H_2/g VS	Ruggeri and Tommasi 2012
Paper and pulp industry effluent	Enzymatic	22.9 g/L 86.8 %[c]	*Enterobacter aerogenes*	35	7.0	2.03	Lakshmidevi and Muthukumar 2010
Reed canary grass	Diluted acid	11.5 %[b]	Enrichment culture	35	nr	1.8	Lakaniemi et al. 2011
Rice straw	Diluted acid	36.7 g/L 12.2 %[b]	Sewage treatment plant	40	6.5	0.95	Chang et al. 2011a
Sugarcane bagasse	Diluted acid	24.5 g COD/L, 36.8 %[b]	*Clostridium butyricum*	37	5.5	1.73 mol H_2/mol TS	Pattra et al. 2008
Waste ground wheat	Diluted acid	nr	Anaerobic sludge	37	6.8	1.46 mol H_2/mol TS	Sagnak et al. 2011
Wheat straw wastes	Diluted acid	nr	Cow dung compost	36	7.0	68.1 mL H_2/g VS	Fan et al. 2006c

[a] g sugars/g dry matter, [b] g RS/g substrate, [c] g RS/g polysaccharides

HY: hydrogen yield, nr: not reported, RS: reduced sugars, TS: total solids, VS: volatile solids

5. FERMENTATION TO SOLUBLE ENERGY CARRIERS

5.1 Ethanol fermentation

In 2005, the annual worldwide bioethanol production was 48.7 million m^3, of which 73 % was produced in Brazil and USA (Antoni et al. 2007). Large-scale ethanol fermentation is at present carried out from sucrose-containing (sugar cane, sweet sorghum) or starchy (corn, wheat) feedstock. However, the supplies of these substrates are decreasing and their utilization competes with food production increasing the costs of raw materials (Gray et al. 2006). Thus, ethanol fermentation from cellulosic substrates, including agricultural and wood residues, has received increased attention. Fermentation of sucrose- or starch-based compounds to ethanol is conducted without pretreatment or with enzymatic hydrolysis (Lin and Tanaka 2006). Lignocellulosic feedstocks, on the other hand, require pretreatment and cellulose hydrolysis before ethanol fermentation. Hydrolysis of lignocellulosic materials may result in the production of inhibitors that either require treatment before fermentation or a resistant ethanologenic strain (Gray et al. 2006). Lignocellulose to ethanol fermentation is at the moment at pilot-scale (Maas et al. 2008, Lin et al. 2012).

At large-scale, *Saccharomyces cerevisiae* yeast is usually used for ethanol fermentation (Lin and Tanaka 2006). However, wild-type *S. cerevisiae* does not convert pentoses to ethanol limiting the fermentation process (Gray et al. 2006), especially from cellulosic feedstocks. Some yeast, e.g., *Candida shehatae* and *Pichia stipitis* and fungi, e.g., *Fomitopsis palustris* ferment pentoses to ethanol (Okamoto et al. 2011, Shupe and Liu 2012). In addition, anaerobic bacteria can convert both hexoses and pentoses to ethanol (Eq. 1,2) (for a review, see Hamelinck et al. 2005). Rao et al. (2007) compared ethanol fermentation from brewery wastewater with *S. cerevisiae* and genetically modified *E. coli* after enzymatic hydrolysis. They concluded that yeast produced ethanol at higher rate but that the final ethanol yields were in the same range. Ethanol tolerances of some ethanologenic strains and ethanol fermentation from different substrates are reported in Tables 8 and 9, respectively. Ethanol fermentation can be improved by metabolic engineering of ethanologenic strains. Progress is required, for example in tolerance of ethanol and inhibitory compounds, substrate versatility, and in ethanol production rates and yields (for a review, see Dien et al. 2003).

$$3\ C_5H_{10}O_5 \rightarrow 5\ C_2H_5OH + 5\ CO_2 \quad (1)$$

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2 \quad (2)$$

Table 8. Ethanol tolerance of some ethanologenic strains.

Strain	EtOH tolerance % (v/v)	Note	Reference
Saccharomyces cerevisiae	10-15	No inhibition	Benjaphokee et al. 2012
	25[a]	Severe growh inhibition	
Escherichia coli	3.8-4.4	50 % growth inhibition	Wang et al. 2011c
C. thermosaccharolyticum	5.1	50 % growth inhibition	Baskaran et al. 2008
Thermoanaerobacterium	3	No inhibition	Koskinen et al. 2008a
aciditolerans	5[a]	Growth completely inhibited	

[a] The highest level of ethanol tested

Table 9. Ethanol or butanol fermentation.

Substrate	Culture	Ethanol (g/L)	Butanol (g/L)	Acetone (g/L)	Note	Reference
Ethanol fermentation						
Glucose	*Zymomonas mobilis*	60[a]	-	-	Continuous production	Rebros et al. 2005
Glucose	*Saccharomyces cerevisiae*	46.6	-	-	-	Benjaphokee et al. 2012
Xylose	*Escherichia coli* KC01	23.5	-	-	-	Wang et al. 2011c
Distiller´s grain	*Saccharomyces cerevisiae*	45	-	-	AFEX and enzymatic hydrolysis	Kim et al. 2008
Brewery wastewater	*Saccharomyces cerevisiae*	14.6	-	-	Enzymatic hydrolysis	Rao et al. 2007
	Escherichia coli KO11	15.5	-	-		
Bamboo	*Saccharomyces cerevisiae*	27.2	-	-	Concentrated acid hydrolysis	Sun et al. 2011a
Sugarcane leaves	*Saccharomyces cerevisiae*	4.2[b]	-	-	Diluted acid and enzymatic hydrolysis	Jutakanoke et al. 2012
Rice straw	*Saccharomyces cerevisiae*	37.0	-	-	AFEX and enzymatic hydrolysis	Zhong et al. 2009
Corn stover	*Amorphotheca resinae*	40	-	-	Steam-explosion pretreatment	Zhang et a. 2010a
Sugar maple wood chips	*Pichia stipitis*	13.5	-	-	Hot water pretreatment	Shupe and Liu 2012
Cornstalk	*Issatchenkia orientalis*	45.9	-	-	Steam-explosion and enzymatic pretreatment	Kwon et al. 2011
Butanol fermentation						
Glucose (continuous fermentation)	*Clostridium acetobutylicum*	0.8	9.8	5.4	One stage	Bankar et al. 2012
		2.5	16.9	5.9	Two stage with solvent extraction	
Glucose	*Clostridium beijerinckii*	0.3	19.6	4.3	-	Qureshi and Blaschek 1999
Glucose	*Clostridium saccharoperbutylacetonicum*	0	20.1	2	Membrane assisted extraction of butanol	Tanaka et al. 2012
Mixed sugars	*Clostridium beijerinckii* BA101	0.2	13.9	3.8	-	Ezeji et al. 2007b
Corn fiber hydrolysate	(hyperbutanol producing strain)	1.0	7.2	4.7	After detoxification	
Cassava flour	*Clostridium beijerinckii* BA101	10.2	31.6	2.7	Enzymatic hydrolysis	Lépiz-Aguilar et al. 2011
	(hyperbutanol producing strain)	20.3	27.5	0.0	Diluted acid hydrolysis	

Note: Ethanol, butanol and acetone concentrations are the final maximum concentrations in the experiments, except for [a] the steady-state ethanol concentrations, [b] the final ethanol concentration

Above described processes produce ethanol as the main metabolite. However, ethanol can also be produced simultaneously, e.g., with hydrogen (Koskinen et al. 2008a,b, Karadag and Puhakka 2010b) or butanol fermentation (Lépiz-Aguilar et al. 2011). In addition, ethanol has been produced from acetate (Steinbusch et al. 2010) or glycerol (Flynn et al. 2010) in the cathode chamber of a bioelectrochemical system (BES), where ethanol was produced by adding a voltage to the cell.

5.2 Butanol fermentation

Butanol fermentation is at present at laboratory scale. Various simple substrates, including glucose, starch, corn and molasses, have been utilized for butanol fermentation (for a review, see Ezeji et al. 2007a). As in ethanol fermentation, cellulosic substrates, such as agricultural biomass, have to be utilized in the future. Before butanol fermentation cellulosic feedstocks have to be pretreated and hydrolyzed (see Section 2). Butanol fermentation proceeds through acetone-butanol-ethanol (ABE) fermentation that consists of acidogenic and solventogenic phases (Figure 7). In the first phase, acetate, butyrate, H_2 and CO_2 are the main metabolites. The second phase takes place after the pH has decreased below critical point, after which remaining carbohydrates and acids are fermented to 1-butanol, acetone, ethanol and CO_2. Various solventogenic clostridial species can be utilized for butanol fermentation (for reviews, see Lee et al. 2008a, Patakova et al. 2012).

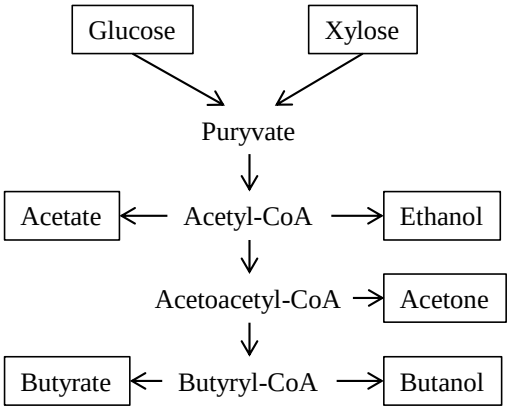


Figure 7. Simplified metabolism of solventogenic *Clostridia* (Adapted from Ezeji et al. 2007b).

Butanol yields have remained relatively low (Table 9). One reason for this is the low butanol tolerance of clostridial strains that is usually in the range of 1-2 % (v/v) (Knoshaug and Zhang 2009). The highest butanol tolerances, between 2.4 and 3.9 % (v/v), have been reported for *Clostridium beijerinckii* and *Clostridium saccharoperbutylacetonicum*, respectively (Qureshi and Blaschek 1999, Lépiz-Aguilar et al. 2011, Tanaka et al. 2012). Butanol yields can be increased by metabolic engineering that may include enhancing butanol production rates and yields, improving butanol tolerance, expanding the substrate range, and directing fermentation to butanol instead of mixed acids, ethanol and acetone (for a review, see Lee et al. 2008a). In addition to clostridial fermentation, Lakaniemi et al. (2012) reported butanol production at the anode of an MFC with simultaneous production of electricity. The highest butanol yield was 1.2 g/L.

6. ELECTRICITY PRODUCTION IN MICROBIAL FUEL CELLS

A traditional MFC consists of anode and cathode compartments that are separated by cation exchange membrane (CEM) (Figure 8). Electrons derived from substrate oxidation are transferred from anode to cathode electrode through external load generating current. Protons migrate from anode to cathode through CEM. The flow of electrons is completed at the cathode electrode, where electrons and protons combine with electron acceptor, such as oxygen. The maximum theoretical voltage obtained from one MFC is in the order of 1.1 V (Logan et al. 2006) depending on anode and cathode reactions as well as cell configuration and conditions. The overall voltage can be enhanced by connecting several MFCs in series (Aelterman et al. 2006, Shin et al. 2006, Oh and Logan 2007).

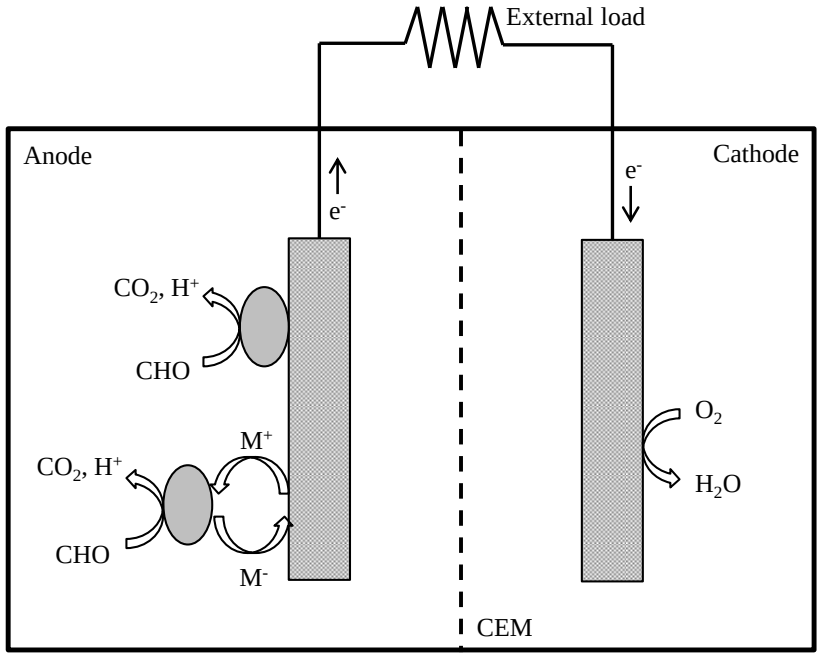


Figure 8. Two-chamber microbial fuel cell. Electrons can be transferred from bacteria to electrode via direct contact or with mediators (M). Protons migrate from anode to the cathode chamber through the membrane. (Adapted from Watanabe 2008)

6.1 Reactions and losses in MFCs

Electricity generation in MFCs is based on oxidation-reduction reactions (Table 10) that either produce or consume protons and electrons (Shukla et al. 2004). Organic electron donors used in MFCs include various volatile fatty acids (Fan et al. 2007a, Jung and Regan 2007), sugars (Rabaey et al. 2003, Catal et al. 2008), wastewaters (Oh and Logan 2005, Cheng et al. 2006a), and cellulose (Rismani-Yazdi et al. 2007, Ren et al. 2008). Sugars and more complex substrates are first fermented into soluble metabolites that are further converted to electricity by exoelectrogens, i.e., bacteria capable of transferring electrons directly to the electrode (Logan and Regan 2006, Logan 2009). Oxygen is the most often used electron acceptor due to its low cost, availability, high oxidation potential, sustainability, and lack of chemical waste products (Logan et al. 2006). However, incomplete oxygen reduction may lead to production of hydrogen peroxide (Rismani-Yazdi et al. 2008) and plain graphite electrode has low oxygen reduction activity (Gil et al. 2003). In laboratory studies, ferricyanide (Rabaey et al.

2003) or permanganate (You et al. 2006a) can be used as electron acceptor due to their high redox potential. However, their use at larger scale is not sustainable.

Table 10. Electron donors and acceptors that can be used at anode or cathode, respectively.

Electron donor/acceptor	Reaction	Reference
At the anode:		
Glucose	$C_6H_{12}O_6 + 6\ H_2O \rightarrow 24\ H^+ + 24\ e^- + 6\ CO_2$	Freguia et al. 2008
Acetic acid	$CH_3COOH + 2\ H_2O \rightarrow 8\ H^+ + 8\ e^- + 2\ CO_2$	Lalaurette et al. 2009
Ethanol	$CH_3CH_2OH + 3\ H_2O \rightarrow 12\ H^+ + 12\ e^- + 2\ CO_2$	Lalaurette et al. 2009
Succinic acid	$COOHCH_2CH_2COOH + 4\ H_2O \rightarrow 14\ H^+ + 14\ e^- + 4\ CO_2$	Lalaurette et al. 2009
Lactic acid	$HC_3CHOHCOOH + 3\ H_2O \rightarrow 12\ H^+ + 12\ e^- + 3\ CO_2$	Lalaurette et al. 2009
Formic acid	$HCOOH \rightarrow H_2 + CO_2$	Lalaurette et al. 2009
Hydrogen	$H_2 \rightarrow 2\ H^+ + 2\ e^-$	Freguia et al. 2008
At the cathode:		
Oxygen	$O_2 + 4\ e^- + 4\ H^+ \rightarrow 2\ H_2O$	Du et al. 2007
Hydrogen peroxide	$O_2 + 2\ H^+ + 2\ e^- \rightarrow H_2O_2$ $H_2O_2 + 2\ e^- + 2\ H^+ \rightarrow 2\ H_2O$	Du et al. 2007
Ferricyanide	$Fe(CN)_6^{3-} + e^- + H^+ \rightarrow Fe(CN)_6^{4-}$	Du et al. 2007
Permanganate	$MnO_4^- + 4\ H^+ + 3\ e^- \rightarrow MnO_2 + 2\ H_2O$	You et al. 2006a

Theoretically, all the biochemical energy in the substrate can be converted into electricity. In practice, however, losses occur due to microbial growth and MFC configuration. Electrons can be lost due to activation, ohmic and/or mass transport losses. Activation losses occur due to the activation barrier present in the substrate or electron acceptor and due to electrolyte resistance (Logan et al. 2006). These losses can be decreased by enhancing the biofilm thickness (Rabaey et al. 2007) or by increasing the electrode surface areas, temperature or substrate/oxidant concentration (Rismani-Yazdi et al. 2008). Ohmic losses are caused by the resistance of electrodes, electrolytes, interconnections (such as membranes), and MFC configuration (Clauwaert et al. 2008a, Rismani-Yazdi et al. 2008). Ohmic losses can be minimized by selecting highly conductive electrodes, improving contacts, decreasing the distance between anode and cathode electrodes, or by increasing solution conductivity (Liu et al. 2005a, Logan et al. 2006, Clauwaert et al. 2008b). Substrate diffusion or product removal close to the electrodes causes mass transport losses (Clauwaert et al. 2008a). For example, a thick biofilm may prevent diffusion at the electrode (Behera et al. 2010). Mass transport losses can be decreased by optimizing the operating conditions and geometry of MFCs or by choosing more efficient electrode materials (Rismani-Yazdi et al. 2008).

6.2 Microorganisms and electron transfer mechanisms

Both pure and mixed cultures have been used for electricity production in MFCs. Bacteria capable of transferring electrons exocellularly to the electrode are called exoelectrogens (Logan and Regan 2006, Logan 2009). Pure exoelectrogenic cultures include, e.g., *Geobacter sulfurreducens* (Bond and Lovley 2003), *Shewanella putrefaciens* (Kim et al. 1999), *Rhodoferax ferrireducens* (Chaudhuri and Lovley 2003), *Comamonas denitrificans* (Xing et al. 2010), *Enterobacter cloacae* (Rezaei et al. 2009), and *Ochrobactrum anthropic* (Zuo et al. 2008). Mixed cultures are often preferred over pure cultures since they (i) are more suitable for wastewater treatment, (ii) allow wider substrate versatility due to presence of both acidophilic and electrophilic microorganisms, (iii) have higher resistance against process disturbances, (iv) often give higher power outputs, and (v) obligate aerobes present minimize the effects oxygen diffusion through membrane (Angenent et al. 2004, Chang et al. 2006, Du et al. 2007). Mixed cultures used for electricity production have been enriched from, e.g., anaerobically digested sludge (Oh and Logan 2005, You et al. 2006a), domestic wastewaters

(Liu and Logan 2004), sediment (Mathis et al. 2008), soil (He and Angenent 2006, Ishii et al. 2008), and compost (Nercessian et al. 2012).

In the electron transfer chain, electrons are first transferred from inside to outside of the microbial cell membrane either physically via reduced compounds (mediators) or via electron hopping across the membrane using membrane bound redox enzymes (for a review, see Schröder 2007). Second, electrons have to be transferred from the bacterial cell to the electrode. This occurs only if no other electron acceptors, e.g. oxygen or nitrate, (Logan et al. 2006) and competing microorganisms, such as fermenters, acetogens and methanogens (Jung and Regan 2007), are present. Bacterial electron transfer can be either mediated or direct. In mediated approach electron shuttles (mediators) undergo redox cycling and transfer electrons from bacteria to electrode (Hernandez and Newman 2001). Exogenous mediators are added to the medium, while endogenous mediators are naturally present or produced by the bacteria (Bond and Lovely 2005). Direct electron transfer occurs by direct contact of bacterial cell wall to electrode by outer membrane redox proteins, such as cytochromes (Schröder 2007), or through nanowires (Reguera et al. 2005, Reguera et al. 2006). Direct electron transfer by bacteria attached to the anode electrode was first reported in the late 1990s by Kim et al. (1999) with *S. putrefaciens*.

6.3 Parameters affecting MFC performance

The MFC architecture is the most important barrier in achieving high power densities (Min et al. 2005, Logan and Regan 2006). In addition, improvements are needed in hardware, operation, and microbial communities (Wrighton and Coates 2009). The materials related to power generation have to be conductive, biocompatible, and chemically stable (Logan et al. 2006). Microbial community structure and activity are affected by environmental parameters, such as pH, conductivity and temperature (Watanabe 2008). Both constructional and operational parameters and their effects on MFC performance are listed in Table 11.

The reactor configuration, i.e., the use of one- or two-chamber MFCs has a huge effect on power generation and losses. The issue is further discussed in Chapter 6.4. Several electrode materials are applicable in MFCs and the main requirement is high surface area (Li et al. 2010). High surface area electrodes used in MFCs include graphite fiber brush (Logan et al. 2007) and activated carbon electrodes (Deng et al. 2010). MFC performance is often limited by the cathodic reactions, which can be improved with catalysts that decrease the activation energy barrier and improve the kinetics of oxygen reduction (Rismani-Yazdi et al. 2008). In addition to expensive platinum, chemicals used as catalysts include manganese (Park and Zeikus 2002, Zhang et al. 2009), iron(II)-phthalocyanine (Zhao et al. 2005), and cobalt tetramethoxyphenyl-phorphyrin (CoTMPP) (Zhao et al. 2005, Cheng et al. 2006b). Furthermore, a decrease in platinum loading from 2 to 0.1 mg/cm^2 resulted in only slight decrease in power density (Cheng et al. 2006b). Another option is to use biocathode, where microorganisms accept electrons from the electrode. Use of biocathode may decrease charge transfer resistance (Zhang et al. 2012), and simultaneously perform denitrification (Jia et al. 2008) or oxidation of iron or manganese (Mao et al. 2010).

Most MFC studies are conducted with mesophilic bacteria, while few studies have investigated the MFC performance at higher temperatures, above 50ºC (Choi et al. 2004, Mathis et al. 2008, Carver et al. 2011). Neutral anodic pH has been used in several MFC studies (Bond and Lovley 2003, Rismani-Yazdi et al. 2007, Borole et al. 2009). Some studies, however, suggest that alkaline pH values favor electrochemical reactions and result in higher

power outputs than neutral or acidophilic pH (Fan et al. 2007b, Yuan et al. 2011b). In addition, power outputs can be increased by increasing solution conductivities (Huang and Logan 2008).

Table 11. Parameters affecting MFC performance.

Factor	Effects	Reference(s)
Electrode material	+ Shortening electrode spacing increases power generation, +/- CE, power generation	
Graphite plates/rods	+ Inexpensive, easy to handle, defined surface area, stable in microbial cultures - Poor catalyst for oxygen reduction	Logan et al. 2006
Carbon cloth/paper	+ Good bacterial adhesion, stable in microbial cultures, - Expensive, difficult to scale up	Logan et al. 2007, Logan 2010
Graphite fiber brush	+ Large surface are, porous structure, easier to scale up	Logan et al. 2007, Logan 2010
Activated carbon	+ Large surface area, excellent adsorption capabilities, relatively inexpensive, high conductivity - Relatively poor oxygen reduction	Deng et al. 2010, Logan 2010, Sun et al. 2011b
Carbon nanotubes	+ Large surface area, fast biofilm formation, rapid stabilization	Mohanakrishna et al. 2012
Electrode catalyst	+/- CE, power generation	
Platinum (Pt)	+ Effective catalyst, low overpotential for oxygen reduction, - Expensive	Cheng et al. 2006b, Rismani-Yazdi et al. 2008
Lead dioxide (PbO_2)	+ Inexpensive, suitable replacement for Pt, - Lead may be toxic to microorganisms	Morris et al. 2007, Zhang et al. 2009
Iron(II)phthalocyanine	+ Effective catalyst, inexpensive, suitable replacement for Pt	Zhao et al. 2005, HaoYu et al. 2007
CoTMPP	+ Effective catalyst, highly porous, inexpensive, suitable replacement for Pt	Zhao et al. 2005, Cheng et al. 2006b
Manganese dioxide (MnO_2)	+ Inexpensive, easy to prepare, good activity towards oxygen reduction	Zhang et al. 2009
Biocathode	+ Reduced construction and operation costs, production of additional values (e.g. denitrification) - Carbon source is required, metabolites and ions that cross the membrane may hinder activity	He and Angenent 2006, Jia et al. 2008, Rismani-Yazdi et al. 2008
Membrane	+ Prevents water leakages, reduces oxygen diffusion to the anode, increases CE, allows closer electrode spacing - Expensive, increases internal resistance, membrane surface can meet fouling, CEM allows transport of other cations than H^+, may result in pH gradient	Jang et al. 2004, Liu and Logan 2004, Logan et al. 2006, Rozendal et al. 2006, Debabov 2008, Clauwaert et al. 2008b, Deng et al. 2010, Logan 2010
pH	+/- Power generation, electrochemical interactions between bacteria and electrodes, biofilm formation, bacterial growth, activity and rate of proton mass transfer through the liquid - Neutral pH reduces reduction kinetics of O_2, pH difference between chambers affects proton flux rate through the CEM	Gil et al. 2003, Liu et al. 2005a, Zhao et al. 2006, Jadhav and Ghangrekar 2009, Yuan et al. 2011b
Conductivity	+/- Power generation - Salts may inhibit proton transport through membrane, salt addition depends on the tolerance of bacteria	Gil et al. 2003, Liu et al. 2005a, Heilmann and Logan 2006, Kim et al. 2007, Huang and Logan 2008, Mohan and Das 2009, Nam et al. 2010a
Temperature	+/- COD removal efficiency, CE, power generation + Increased temperature: increases electricity yields and chemical and biochemical reactions, reduces losses at the cathode, and enables treatment of high-temperature industrial wastewaters - Increased temperature has larger energy requirements, affects tolerance of bacteria, and may result in water evaporation	Choi et al. 2004, Jong et al. 2006, Du et al. 2007, Rismani-Yazdi et al. 2008, Mathis et al. 2008, Jadhav and Ghangrekar 2009
External resistance	+/- CE, power generation, anode availability as an electron acceptor, changes anode bacterial communities and total substrate consumption	Gil et al. 2003, Jadhav and Ghangrekar 2009, Jung and Regan 2011

+ positive effect, - negative effect, +/-: can have either positive or negative effect, CE: Coulombic efficiency

6.4 Electricity generation in microbial fuel cells

Most of the MFC studies have been conducted in one- or two-chamber MFCs. Two-chamber MFCs usually have higher losses and thus, higher internal resistance. However, the electricity production can be combined with, e.g., denitrification (Jia et al. 2008) or copper recovery (Ter Hejne et al. 2010, Tao et al. 2011) at the cathode. One-chamber MFCs consist of anodic chamber, where cathode electrode is deposited to one side and is directly exposed to air. The air-cathode can be constructed with or without a membrane (Fan et al. 2007a). The advantages of one-chamber MFCs include simpler design, elimination of the use of catholyte, prevention of cathode aeration, and lower internal resistance (Du et al. 2007, Fan et al. 2007a, Rismani-Yazdi et al. 2008). Some of the highest power yields obtained in one- or two-chamber MFCs are presented in Table 12. The highest power densities have been reported in single-chamber MFCs having low anodic volume and platinum as cathode catalyst. Small electrode spacing improves proton transfer from anode to cathode electrode (Liu et al. 2005a) and platinum as catalyst promotes cathodic reaction (Cheng et al. 2006b).

The MFC performance is greatly affected by the type, concentration and feeding rate of substrate (Du et al. 2007). Using fermentable instead of non-fermentable substrates results in decreased Coulombic efficiencies (CEs), since proportion of the electrons are often directed to production of soluble metabolites instead of electricity (Lee et al. 2008b, Huang and Logan 2008). Wastewaters, on the other hand, may also contain some inorganic or non-biodegradable compounds that interfere with electricity production and decrease power densities and CEs (Nam et al. 2010b). Simple substrates, such as acetate and glucose, have been used to study the MFC performance with different reactor configurations, anode or cathode materials, pH values, temperatures, ionic strengths, or microbial communities. The goal is, however, to utilize wastewaters or other complex substrates in MFCs. Wastewaters from food-processing industries, breweries, and animal confinements contain high levels of easily degradable organic material and have high water content and thus, are especially suitable for MFCs (Angenent et al. 2004). Pant et al. (2010) have reviewed the substrates utilized for electricity production in MFCs.

Table 12. Electricity yields from different substrates utilized in one- or two-chamber MFCs.

e^--donor	**e^--acceptor**	**Culture**	**Anode material**	**Membrane**	**PD (mW/m^2)[a]**	**PD (W/m^3)[c]**	**CE (%)**	**Reference**
One-chamber MFC								
Acetate	Air, Pt	Domestic WW	Carbon paper	-	1330	nr	61	Liu et al. 2005b
Acetate	Air, Pt	Enriched culture	Carbon cloth	-	1120	627	71	Fan et al. 2007a
Acetate	Air, Pt	Enriched culture	Carbon cloth	-	2770	1550	nr	Fan et al. 2007b
Acetate	Air, Pt	Enriched culture	Graphite brush	-	2400[b]	73	60	Logan et al. 2007
Acetate	Air, Pt	Enriched culture	Carbon cloth	-	6860	nr	nr	Fan et al. 2008
Xylose	Air, Pt	Enriched culture	Graphite-fiber brush	Plastic mesh	673	13	50	Huang and Logan 2008
Xylose	Air, Pt	Enriched culture	Carbon cloth	-	2330	136	31	Catal et al. 2008
Glucose					2160	126	28	
Glucuronic acid					2770	162	24	
Domestic WW	Air, Pt	Domestic WW	Carbon paper	Nafion	28	0.7	28	Liu and Logan 2004
				-	146	3.7	20	
Paper recycling WW	Air, Pt	Paper recycling WW	Graphite-fiber brush	-	672[b]	nr	nr	Huang and Logan 2008
$BioH_2$ effluent	Air, Pt	Sludge	Carbon paper	-	371	nr	nr	Oh and Logan 2005
Domestic WW	Air, Pt	Domestic WW	Carbon cloth	-	464	15.5	27	Cheng et al. 2006a
Meat packing WW	Air	Meat packing WW	Carbon paper	-	139	14.0	nr	Heilmann and Logan 2006
		Domestic WW			93	9.4		
Distillery WW	Air	UASB reactor	Carbon nanotube	Nafion	245	3.4	nr	Mohanakrishna et al. 2012
Two-chamber MFC								
Acetate	HCF, Pt	Digested sludge	Carbon felt	Nafion	3650	345	nr	Borole et al. 2009
Acetate	HCF	*G. sulfurreducens*	Carbon fibre	Nafion	1900	43.3	100	Nevin et al. 2008
		Digested sludge			1600	1.4	40-45	
Acetate	O_2, Pt	Digested sludge	Graphite felt	Nafion	1030	nr	80	Jong et al. 2006
Glucose	HCF	Enriched culture	Graphite	Ultrex	3600	216	65-89	Rabaey et al. 2003
Glucose	HCF	Digested sludge	Graphite rod	Ultrex	4310	nr	81	Rabaey et al. 2004
Landfill leachate	HCF	Digested sludge	Activated carbon	Nafion	nr	2.1	nr	You et al. 2006b
Food processing WW	Air, Pt	Sludge	Carbon paper	Nafion	81	0.59	nr	Oh and Logan 2005
Cellulose	HCF	Rumen microbes	Graphite plate	Ultrex	55	1.2	nr	Rismani-Yazdi et al. 2007

[a] power density against the anode area, [b] power density against the cathode area, [c] power density against the anode volume

nr: not reported, -: no membrane, WW: wastewater, air: air-cathode, HCF: hexacyanoferrate, Pt: platinum, UASB: upflow anaerobic sludge blanket reactor

In future, MFCs may not be applicable solely for electricity production and/or wastewater treatment (Rozendal et al. 2008). Pham et al. (2006) suggested that to be competitive with anaerobic digestion for wastewater treatment, MFCs should produce at least 400 W/m^3 when scaled-up. As Table 12 suggests these power densities are at the moment obtained only at small, laboratory scale MFCs. However, MFCs may become viable at lower power densities, if they are combined with other value having processes, such as bioremediation, denitrification or hydrogen production at the cathode (Jia et al. 2008, Lovley and Nevin 2011). Before commercialization MFCs have to be scaled-up. Challenges that need to be overcome before scaling-up include reducing costs of electrode materials as well as construction and operation, replacing precious metal catalysts with other materials, and maximizing power densities (Rabaey et al. 2003, Angenent et al. 2004, Liu and Logan 2004, Logan 2010). In addition, the longevity of materials needs to be determined (Logan 2010).

At least two pilot-scale MFC configurations have been reported so far. Jiang et al. (2011) operated a 20 L MFC consisting of 12 anode/cathode compartments with domestic wastewater. The highest power density was 500 mW/m^2 with Co-MnO_2 cathodes and the maximum contaminant removal was 80 % with HRT of 20 h. Problems were encountered with calcium and sodium precipitation on the cathode. University of Queensland and Foster´s Yatala Brewery have run in co-operation a 1000 L MFC consisting of 12 modules (www.microbialfuelcell.org). They reported that MFC operation for electricity production may not be sustainable but simultaneous production of caustic soda at the cathode could make the process economical. In addition to these two MFCs, Cusick et al. (2011) operated a 1000 L MEC for the production of hydrogen from winery wastewater. The reactor consisted of 24 modules (144 electrode pairs) and produced 0.19 L gas/L/d. However, most of the biogas consisted of methane, which requires further process development in the future.

7. SEQUENTIAL PROCESS OPTIONS FOR PRODUCTION OF MULTIPLE ENERGY CARRIERS

Dark fermentative hydrogen production converts at the highest 33 % of the substrate to hydrogen, whilst the rest of the substrate is converted into soluble metabolites. The effluents from dark fermentation can be further utilized for hydrogen production through photofermentation or with microbial electrolysis cells, for methane production through anaerobic digestion, or for electricity production with microbial fuel cells. In addition, the effluents from ethanol fermentation may contain some organics that can be further utilized, for example for hydrogen or methane production (Juang et al. 2011). Potential sequential processes for the production of different energy carriers are presented in Figure 9.

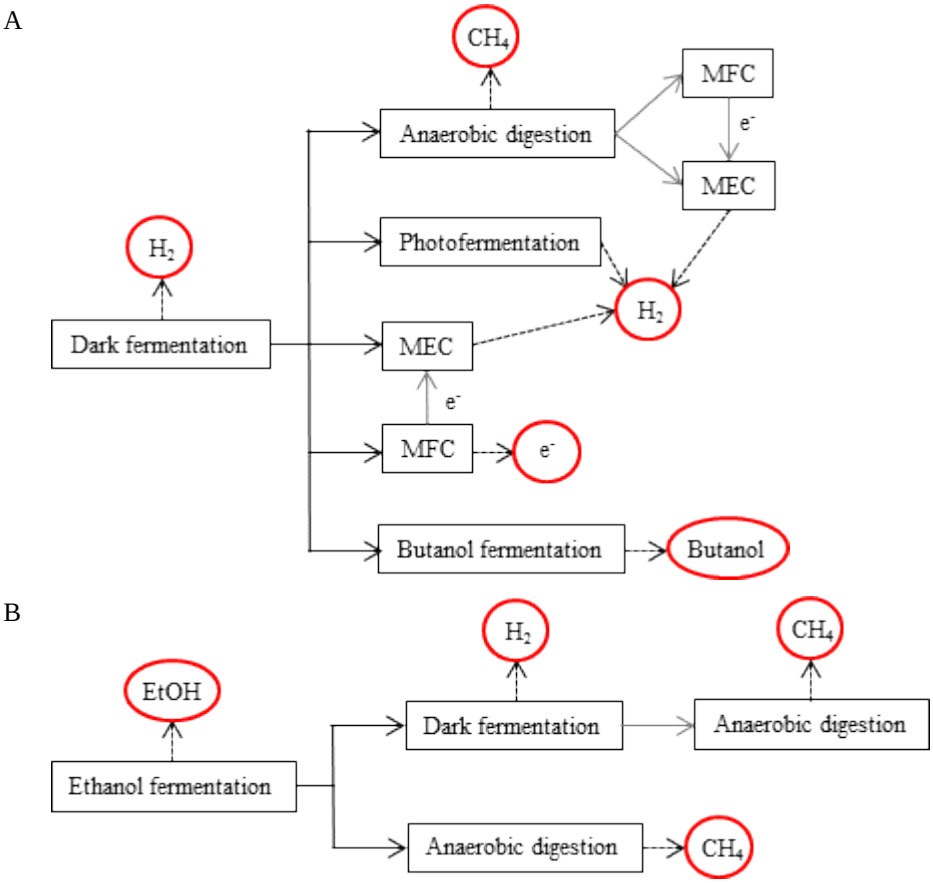


Figure 9. Sequential production of different energy carriers. Dark fermentation effluents can be utilized in anaerobic digestion, photofermentation, butanol fermentation, MFCs or MECs (A). Effluents from ethanol fermentation can be used for methane or hydrogen production, or for two-step H_2 and CH_4 production (B). Possible additional routes are presented in grey and the produced energy carriers are marked with red circles.

Sequential processes have the potential of efficient energy recovery from wastewaters. Furthermore, dividing the process into two different steps enables the optimization of each sub-process separately (Guwy et al. 2011). Anaerobic digestion process is already used at large scale and it could be added promptly after dark fermentation step. This two-stage process produces a mixture of H_2 and CH_4 gases that is shown to result in cleaner conversion

in internal combustion engines than methane alone (Bauer et al. 2001). Using two-stage process instead of one-stage methanogenic reactor allows the use of higher organic loading rates and reduces the retention time of the methanogenic reactor (Ueno et al. 2007a). There are already pilot-scale studies on two-step H_2 and CH_4 producing processes (Ueno et al. 2007b, Lee and Chung 2010, Cavinato et al. 2012). Lee and Chung (2010) reported a 25 % increase in the energy production when two-step process was compared to one-stage methane production. Furthermore, Liu et al. (2006) obtained 21 % higher methane yield in two- than one-stage process.

Dark fermentation effluents have also been used for the production of hydrogen either through photofermentation (Chen et al. 2008, Özgur et al. 2010) or with MECs (Lu et al. 2009, Wang et al. 2011c). Both processes can theoretically convert all the organics into hydrogen with a yield of 12 mol H_2/mol glucose (Hallenbeck and Ghosh 2009, Guwy et al. 2011). In practice, the light conversion efficiencies in photofermentation are low and the photobioreactors are expensive (for a review, see Hallenbeck et al. 2012). In MECs, energy has to be added to the process, the current densities are low due to high losses, and expensive metal cathodes are often required (Hallenbeck et al. 2012). Lalaurette et al. (2009) obtained 9.95 mol H_2/mol glucose from cellobiose with two-step process combining dark fermentation and MEC.

In addition to above mentioned processes, electricity can be produced from dark fermentation effluent in MFCs. Mohanakrishna et al. (2010) reported a 71 % increase in the COD removal efficiency when utilizing the hydrogen fermentation effluents in MFC compared to one-stage dark fermentative hydrogen production. Wang et al. (2011c) added two MFCs and a MEC after dark fermentation and utilized the electricity produced in MFCs for the production of H_2 in MEC. This process resulted in 41 % increase in hydrogen yield when compared to one-step dark fermentation with the highest yield of 2.58 mol H_2/mol hexose without added electricity.

In ethanol fermentation from energy crops about 75-80 % of the organics is converted into ethanol and thus, 20-25 % of organics remain in the effluent (Juang et al. 2011). The energy from these effluents can be utilized for the production of hydrogen and/or methane (Zhang et al. 2010d, Juang et al. 2011). Dererie et al. (2011) produced 9.8 MJ/kg dry oat straw (85-87 % of the maximum energy yield) from two-step ethanol and methane production process. They obtained 32 % more energy by adding an ethanol fermentation step before methane reactor. In addition, 30 % higher carbon utilization has been reported in two-step ethanol-CH_4 process when compared to one-step methane fermentation (Uellendahl and Ahring 2010). A two-step process producing ethanol and methane has been studied at pilot-scale (Uellendahl and Ahring 2010) and is at the moment under commercialization (Ahring and Langvad 2008, Langvad et al. 2010). Results from different sequential processes are reported in Table 13.

Table 13. Sequential two-step processes.

	1st energy carrier		2nd energy carrier		
Substrate	**Target**	**Yield**	**Target**	**Yield**	**Reference**
Food waste	H_2	1.8 mol H_2/mol hexose 3.9 mL H_2/L/d	CH_4	5.7-6.5 L CH_4/L/d	Lee and Chung 2010
Food waste	H_2	66.7 L H_2/kg VS	CH_4	720 L CH_4/kg VS	Cavinato et al. 2012
Household solid waste	H_2	43 L H_2/kg VS	CH_4	500 L CH_4/kg VS	Liu et al. 2006
Garbage slurry and milled paper	H_2	4.8 L H_2/L/d	CH_4	10.7 L CH_4/L/d	Ueno et al. 2007a
Garbage and paper waste	H_2	5.4 L H_2/L/d 2.4 mol H_2/mol hexose	CH_4	6.1 L CH_4/L/d	Ueno et al. 2007b
Glucose	H_2	3.3 mol H_2/mol hexose	H_2 (PF)	1.7 mol H_2/mol acetate	Nath et al. 2008
Sucrose	H_2	3.7 mol H_2/mol sucrose	H_2 (PF)	3.0 mol H_2/mol sucrose	Tao et al. 2007
Sucrose	H_2	3.8 mol H_2/mol sucrose	H_2 (PF)	10.2 mol H_2/mol sucrose	Chen et al. 2008
Glucose Molasses	H_2	2.3 mol H_2/mol hexose 2.1 mol H_2/mol hexose	H_2 (PF)	1.3 mol H_2/mol hexose [a] 3.7 mol H_2/mol hexose	Özgur et al. 2010
Corn stover Cellobiose	H_2	1.7 mol H_2/mol hexose 1.6 mol H_2/mol hexose	H_2 (MEC)	2.4 mol H_2/mol hexose 3.0 mol H_2/mol hexose	Lalaurette et al. 2009
Molasses wastewater	H_2	0.3 mol H_2/mol hexose, 0.70 L H_2/L/d	H_2 (MEC)	1.8 mol H_2/mol hexose, 1.41 L H_2/L/d	Lu et al. 2009
Glucose	H_2	2.7 mol H_2/mol hexose	e^-	4.2 W/m^3	Sharma and Li 2010
Food processing wastewater	H_2	0.8 mol H_2/mol hexose, 210 mL H_2/L	e^-	0.37 W/m^2	Oh and Logan 2005
Vegetable waste	H_2	0.24 L H_2/d	e^-	1.56 W/m^3, 0.11 W/m^2	Mohanakrishna et al. 2010
Oat straw (steam explosion and enzymatic hydrolysis)	EtOH	0.19 L EtOH/kg DM	CH_4	0.23 L CH_4/g VS	Dererie et al. 2011
Straw (wet explosion and enzymatic hydrolysis)	EtOH	300 L EtOH/L	CH_4	67-79 m^3 CH_4/L	Uellendahl and Ahring 2010

[a] No buffer used, DM: dry matter, VS: volatile solids

8. HYPOTHESES AND AIMS OF THE PRESENT WORK

The main objective of this work was to utilize cellulosic materials for the biological production of different energy carriers (hydrogen, methane, ethanol, butanol and electricity). Furthermore, microbial communities enriched from different sources for energy carrier(s) production were characterized. Cellulosic and lignocellulosic materials consist of hexose and pentose sugars (Kumar et al. 2008) that are amenable to fermentation to hydrogen (Calli et al. 2008b, Karadag and Puhakka 2010b), ethanol (Rebros et al. 2005, Wang et al. 2011c) or butanol (Ezeji et al. 2007b, Tanaka et al. 2012), or for the production of bioelectricity (Rabaey et al. 2004, Catal et al. 2008). Previously, it was reported that a hot spring enrichment culture fermented glucose to hydrogen in a continuous reactor with high H_2 yields (Karadag and Puhakka 2010a). However, for hydrogen fermentation to be feasible, the hydrogen producing culture should utilize all the common hexoses and pentoses that may be present in, e.g., hydrolysates. It was hypothesized that the hot sping culture can also ferment other hexose and pentose sugars to hydrogen in batch and continuous mode (Paper I). To obtain hexose and pentose sugars, cellulosic materials can be pretreated and/or hydrolyzed, e.g., with chemical or enzymatic treatments (Cao et al. 2009, Guo et al. 2010b). It was hypothesized that hydrolyzation of cellulosic pulp materials with concentrated sulfuric acid would result in a mixture of sugars amenable to hydrogen fermentation with the hot spring culture (Paper IV).

Adding pretreatment-step prior to hydrogen fermentation increases the process costs and complexity. Another option for hydrogen production from cellulosic materials would be consolidated bioprocessing, where cellulose is hydrolyzed to sugars and sugars are simultaneously fermented to hydrogen (Lynd et al. 2005). It was hypothesized that cellulolytic and hydrogenic cultures can be enriched from generally available sources, such as compost and rumen fluid (Papers II-IV). Some renewable substrates may already contain cellulolytic and hydrogenic bacteria. In these cases bioaugmentation may not be needed. Thus, it was hypothesized that grass silage would contain indigenous bacteria capable of cellulose hydrolysis and hydrogen fermentation, which was tested at laboratory scale (Paper V). Growth conditions affect considerably both bacterial cellulose hydrolysis (Hu et al. 2004, Lo et al. 2008) and hydrogen fermentation (Kim et al. 2006a, Karadag and Puhakka 2010a,b). However, the optimal conditions for cellulose hydrolysis and hydrogen fermentation often differ. It was hypothesized that enrichment of cellulolytic and hydrogenic cultures would be influenced by temperature (Papers II,III), pH (Papers III,IV), and substrate concentration (Paper V). Furthermore, the effects of enrichment conditions on microbial communities were evaluated. It was hypothesized that operational conditions would change the microbial community composition.

Mixed cultures often contain both hydrogen producing and hydrogen consuming microorganisms, such as methanogens, acetogens or sulfate-reducers (Baghchehsaraee et al. 2008, Calli et al. 2008b, O-Thong et al. 2009). The growth of hydrogen consuming microorganisms can be inhibited, e.g., by heat treatment (O-Thong et al. 2009, Ravindran et al. 2010), acid or base treatment (Mu et al. 2007, Lee et al. 2009), or by controlling the process parameters, such as pH or HRT (Oh et al. 2003, Karadag and Puhakka 2010a). However, culture pretreatment may also inhibit some cellulolytic bacteria and thus, decrease hydrogen yields (Lin and Hung 2008). It was hypothesized that the severity of heat treatment and the following elevated growth temperature would affect the hydrogen production potentials and microbial community compositions (Papers II,III). It was further hypothesized

that a chemical inhibitor specific to methanogens (BESA) could enhance hydrogen production (Papers IV,V).

Theoretically, dark fermentative hydrogen production can utilize only 33 % of the chemically boung organic matter to hydrogen (Hallenbeck 2005). Thus, the remaining effluent contains a lot of energy that can be converted, for example, to methane through anaerobic digestion (Ueno et al. 2007a,b, Cavinato et al. 2012) or to bioelectricity in MFCs (Oh and Logan 2005, Sharma and Li 2010). The second-step processes have to convert both sugars and soluble metabolites into energy carrier(s). Bioelectricity production from glucose and volatile fatty acids has been studied extensively (e.g., Rabaey et al. 2004, Logan et al. 2007, Catal et al. 2008). It was hypothesized that exoelectrogenic cultures converting a pentose sugars, i.e. xylose, to electricity and alcohol(s) could be enriched in two-chamber microbial fuel cells (Paper VI).

Based on the hypothesis described above, the specific aims of the thesis were as follows:

- To study the ability of a hot spring enrichment culture to ferment different sugars to hydrogen and to determine the hydrogen production potential of a hot spring culture from xylose in a continuous completely stirred tank reactor (CSTR) (Paper I).
- To enrich cellulolytic and hydrogenic cultures from locally available source at different operational conditions, including
    - pH (Papers III,IV),
    - elevated temperature (Papers II,III) and
    - substrate concentration (Paper V).
- To study the effects of culture pretreatments, i.e., heat treatment (Papers II,III) and chemical inhibitor (Papers IV,V) on cellulose fermentation to hydrogen.
- To examine the potential of using indigenous silage bacteria for the simultaneous silage hydrolysis and hydrogen fermentation (Paper V).
- To optimize concentrated sulfuric acid hydrolysis of different pulp materials and to determine the amenability of the hydrolysates for hydrogen fermentation with a hot spring enrichment culture (Paper IV).
- To characterize the microbial communities responsible for hydrogen production from sugars (Paper I) and cellulosic substrates (Papers II-V).
- To enrich and characterize electricity and alcohol(s) producing cultures on xylose in two-chamber microbial fuel cells and to study the effect of xylose concentration on electricity and alcohol(s) production (Paper VI).

9. MATERIALS AND METHODS

9.1 Enrichment cultures

Hydrogen producing culture growing on glucose was previously enriched from Hisarkoy hot spring, Turkey (Karadag and Puhakka 2010a). Cellulolytic and hydrogenic cultures were enriched from rumen fluid obtained from a fistulated cow (MTT Agrifood Research Institute, Jokioinen, Finland), compost obtained from a Solid Waste Management Site (Tarastenjärvi, Tampere, Finland) treating municipal biodegradable wastes, and from grass silage (MTT Agrifood Research Institute, Jokioinen, Finland). Exoelectrogenic cultures growing on xylose were enriched from compost (Tarastenjärvi, Tampere, Finland) or from anaerobically digested sludge of a sewage treatment plant (Viinikanlahti, Tampere, Finland). Cultures enriched for the production of different energy carriers at different process conditions (Figure 10).

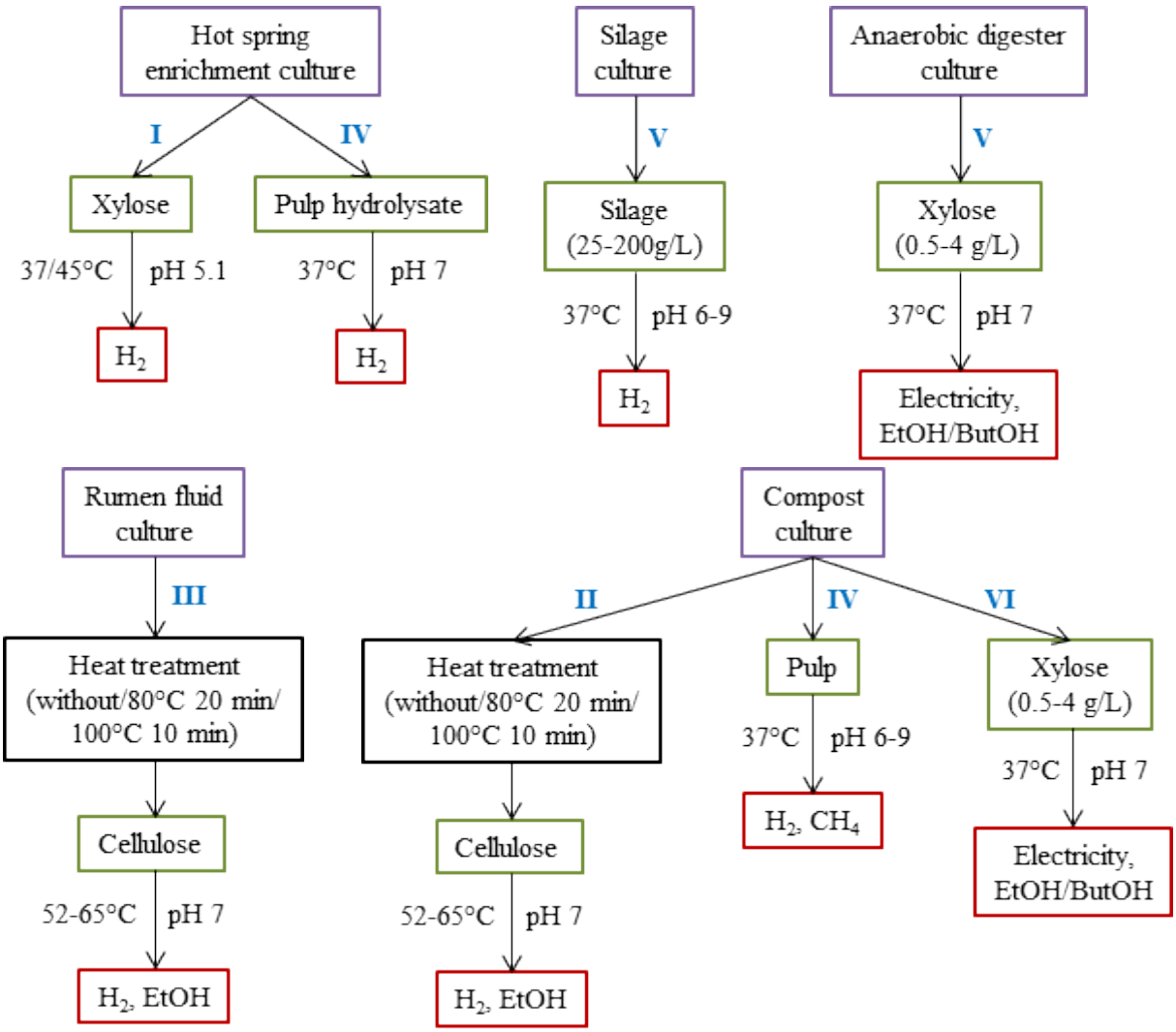


Figure 10. Outline of the experimental designs of this stydy. The cultures and substrates used are emphasized with purple and green lines, respectively. Energy carriers produced are emphasized with red lines and possible culture pretreatments with black lines. Related papers are highlighted in blue.

9.2 Hydrogen fermentation

The ability of a Hisarkoy hot spring culture to ferment different sugars to hydrogen was studied in 120 mL batch bottles with a working volume of 50 mL (Paper I). Experiments were conducted in triplicate at 45ºC, with initial pH of 6.8, and with 2 % (v/v) inoculum. Hydrogen fermentation from xylose was further studied in a continuous reactor (CSTR, 0.9 L) at 37 or 45ºC (Figure 11). Continuous on-line titration maintained the pH at 5.1, HRT was kept at 10 or 12.5 h, and 17 % (v/v) inoculum was added in the beginning of the experiment (Paper I).

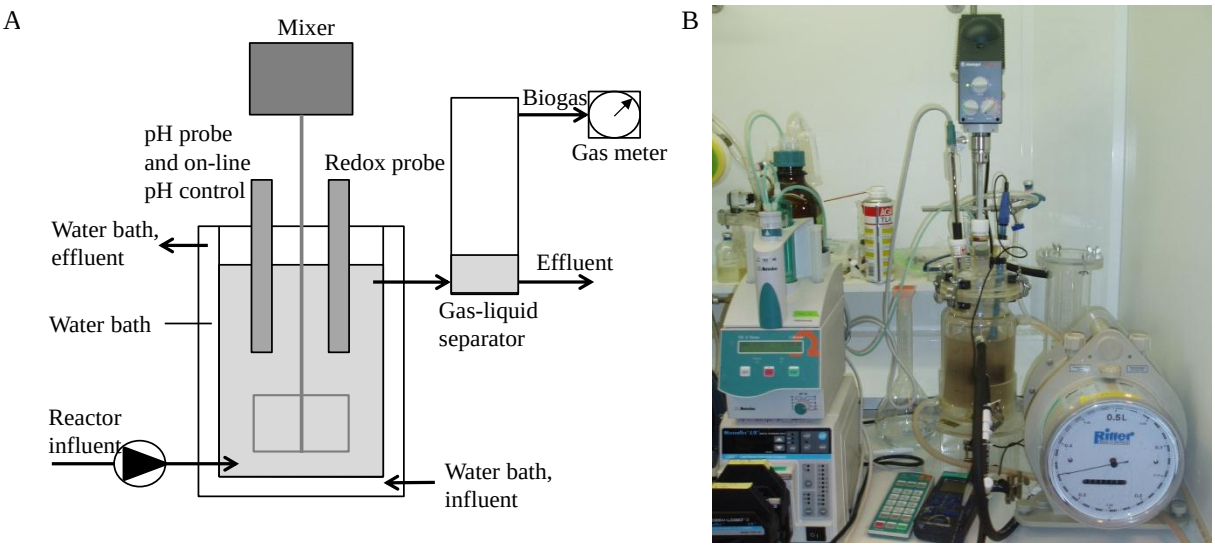


Figure 11. Shematic diagram (A) and photograph (B) of CSTR configuration used in Paper I.

Dry pulp obtained from a paper factory (UPM-Kymmene Oyj, Jämsänkoski, Finland) was hydrolyzed with 55 % H_2SO_4 at 37°C (Paper IV). The hydrolysis conditions were optimized for the reaction time and the hydrolysate was used for hydrogen production in 120 mL batch bottles (50 mL working volume) at 37°C. Hot spring culture able to utilize various sugars was used as inoculum (4 % v/v). Initial total sugar concentration and pH were 10 g/L and 7.0, respectively. The optimal pH (5-9) and temperature (25-43°C) for hydrogen production from hydrolysate were determined.

Fermentation of cellulosic materials, including cellulose (Papers II,III), pulp (Paper IV) and silage (V), to hydrogen was studied with different enrichment cultures (Figure 10). The effect of culture heat treatment on thermophilic hydrogen production potential from 5 g/L cellulose was studied (Papers II, III) with following procedures: no heat treatment, heat treatment at 80°C for 20 min, or heat treatment at 100°C for 10 min. Experiments were conducted in 120 mL batch bottles having a working volume of 50 mL, and with 2 % (v/v) inoculum. Furthermore, the optimal temperature (45-75°C) and pH (5.2-7.3) for hydrogen fermentation from cellulose were determined with rumen fluid enrichment culture (Paper III).

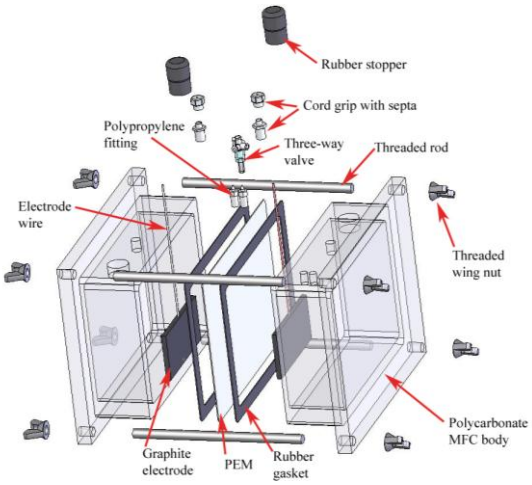
The effect of pH on the hydrogen production potential from wet or dry pulps made from conifer or birch was studied (Paper IV). The enrichment of cellulolytic and hydrogenic cultures was done in triplicate at 37°C, in 500 mL batch bottles with a working volume of 150 mL, and at different pH values (6-9). The concentrations of dry pulps, wet birch and wet conifer pulps were 5, 1.1 and 1.2 g TS/L. The second enrichment step was conducted in triplicate at pH 7 and in 500 mL batch bottles (working volume 250 mL) with substrate concentration of 5 g TS/L. Methanogens were inhibited with the addition of 20 mM BESA.

Hydrogen fermentation from silage was performed with the indigenous bacteria present in the silage by increasing the pH from below 4.0 to 7.0 (Paper V). Hydrogen production potential was studied at different silage concentration (25-200 g/L) and at 37°C. 500 mL batch bottles having a working volume of 150 mL were used and no buffer was added. In the second enrichment step, working volume was increased to 250 mL, buffered medium was used, silage concentration was 25 g/L, and methanogens were inhibited with 20 mM BESA.

9.3 Bioelectricity production in microbial fuel cells

Exoelectrogens were enriched on xylose in fed-batch two-chamber MFCs (Figure 12) at 37°C (Paper VI). Anode and cathode had total and working volumes of 95 and 75 mL and were separated with a cation exchange membrane (CMI-7000S). Electrodes were made from graphite, external resistance was 100 Ω, anodic pH was adjusted to 7.0±0.1, and buffered 50 mM potassium ferricyanide was used as catholyte.

A



B

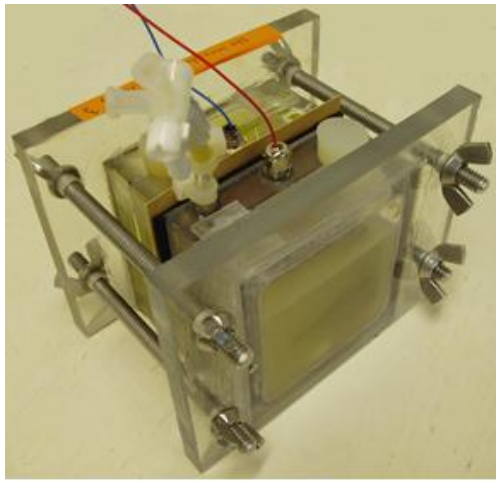


Figure 12. Assembly (A) and photograph (B) of the two-chamber MFC used in Paper VI (http://digitalunion.osu.edu/r2/summer07/nskrinak/assembly.html).

9.4 Analyses

Physicochemical and microbiological analyses conducted in the experiments were as presented in Table 14. The composition and characteristics of silage (Paper V) were analyzed by MTT Agrifood Research, Finland.

Table 14. Physicochemical and microbiological analysis used in the experiments.

Analysis	Method / Instrument	Paper(s)
Physicochemical		
pH	pH paper	I-III
	pH electrode	I, IV-VI
Oxidation-reduction potential	Redox electrode	I
Volume of biogas	Syringe	I-V
	Gas meter	I
	Gas-bag, water displacement method	VI
Gas composition (H_2, CH_4, CO_2)	GC-TCD	I-VI
Volatile fatty acids and alcohols	HPLC	I-V
	GC-FID	VI
Sugar distribution	HPLC	I, IV-V
Total (soluble) sugars	Spectrophotometer (phenol-H_2SO_4 method)	IV-VI
Total solids (TS)	Oven, balance	IV-V
Volatile suspended solids (VSS)	Oven, furnace, balance	I
Voltage	Data logger	VI
Performance analysis	Voltage meter and resistance box	VI
Microbial diversity, identification and analysis		
DNA extraction	VIOGENE Blood and Tissue Genomic DNA kit	II-III
	PowerSoil™ DNA isolation kit	I, IV-VI
Polymerase chain reaction (PCR)	Primer pair GC-BacV3f and 907r	I-VI
Denaturing gradient gel electrophoresis (DGGE)		I-VI
Sequence data analysis	Bioedit-software (version 7.0.5)	I-VI
Sequence identification	Comparison in GenBank	I-VI
Phylogenetic tree	MUSCLE, PhyML, TreeDyn (www.phylogeny.fr)	VI

9.5 Calculations

Cumulative gas productions were calculated according to Logan et al. (2002) after correcting the gas volumes to standard pressure (760 mm Hg) and temperature (0°C). The energy yields were calculated based on the lower heating values that are 120 MJ/kg for H_2 (Perera et al. 2012), 50.1 MJ/kg for CH_4 (Kedia and Ghoniem 2012), and 26.8 and 33.1 MJ/kg for ethanol and butanol, respectively (Arnas et al. 2012). COD in degradation products were calculated with Eq. 3 (van Haandel and van der Lubbe 2007), and was further used to calculate cellulose degradation in Papers II and III.

$$COD = 8 \times (4x + y - 2z) / (12x + y + 16z) \text{ g COD / g } C_xH_yO_z \quad (3)$$

Electricity production in MFCs was monitored by recording the voltage every 2 min. Voltage (U) was used to calculate current (I) (Eq. 4) and power (P) (Eq. 5). Current and power densities were calculated against the area or working volume of the anode. Coulombic efficiency (CE) was calculated according to Eq. 6, where C_p is calculated by integrating the current over time, F is Faraday´s constant (96 485 C/mol e^-), n is the molar amount of substrate, and z is the number of electrons produced per mol substrate. Complete Coulombic analysis was done as described by Huang and Logan (2008). The energy produced in the MFCs was calculated by integrating the power over time.

$$I = U / R \quad (4)$$

$$P = U \times I \quad (5)$$

$$CE = (C_p / C_t) \times 100\ \% = (\int I\, dt / Fnz)) \times 100\ \% \quad (6)$$

10. RESULTS AND DISCUSSION

10.1 Hydrogen production from cellulosic substrates

10.1.1 Effects of culture pretreatment

Mixed cultures usually contain both hydrogen producing and consuming microorganisms. Microbial communities have been pretreated to increase the hydrogen production potentials. Methanogens can be inhibited, e.g., by heat treatment or by extreme acidic or alkaline pH (Chang et al. 2011b, Wang et al. 2011a). In addition, a chemical inhibitor (BESA) specific to methanogens (Zhu and Beland 2006) has been used to enhance hydrogen production (O-Thong et al. 2009). Heat treatment has been reported more efficient than acid or base additions (Mu et al. 2007, Wang et al. 2011a, Zhang et al. 2011a). Although, Chang et al. (2011b) and Chaganti et al. (2012) obtained higher H_2 yields with acid pretreatment. Alkaline pretreatments have not always been successful in inhibiting methanogens (O-Thong et al. 2009, Wang et al. 2011a, Chaganti et al. 2012). In this study, the effects of different heat treatments (Papers II,III) and addition of BESA (Papers IV,V) on the hydrogen production potentials were examined. In Table 15, the highest hydrogen yields after pretreatments are reported and compared to the results obtained in other studies.

Compost (Paper II) and rumen fluid (Paper III) cultures were pretreated at 80°C for 20 min or at 100°C for 10 min and used for hydrogen production from cellulose at elevated temperatures (52-70°C). The hydrogen yields were compared to results obtained with cultures that were not pretreated. Hydrogen producers were successfully enriched at 52°C from compost (Paper II) and at 60°C from rumen fluid (Paper III). At 52°C, heat-treating the compost culture at 80°C for 20 min resulted in a hydrogen yield of 2.4 mol H_2/mol $hexose_{degraded}$ (1.4 mol H_2/mol hexose) (Paper II). This H_2 yield is 60 % from the maximum H_2 yield of 4 mol H_2/mol hexose produced with acetate as the only soluble metabolite. With both compost and rumen fluid cultures the most severe pretreatment at 100°C resulted in low hydrogen yields. Wang et al. (2011a) and Baghchehsaraee et al. (2008) also reported decreased hydrogen yields with heat treatments above 80°C and 65°C, respectively. Hydrogen yields after soil heat treatment were increased with increased treatment temperature from 65 to 105°C, whereas heat treatment at 120°C decreased H_2 yields (Ravindran et al. 2010). In addition, use of BESA decreased hydrogen yields (Papers IV,V, Chang et al. 2011b). BESA may inhibit some Clostridial species (Wang et al. 2003b), which likely decreased hydrogen yields.

Pretreatment is not always advantageous. Enrichment of heat-treated rumen fluid culture resulted in lower H_2 yields than non-heat-treated culture (Paper III). These results agree with the conclusions by Lin and Hung (2008) who reported two times higher H_2 yields from cellulose without heat treatment. In addition, Kawagoshi et al. (2005) and Yossan et al. (2012) attained similar H_2 yields with and without heat treatment. Many hydrogen producing bacteria are spore-formers that can be enriched with heat treatment, but heat treatment may also inhibit some non-spore-forming cellulolytic bacteria (Lin and Hung 2008). Inhibition of cellulolytic bacteria likely resulted in the low H_2 yields reported from cellulose after heat treatment with rumen fluid culture (Paper III). Heat treatment has enhanced the hydrogen yields from sugars (Table 15) suggesting that heat treatments successfully enrich for saccharolytic hydrogen

producers. However, effects of heat treatments on hydrogen production are highly dependent on the original microbial community. For example, heat treatment of rumen fluid culture did not affect the bacterial diversity (Paper III), whilst the diversity of microbial communities decreased when compost culture was heat-treated (Paper II). The enrichment of cellulolytic and hydrogenic communities, on the other hand, had similar effects within both cultures resulting in decreased bacterial diversities in sequential enrichments (Papers II,III).

Table 15. Effects of different pretreatments of hydrogen yields by dark fermentation (hydrogen yield without pretreatment is given in parantheses). The better yields are marked in bold.

Pretreatment	Culture	Substrate	H_2 yield (mol H_2/mol hexose)	Reference
Heat treatment				
65°C, 30 min	Digested sludge	Glucose	**2.30** (0.43)	Baghchehsaraee et al. 2008
80°C, 20 min	Compost	Cellulose	**1.41** (0.62)	Paper II
80°C, 20 min	Rumen fluid	Cellulose	0.32 (**0.44**)	Paper III
80°C, 30 min	Anaerobic sludge	Glucose	**3.58** (2.34)	Wang et al. 2011a
95°C, 30 min	Activated sludge	Glucose	**0.90** (0.38)	Chang et al. 2011b
95°C, 1 h	Soil	Glucose	**1.93** (0.54)	Ravindran et al. 2010
100°C, 1 h	Aerobic sludge	Corn stover hydrolysate	**0.91** (0.49)	Zhang et al. 2011a
100°C, 1 h	Digested sludge	Sucrose	**1.16** (0.30)	O-Thong et al. 2009
100°C, 2 h	Digested sludge	Glucose	**1.38** (1.32)	Kawagoshi et al. 2005
104°C, 2 h	Digested sludge	Glucose	**0.97** (0.60)	Oh et al. 2003
Acid pretreatment				
1 M HCl, pH 4, 3 h	Anaerobic sludge	Glucose	**3.18** (2.34)	Wang et al. 2011d
1 M HCl, pH 3-4, 1 d	Digested sludge	Sucrose	**0.65** (0.30)	O-Thong et al. 2009
1 M HCl, pH 3, 1 d	Aerobic sludge	Corn stover hydrolysate	**0.58** (0.49)	Zhang et al. 2011a
1 M HCl, pH 3, 1 d	Activated sludge	Glucose	**1.51** (0.38)	Chang et al. 2011b
Alkaline pretreatment				
1 M NaOH, pH 10, 3 h	Anaerobic sludge	Glucose	**3.43** (2.34)	Wang et al. 2011a
1 M NaOH, pH 10, 1 d	Activated sludge	Glucose	**1.34** (0.38)	Chang et al. 2011b
1 M NaOH, pH 12, 1 d	Aerobic sludge	Corn stover hydrolysate	**0.80** (0.49)	Zhang et al. 2011a
pH 12, 1 d	Digested sludge	Sucrose	**0.51** (0.30)	O-Thong et al. 2009
6 M KOH, pH 12.5, 1 d	Digested sludge	Food waste	**0.87** (0.10)	Kim and Shin 2008
BESA pretreatment				
10 mmol BESA	Digested sludge	Sucrose	**1.01** (0.30)	O-Thong et al. 2009
10 mM BESA	Activated sludge	Glucose	0.33 (**0.38**)	Chang et al. 2011b
20 mM BESA	Compost	Birch pulp	66 (**560**)[a]	Paper IV
20 mM BESA	Silage	Silage	140 (**160**)[a]	Paper V

[a] mL H_2/g TS

10.1.2 Effects of process parameters

Hydrogen yields and production rates are affected by process parameters, including temperature (Yokoyama et al. 2007a, Gadow et al. 2012), pH (Lin and Hung 2008, Tang et al. 2008) and substrate concentration (Liu et al. 2003, Zhang et al. 2003). Process conditions also influence substrate degradation efficiency (Hu et al. 2004, Antonopoulou et al. 2011), soluble metabolites production (Wang et al. 2006, Lin and Hung 2008), and microbial community distributions (Yokoyama et al. 2007a, Yossan et al. 2012). The optimal temperatures (Papers II,III), pH (Paper IV) and substrate concentration (Paper V) for hydrogen production from cellulosic substrates obtained in this study and in other studies are presented in Table 16.

Table 16. Effects of different process parameters on dark fermentation hydrogen yields and degradation efficiencies from complex substrates.

Culture	Substrate	T (°C)	pH	Concentration (g/L)	H_2 yield (mol H_2/mol hexose)	DE (%)	Reference
Temperature							
Sewage sludge	Cattle wastewater	**45** (30-55)	nr	2.6 g COD/L	2.55	nr	Tang et al. 2008
Compost	Cellulose	**52** (52-70)	7	5.0	1.41	57	Paper II
Pig slurry	Water hyacinth	**55** (25-65)	7.0	40	0.41	nr	Chuang et al. 2011
Anaerobic sludge	Starch	**55** (37/55)	7.0	4.6	78 mL H_2/g VS	nr	Zhang et al. 2003
Settling tank	Rice winery WW	**55** (20-55)	5.5	34 g COD/L	1.9	nr	Yu et al. 2002
Rumen fluid	Cellulose	**60** (52-65)	7	5.0	0.44	21	Paper III
Anaerobic sludge	Cassava stillage	**60** (37-70)	7	28 g VS/L	53.8 mL H_2/g VS	nr	Luo et al. 2010
Digested sludge	Cellulose	**80** (37-80)	5.7	5	3.4	77	Gadow et al. 2012
pH							
Cow dung	POME	nr	**5.0** (4.0-7.0)	10 g COD/L	1.91	67	Vihayaraghavan and Ahmad 2006
Settling tank	Rice winery WW	35	**5.5** (4.0-6.0)	34 g COD/L	1.74	nr	Yu et al. 2002
Sewage sludge	Cattle wastewater	nr	**5.5** (4.5-7.5)	2.6 g COD/L	2.55	nr	Tang et al. 2008
Anaerobic sludge	Cassava stillage	60	**6.0** (4.0-10)	28 g VS/L	0.78	nr	Luo et al. 2010
Hot spring	Dry conifer pulp hydrolysate	37	**6.0** (5.0-9.0)	10 g sugars/L	0.77	86.8	Paper IV
Enriched culture	Cellulose	55	**6.5** (5.5-8.5)	5.0	0.76	nr	Liu et al. 2003
Rumen fluid	Cellulose	39.5	**7.0** (5.5-7.5)	10	nr	71	Hu et al. 2005
Rumen fluid	Cellulose	40	**7.3** (4.8-7.3)	10	nr	78	Hu et al. 2004
Rumen fluid	Cellulose	60	**7.3** (5.2-7.3)	5	2.4	nr	Paper III
Cow dung sludge	Cellulose	55	**7.5** (5.5-9.0)	10	0.50	nr	Lin and Hung 2008
Compost	Dry birch pulp	37	**6.0** (6.0-9.0)	5 g TS/L	150 mL H_2/g TS	nr	Paper IV
	Dry conifer pulp		**6.0** (6.0-9.0)		160 mL H_2/g TS		
	Wet birch pulp		**7.0**(6.0-9.0)		620 mL H_2/g TS		
	Wet conifer pulp		**9.0** (6.0-9.0)		540 mL H_2/g TS		

DE: degradation efficiency, POME: palmo oil mill effluent, WW: wastewater, nr: not reported, COD: chemical oxygen demand, TS: total solids, VS: volatile solids

Table 16. Continued

Culture	Substrate	T (°C)	pH	Concentration (g/L)	H_2 yield (mol H_2/mol hexose)	DE (%)	Reference
Substrate concentration							
Pig slurry	Water hyacinth	55	7.0	**10** (10-80)	0.64	nr	Chuang et al. 2011
Enriched culture	Cellulose	55	6.5	**10** (10-40)	0.57	nr	Liu et al. 2003
Cow dung compost	Cellulose	37	6.8	**10** (5-30)	2.09	56	Ren et al. 2010
Settling tank	Rice winery WW	35	5.5	**13** g COD/L (13-36)	1.89	nr	Yu et al. 2002
Sweet sorghum extract	Sweet sorghum extract	35	7.5	**17.5** g/L[a] (9.9-21)	0.74	99.4	Antonopoulou et al. 2011
Silage	Silage	37	7.0	**25** (25-200)	163 mL H_2/g TS	nr	Paper V

[a] in glucose equivalents

DE: degradation efficiency, POME: palmo oil mill effluent, WW: wastewater, nr: not reported, COD: chemical oxygen demand, TS: total solids, VS: volatile solids

Temperature

Hydrogen production has been widely studied with mesophilic (20-40°C), thermophilic (50-65°C) and hyperthermophilic (≥70°C) cultures. Li and Fang (2007) reviewed 98 studies on hydrogen production from wastewaters and solid wastes, from which 85 and 13 % were conducted with mesophilic and thermophilic cultures, respectively. In this study, cellulolytic and hydrogenic cultures were enriched from compost (Paper II) and rumen fluid (Paper III) at elevated temperatures on cellulose. No hydrogen was produced at 70 or 65°C, respectively. Compost culture could not produce H_2 at 60°C, while rumen fluid culture produced only negligible amounts of H_2 at 52°C. The highest hydrogen yields with compost culture were obtained at 52°C, 1.4 mol H_2/mol hexose (2.4 mol H_2/mol $hexose_{degraded}$), and with rumen fluid culture at 60°C, 0.4 mol H_2/mol hexose (1.9 mol H_2/mol $hexose_{degraded}$). It has been indicated that increasing temperature increases H_2 production in a suitable range, while too high temperatures inhibit H_2 evolution (Tang et al. 2008, Wang and Wan 2009).

In most of the studies evaluating optimal temperature with complex substrates, the highest H_2 yields have been obtained with thermophilic cultures (≥ 50°C) (Table 17). Furthermore, Gadow et al. (2012) reported the highest H_2 yield with a hyperthermophilic culture (80°C). The advantages of higher temperatures include increased chemical and enzymatic reaction rates, lower solubility of gases, and decreased effect of hydrogen partial pressure on hydrogen production (Levin et al. 2004, Hallenbeck 2005). In addition, high temperatures may enhance hydrolysis of complex substrates (Liu et al. 2003, Guo et al. 2010a) and inhibit the growth of methanogens (Yokoyama et al. 2007a, Chuang et al. 2011). Homoacetogenesis has been reported to occur at temperatures as high as 60°C, above which homoacetogenesis has been inhibited (Yokoyama et al. 2007a, Luo et al. 2010). On the other hand, the net energy gain (calculated based on energy obtained from hydrogen and energy used for heating) of thermophilic processes often remains negative (Gadow et al. 2012) due to heating requirements (Perera et al. 2012). Elevated temperatures are, however, acceptable if process heat is available (Hawkes et al. 2002) or if high-temperature wastewaters are used (Luo et al. 2010).

In this study, the microbial communities with and without heat treatments were enriched at different elevated temperatures (52-70°C) for the first time (Papers II,III). The microbial communities between the enrichment cultures varied considerably. The rumen fluid enrichment culture consisted mainly of Clostridial species, while compost enrichment culture contained bacteria from families *Clostridiales* and *Thermoanaerobacteriales*. The heat treatments affected the microbial communities of compost and rumen fluid cultures distinctly at different temperatures. The heat treatment of compost culture did not affect the bacterial diversities much at 52°C, at which temperature the highest hydrogen yield was obtained. At 60°C, however, the number of bacterial strains decreased after heat treatments suggesting that fewer spore formers were present that would have tolerated higher growth temperature (Paper II). The rumen fluid culture consisted of considerably more bacterial strains at 52 than 60°C. Tha bacterial diversity was decreased at 52°C after heat treatments, while at 60°C heat treatments did not affect the number of bacterial strains. These results indicate that the microbial communities consisted mainly of spore formers at 60°C resulting in the highest hydrogen yields (Paper III).

pH

The pH affects cellulase production (for a review, see Kumar et al. 2008) and cellulose hydrolysis (Russel and Wilson 1996, Hu et al. 2005), soluble metabolites production (Yu et al. 2002), and microbial community composition (Luo et al. 2010). The effect of pH on

hydrogen and methane production potential from four different pulp materials was studied with a compost culture in batch bottles (Paper IV). Hydrogen was produced at all pH values between 6 and 9, while methane was detected at other pH values than at pH 9 and at pH 6 with dry conifer pulp as substrate. The optimal pH for methanogens is between 6.8 and 7.5 (Zhu et al. 2008) and methanogens are inhibited at pH values below 6.0 and above 8.5 (Chandra et al. 2012). Thus, maintaining pH at appropriate range enriched for hydrogen producers and inhibited the growth of methanogens (Paper IV, Chandra et al. 2012). From dry pulps the highest H_2 yields (150-160 mL H_2/g TS) were obtained at pH 6, while the optimal pH values for H_2 production from wet birch and conifer pulps were 7 (620 mL H_2/g TS) and 9 (540 mL H_2/g TS), respectively (Table 16). Only a few reports exist on hydrogen production at pH 9.0 from cellulosic and lignocellulosic materials. Luo et al. (2010) obtained 39 mL H_2/g VS from cassava stillage.

In addition to culture and operational conditions, the optimal pH is highly dependent on the substrate. Optimal pH for pure cellulose has been between 6.5 and 7.5 (Table 16). In this study, hydrogen was produced from cellulosic pulps at a wide pH range from 6 to 9 (Paper IV). The pH was adjusted regularly to prevent deep decreases in pH. Maintaining pH at desired level is important especially with cellulosic substrates, since drop in pH may inhibit cellulolytic bacteria (Russel and Wilson 1996, Lynd et al. 2002). Furthermore, even a small change of 0.5 from the optimal pH may result in sharp decreases in H_2 production (Lin and Hung 2008). Less complex substrates, such as carbohydrate-rich cassava stillage, rice winery and cattle wastewaters as well as starch and pulp hydrolysate containing sugars (Paper IV), produced hydrogen at lower optimal pH of 5.5-6.0 (Table 16). Li and Fang (2007) reported optimal pH for hydrogen fermentation from carbohydrates and wastewaters to be in the range of 5.2-7.0 and 5.2-5.6, respectively.

Substrate concentration

In this study, the indigenous grass silage bacteria were reported to produce hydrogen from silage for the first time. The fermentation of silage was continued by neutralizing the pH and hydrogen production potential was studied at silage concentrations from 25 to 200 g silage/L (Paper V). The highest hydrogen yield of 163 mL H_2/g TS was obtained at 25 g/L silage and the H_2 yield decreased when silage concentration increased. Similar results indicating that increasing substrates concentrations decrease H_2 yields were reported by Liu et al. (2003) and Chuang et al. (2011). Decreased H_2 yields at higher substrate concentrations have been associated with a change in soluble metabolites from acids to alcohols (Yu et al. 2002, Wang et al. 2006, Antonopoulou et al. 2011). In addition, accumulation of VFAs may inhibit microorganisms (van Ginkel et al. 2001) or result in pH decrease affecting H_2 yields and substrate degradation at higher substrate concentrations (Ren et al. 2010). It has been suggested that enriching microorganisms with higher substrate concentrations may increase the H_2 yields by enhancing the hydrogen production activity of the culture (Lin and Cheng 2006). Opposite to H_2 yields, the cumulative hydrogen production (Paper V) and hydrogen production rates (Yu et al. 2002, Liu et al. 2003) often increase with increasing substrate concentrations. There is, anyhow, an upper substrate concentration, above which the hydrogen production (Zhang et al. 2003, Antonopoulou et al. 2011) and substrate degradation (Ren et al. 2010) decline.

10.1.3 Simultaneous H_2 and EtOH fermentation

During thermophilic hydrogen production from cellulose ethanol was also produced at high yields (Papers II, III). Dark fermentation can proceed through (i) butyric acid-type fermentation that results in acetate and butyrate production associated with hydrogen production, (ii) ethanol-type fermentation that produces mainly acetate and ethanol with smaller amounts of hydrogen, or through (iii) propionic acid-type fermentation that does not promote H_2 production (Ren et al. 2007). Acetate and ethanol were the main metabolites during H_2 production with compost and rumen fluid cultures followed by smaller amounts of butyrate (Paper II) or lactate (Paper III), respectively. Simultaneous production of acetate and ethanol results in the theoretical maximum yields of 2 mol H_2/mol hexose and 1 mol EtOH/mol hexose (Eq. 7) (Barros and Silva 2012). The hydrogen and ethanol yields of 1.93 mol H_2/mol $hexose_{degraded}$ (0.4 mol H_2/mol hexose) and 0.99 mol EtOH/mol $hexose_{degraded}$ (0.2 mol EtOH/mol hexose), respectively, with rumen fluid culture are close to the maximum yields (Paper III). With compost culture the hydrogen yield was higher, 2.4 mol H_2/mol $hexose_{degraded}$ (1.4 mol H_2/mol hexose), followed by 0.75 mol EtOH/mol $hexose_{degraded}$ (0.4 mol EtOH/mol hexose) (Paper II). Thus, the metabolism with compost enrichment culture was likely directed more towards H_2 production through acetate (Eq. 8), while some ethanol may have been produced through Eq. 9. In conclusion, the hydrogen and ethanol yields with compost and rumen fluid enrichment cultures from hydrolyzed cellulose were high and close to the maximum theoretical yields.

$$C_6H_{12}O_6 + H_2O \rightarrow C_2H_5OH + CH_3COOH + 2\ H_2 + 2\ CO_2 \quad (7)$$
$$C_6H_{12}O_6 + 2\ H_2O \rightarrow CH_3COOH + 4\ H_2 + 2\ CO_2 \quad (8)$$
$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2 \quad (9)$$

Hydrogen production accompanied with ethanol production is beneficial, since both H_2 and EtOH are high-energy compounds that can be utilized for bioenergy production. Furthermore, in a reactor they exist in two different phases that can be easily separated (Zhao et al. 2009). Simultaneous H_2 and EtOH production has been studied mainly from sugars, but a few studies from more complex sugars also exist (Table 17). The results in Table 17 have been chosen at operational parameters resulting in the highest ethanol yields. The proportions of hydrogen and ethanol depend on the operational parameters, such as pH (Lin and Hung 2008, Zhao et al. 2009, Karadag and Puhakka 2010a), HRT (Koskinen et al. 2008b, Barros and Silva 2012), and substrate loading rate (Koskinen et al. 2008a), type (Wu et al. 2007) or concentration (Lay et al. 2012).

Table 17. Simultaneous hydrogen and ethanol production by bacteria. Hydrogen and ethanol yields are given per moles of hexoses (glucose, fructose) or pentoses (xylose).

Substrate	Culture	H_2 yield (mol H_2/ mol sugar)	EtOH yield (mol EtOH/mol sugar)	Reference
Glucose	*Thermoanaerobacter thermohydrosulfuricus*	0.68	1.21	Koskinen et al. 2008b
Glucose	Enrichment culture	0.72	1.43	Zhao et al. 2009
Glucose	*Thermoanaerobacterium aciditolerans*	1.2	1.6	Koskinen et al. 2008a
Xylose		1.0	1.1	
Glucose	AK_{54}[a]	1.02	1.03	Sigurbjornsdottir and Orlygsson 2012
Xylose	Cow dung	0.3	1.0	Lin and Hung 2008
Fructose	Anaerobic sludge	0.56	0.65	Wu et al. 2007
Cellulose	Cow dung	0.5	1.0	Lin and Hung 2008
Cellulose	Compost	1.4	0.4	Paper II
Cellulose	Rumen fluid	0.4	0.2	Paper III
Sweet potato	Sweet potato	0.4	0.45	Lay et al. 2012

[a] 99.0 % similarity to *Thermoanaerobacterium aciditolerans*

10.1.4 Metabolic pathways

As discussed in the Chapter 10.1.3, three main fermentation types are associated with dark fermentation: butyric acid-, ethanol- and propionic acid-type fermentations (Ren et al. 2007). In this study, hydrogen was produced from cellulosic substrates (Papers II-V) and the soluble metabolite distributions of the cultures producing the highest hydrogen yields were as presented in Figure 13. At the highest, 33 % of hexose sugars can be converted to hydrogen (4 mol H_2/mol hexose) provided that acetate is the only soluble metabolite. Thermophilic hydrogen production with rumen fluid culture proceeded through ethanol-type fermentation, resulting in high electron recoveries as ethanol (43 % from the produced metabolites) and hydrogen (15 %) (Paper III). Cellulose fermentation with compost culture at elevated temperature was a mixture of butyric acid- and ethanol-type fermentations, which also directed large amounts of electrons to ethanol (31 %) and hydrogen (17 %) (Paper II). In addition, fermentation of dry conifer pulp followed both butyric acid- and ethanol-types with slightly lower hydrogen and ethanol yields of 14 and 3 %, respectively. Acetate was the main soluble metabolite of wet conifer pulp fermentation, which recovered 8 % electrons as hydrogen (Paper IV).

Fermentation of pulp materials generally resulted in the production of methane. Absence of methane in compost culture grown on dry and wet conifer pulps (Figure 13) may have resulted from the growth pH values of 6.0 and 9.0, respectively. The optimal pH of methanogens is in the range of 6.5-8.2 (Agdag and Sponza 2005) and the low and high pH values likely inhibited methane production. In anaerobic digestion, VFAs and alcohols are converted into acetate through acetogenesis. Methane is mainly produced from acetate with acetoglastic methanogens (Eq. 10) or from hydrogen and carbon dioxide with hydrogenotrophic methanogens (Eq. 11) (Chandra et al. 2012). Anaerobic degradation of wet conifer pulp at pH 7 resulted in almost complete conversion of degraded substrate into methane (88 % electron recovery). Methane production from other dry and wet pulps was incomplete and acetate was the main soluble metabolite remaining in the solution (Figure 13, Paper IV) suggesting decreased performance of acetoglastic methanogens.

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad (10)$$

$$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O \quad (11)$$

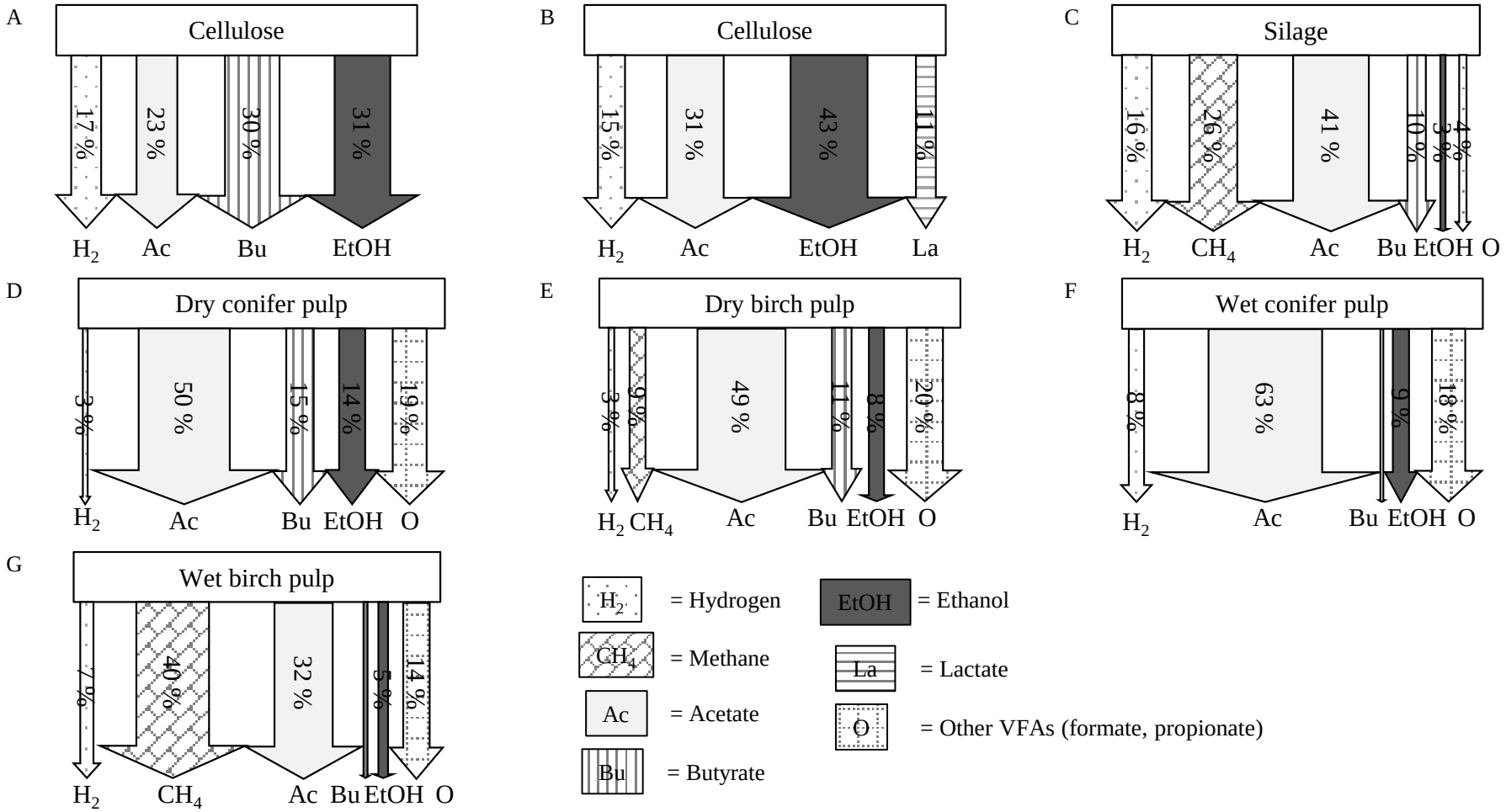


Figure 13. Electron distribution from degraded cellulosic substrates. Diagrams are based on electrons present in each metabolic product and are compared to mol substrate utilized (A,B) or to g TS added to the medium (C-G). (A) Compost culture enriched on cellulose at 52°C (Paper II); (B) Rumen fluid culture enriched on cellulose at 60°C (Paper III); (C) Indigenous silage bacteria grown on 25 g/L silage (Paper V); Compost culture grown on (D) dry conifer pulp at pH 6, (E) dry birch pulp on pH 6, (F) wet conifer pulp at pH 9, or on (G) wet birch pulp at pH 7 (Paper IV). The width of the arrow is proportional to the electron flux.

10.1.5 Bacteria enriched on cellulose

Cultures fermenting cellulosic substrates into hydrogen are often composed of several different bacteria (Ueno et al. 2001). In this sudy, the microbial community compositions were characterized extensively to discover the bacteria responsible for cellulose hydrolysis and hydrogen production. Table 18 presents the main bacteria enriched on cellulosic substrates in this study (Papers II-V). Thermophilic cultures enriched from compost or rumen fluid materials on pure cellulose contained two known cellulosic bacteria, i.e., *C. cellulosi* (Paper II) and *C. stercorarium* (Papers II,III). H_2 producers enriched at elevated temperatures from compost mainly consisted of *Thermoanaerobacteria* (Paper II), while from rumen fluid *Clostridium caenicola* and *Symbiobacterium thermophilum* were enriched (Paper III). The presence of non-cellulolytic bacteria shows that at least some of the cellulose was degraded into sugars (Ueno et al. 2001) that were further degraded by other bacteria. The presence *Thermoanaerobacteria* (Liu et al. 2003, O-Thong et al. 2007, Prasertsan et al. 2009) and cellulolytic *C. cellulosi* (Ueno et al. 2001, Yokoyama et al. 2007b) or *C. stercorarium* (Ueno et al. 2006, Yokoyama et al. 2007a) in thermophilic cellulolytic and hydrogenic cultures has been reported earlier. Mesophilic compost culture enriched on pulp materials (Paper IV) and indigenous silage bacteria (Paper V) contained only one cellulolytic bacterium, *C. populeti*, which also produces hydrogen. Other H_2 producers were also present (Table 18). In all the enrichment cultures, bacteria that do not degrade cellulose or produce H_2 were also detected. For example, compost and rumen fluid enrichment cultures contained many ethanol producers, which is in accordance with the high ethanol yields (Table 17, Papers II,III).

Enrichment conditions affect the characteristics of enrichment cultures (for a review, see Hung et al. 2011). Enrichment of different dark fermentative cultures at different temperatures (Shin et al. 2004, Yokoyama et al. 2007a) or on different substrates (Lin and Hung 2008) has been reported. In this study, a culture of compost origin was enriched on cellulose at 52 or 60°C (Paper II) and on pulp materials at 37°C (Paper IV). The resulting bacterial communities differed considerably (Table 18). At elevated temperatures, both thermophilic cellulose degraders and hydrogen producers were detected, while at 37°C mesophilic bacteria dominated. In addition, rumen fluid enrichment cultures at 52 and 60°C were similar but the hydrogen production and cellulose degradation at 52°C were negligible (Paper III). This likely resulted from the reduced activity of the only cellulolytic species, *C. stercorarium*, at 52°C. Different pH values (Ueno et al. 2006, Lin and Hung 2008) and HRTs (Prasertsan et al. 2009, Lay et al. 2010) also enrich for different bacterial communities. For example, there was only one same bacterium in compost cultures enriched on pulp materials at pH values 6 and 9 (Paper IV). Furthermore, the results indicate that at different pH values different cellulolytic bacteria dominated. Hydrogenic bacteria were pH dependent and, e.g., at pH 9 *Clostridium ultunense* that was absent at other pH values was the main hydrogen producer. Characterization of the microbial communities improves understanding of the community dynamics and the effects of operational conditions on hydrogen production potentials. Further, it promotes the selection of suitable cultures and studying their growth conditions for continuous and pilot-scale hydrogen production research.

Table 18. Characteristics of bacteria enriched on cellulosic substrates in this study (Papers II-V) (modified from Lay et al. 2012).

Bacterium	opt.T (°C)	opt.pH	Cellulolytic	Hydrogenic	Metabolites / Notes	Paper(s)	Reference
Clostridium cellulosi	55-60	7.3-7.5	+	+	EtOH,A	II	Yanling et al. 1991
Clostridium stercorarium	65	7.0-7.5	+	-	EtOH,A,L / H_2 from lactose	II, III	Madden 1983, Fardeau et al. 2001, Collet et al. 2004
Thermoanaerobium thermosaccharolyticum	60	5-6	-	+	A,B	II	Ueno et al. 2001, O-Thong et al. 2009
Thermoanaerobacterium mathranii	70-75	7.0	-	+	EtOH,A,L	II	Larsen et al. 1997
Thermoanaerobacterium italicus	70	7.0	-	+	EtOH,L	II	Kozianowaski et al. 1997
Coprothermobacter proteolyticus	63	7.5	nr	+	A	II	Ollivier et al. 1985
Clostridium caenicola	60	6.5	-	+	EtOH,A,L	II, III	Shiratori et al. 2009
Symbiobacterium thermophilum	60	7.5	-	+	nr / Grows in co-culture with *Bacillus* sp.	III	Ohno et al. 2000, Ueda et al. 2004
Clostridium populeti	35	7.0	+	+	A,B,L	IV, V	Sleat and Mah 1985
Comamonas denitrificans	30	7.5	nr	nr	nr / Denitrifying	IV	Gumaelius et al. 2001
Parabacteroides goldsteinii	nr	nr	nr	nr	A,S	IV	Sakamoto and Benno 2006
Eshcerichia coli	nr	nr	nr	+	EtOH,A,L,S / Has H_2 consuming hydrogenases	IV	Laurinavichene and Tsygankov 2003, Seppälä et al. 2011
Clostridium ultunense	37	7	nr	+	A,(F)	IV	Schnürer et al. 1996
Gracilibacter thermotolerans	43-47	6.8-7.8	nr	nr	EtOH,A,L	V	Lee et al. 2006
Acetanaerobacterium elongatum	37	6.5-7.0	nr	+	EtOH,A	V	Chen and Dong 2004

nr: not reported, opt.T: optimal temperature, opt.pH: optimal pH, A: acetic acid, B: butyric acid, EtOH: ethanol, F: formic acid, L: lactic acid, S: succinic acid

10.2 Dark fermentative H_2 production from sugars

10.2.1 Hydrolysis of cellulosic materials

Cellulosic substrates are often pretreated prior to hydrogen fermentation. Acid hydrolysis can be done with diluted acids at elevated temperature and/or pressure (Pattra et al. 2008, Chang et al. 2011a) or with concentrated acid hydrolysis that takes place at milder conditions (Chu et al. 2011, Li et al. 2011). After acid hydrolysis, the acid has to be recovered and/or neutralized. In this study, conifer and birch pulps were hydrolyzed with concentrated sulfuric acid, neutralized and utilized for hydrogen production (Paper IV, Figure 14). The optimal time for concentrated acid hydrolysis was determined and was 90 min for wet and 180 min for dry pulps resulting in sugar yields of 33-37 % and 70-84 % (g sugars/g substrate), respectively. The sugar yield determines the conversion efficiency of acid pretreatment and thus, is an important parameter (Panagiotopoulos et al. 2011). Concentrated acid hydrolysis has generally resulted in higher sugar yields and conversion efficiencies than diluted acid hydrolysis (Table 19).

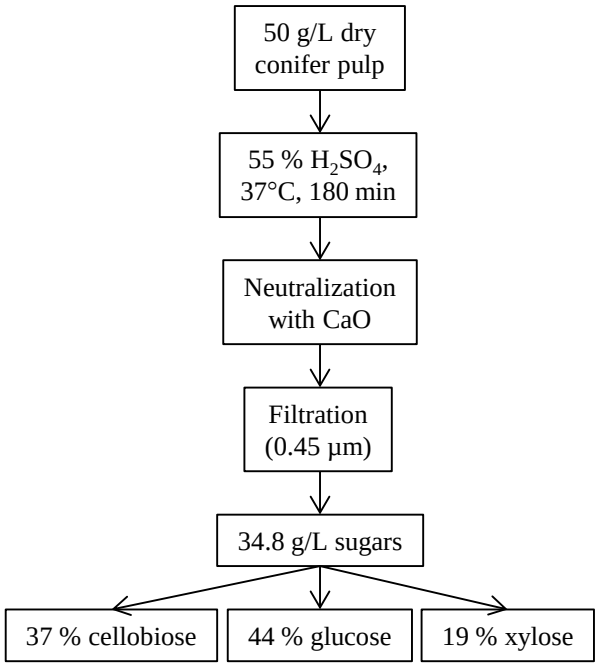


Figure 14. Acid hydrolysis of dry conifer pulp and the corresponding sugar yields (Paper IV).

Glucose and xylose are the main sugars after acid hydrolysis followed by smaller amounts of arabinose and cellobiose (Table 19). The composition and ratio of sugars affects the hydrogen fermentation efficiency depending on the type of bacteria used (Panagiotopoulos et al. 2011). Different cultures prefer different sugars and/or sugar compositions. Hydrogen fermentation from different sugars was studied with a hot spring enrichment culture (Paper I) and the highest hydrogen yields were obtained from xylose, arabinose and glucose. Substrate conversion was also affected by the sugar type; most of the sugars were degraded at high efficiency (>91 %) whilst mannose as substrate resulted in only 59 % degradation (Paper I). The substrate utilization efficiency of many pure cultures has been reported. *Thermoanaero-bacterium* AK_{54} is known to degrade all other sugars than arabinose and ribose (Sigurbjornsdottir and Orlygsson 2012), while three *Caldicellulosiruptor* species were found to prefer xylose over glucose for hydrogen fermentation (Zeidan and van Niel 2009). The

sugar composition also affects the metabolic pathways. The main metabolite with a hot spring enrichment culture from hexoses was lactate, while fermentation of pentoses was directed towards butyrate and acetate or formate (Paper I). Rosales-Colunga et al. (2012) reported that glucose degradation with *E. coli* WDHL was directed towards lactate production and that the highest H_2 yield was obtained from galactose.

Table 19. Sugar yields and compositions obtained from acid hydrolyzed substrates.

Substrate	Hydrolysis	Sugar yield (g/L / %)	Cel (%)	Glu (%)	Xyl (%)	Ara (%)	Reference
Rice straw	3 wt%[a] H_2SO_4, 150°C, 1 h	31.1 / nr	5.5	6.1	34.4	13.2	Chang et al. 2011a
Sugarcane bagasse	0.5 % H_2SO_4, 121°C, 1 h, 1.5 kg/cm^3	24.5 / nr	nr	44.9	46.1	9.1	Pattra et al. 2008
Corn stover	2.1 % H_2SO_4, 121°C, 105 min	12.2 / nr	nr	17.3	73.7	9.1	Cao et al. 2009
Barley straw	2 % H_2SO_4, 180°C, 30 min	21.1 / 63[b],50[c]	nr	73.4	24.0	0.0	Panagiotopoulos et al. 2011
Reed canary grass	3 % HCl, 121°C, 90 min	nr / 11.5	nr	nr	nr	nr	Lakaniemi et al. 2011
Bagasse	86 % H_3PO_2, 50°C, 30 min	1.30 / nr	65	24	0	0	Lo et al. 2011
Cotton cellulose	55 % H_2SO_4, 40°C, 1.5 h	nr / 73.9	nr	nr	nr	nr	Chu et al. 2011
Mushroom farm waste	55 % H_2SO_4, 40°C, 20 min	nr/ 73.7	nr	nr	nr	nr	Li et al. 2011
Dry birch pulp	55 % H_2SO_4,	42.2 / 84.3	30	38	32	0	Paper IV
Dry conifer pulp	37°C, 3 h	34.8 / 69.6	37	44	19	0	

[a] acid/biomass, [b] cellulose, [c] hemicellulose, Cel: cellobiose, Glu: glucose, Xyl: xylose, Ara: arabinose, nr: not reported

10.2.2 Hydrogen yields from sugars

Lignocellulosic materials are hydrolyzed into hexoses (glucose, mannose, galactose, fructose) and pentoses (xylose, arabinose, ribose) (Kumar et al. 2008). Thus, it is important to seek for microbial communities that can ferment all of these sugars into hydrogen. Hydrogen production from different sugars, especially from glucose and xylose, has been extensively studied and some of the results are presented in Table 20. Hot spring culture was enriched on above mentioned hexose and pentose sugars and the hydrogen production potentials form these sugars were evaluated in batch bottles (Paper I). In the end of the enrichment, hydrogen was not produced from galactose, mannose, fructose and sucrose. The highest hydrogen yield was obtained from xylose (0.71 mol H_2/mol xylose) and the hot spring culture favored pentoses over hexoses for H_2 fermentation.

Table 20. Hydrogen yields from different sugars in batch or continuous mode by pure or mixed cultures.

Sugar	Culture	T (°C)	pH	Hydrogen yield		Reference
				mol H_2/mol sugar	%[a]	
Batch						
Glucose	Digested sludge	nr	6.2	0.97	24.2	Oh et al. 2003
Glucose	Digested sludge	35	5.5	2.00	50.0	Mu et al. 2007
Glucose	Digested sludge	65	6.7	2.3	57.5	Baghchehsaraee et al. 2008
Glucose	Hot spring	52	6.5	1.59	39.8	Karadag et al. 2009
Galactose	*E. coli* WDHL	nr	nr	1.12	28.0	Rosales-Colunga et al. 2012
Fructose	*E. coli* DJT135	35	6.5	1.27	31.8	Ghosh and Hallenbeck 2009
Arabinose	Anaerobic sludge	37	6.5	1.98	59.5	Danko et al. 2008
Xylose	Sewage sludge	35	6.5	1.3	39.0	Lin et al. 2006
Xylose	Sewage sludge	35	6.0	2.25	67.7	Lin and Cheng 2006
Xylose	Pure culture[b]	75	7.0	2.8	84.1	Ngo et al. 2012
Xylose	Hot spring	37	6.8	0.71	21.3	Paper I
Glucose				0.54	13.5	
Arabinose				0.61	18.3	
Continuous						
Glucose	Enrichment culture	36	5.5	2.1	52.5	Fang and Liu 2002
Glucose	Hot spring	45	5.0	1.71	42.8	Karadag and Puhakka 2010b
Arabinose	Enrichment culture	70	5.5	1.10	33.0	Abreu et al. 2012
Xylose	Sewage sludge	50	7.1	1.4	42.0	Lin et al. 2008
Xylose	Compost	55	5.0	1.70	51.2	Calli et al. 2008
Xylose	Hot spring	37	5.1	0.3	10.2	Paper I
		45		1.46	43.8	

[a] percentage of the theoretical maximum, [b] *Thermoanaerobium thermosaccharolyticum*, nr: not reported

Continuous hydrogen fermentation from xylose was further studied in a CSTR at two different temperatures (Paper I). The highest hydrogen yield and production rate of 1.97 mol H_2/mol xylose and 7.3 mmol H_2/L/h, respectively, were obtained at suboptimal temperature of 45°C. Considerably lower maximum hydrogen yield and production rate of 1.18 mol H_2/mol xylose and 1.7 mmol H_2/L/h, respectively, were achieved at 37°C. The results suggest that suboptimal temperature of 45°C may be used to selectively enrich efficient hydrogen producing bacteria. The distribution of soluble metabolites from glucose in batch bottles and from xylose both in batch and continuous processes are presented in Figure 15. Xylose fermentation in batch and continuous mode proceeded through butyric acid-type fermentation (Ren et al. 2007) with butyrate and acetate as the main metabolites (Paper I). More electrons were directed to hydrogen in continuous mode, which likely resulted from the high electron recoveries as ethanol and propionate in batch bottles that decreased H_2 yields. The difference between pentose and hexose fermentations can also be seen from Figure 15; 27 % of the electrons from glucose were directed into lactate production, while negligible amounts of lactate were produced from xylose (Paper I). Lactate production directs electrons from hydrogen production (Hallenbeck et al. 2009) decreasing hydrogen yield.

Many recent studies have focused on hydrogen fermentation from hydrolysates (Table 7). Hot spring enrichment culture (Paper I) was used to produce hydrogen from dry conifer pulp hydrolysate consisting of glucose, cellobiose and xylose. The highest hydrogen yield was 63 ml H_2/g TS (Paper IV). Various soluble metabolites were produced (Figure 15), from which high lactate production (38 %) likely decreased hydrogen yields (Hallenbeck et al. 2009). The H_2 yield from concentrated acid hydrolysate was similar to 0.49 mol H_2/mol hexose obtained

from mushroom farm waste after concentrated acid hydrolysis (Li et al. 2011). Higher hydrogen yields have been obtained from diluted acid hydrolysates (Table 7), which may be due to the larger inhibitory effects of concentrated acid hydrolysates on dark fermentative hydrogen production. Concentrated sulfuric acid hydrolysates may contain sulfate ions that enhance the growth of sulfate-reducing bacteria (Lin and Chen 2006). In addition, hydrolysates may contain furfural, HMF or phenolic compounds that inhibit hydrogen fermentation (Ren et al. 2009). The hydrogen yield from dry conifer pulp hydrolysate (63 mL H_2/g TS) was considerably lower than the yield obtained with simultaneous cellulose fermentation and hydrogen production (120 mL H_2/g TS). Thus, chemical hydrolysis resulted in lower hydrogen yields although hydrogen was produced faster from chemically hydrolyzed pulp (10 days) than from direct cellulose fermentation to hydrogen (28 days) (Paper IV).

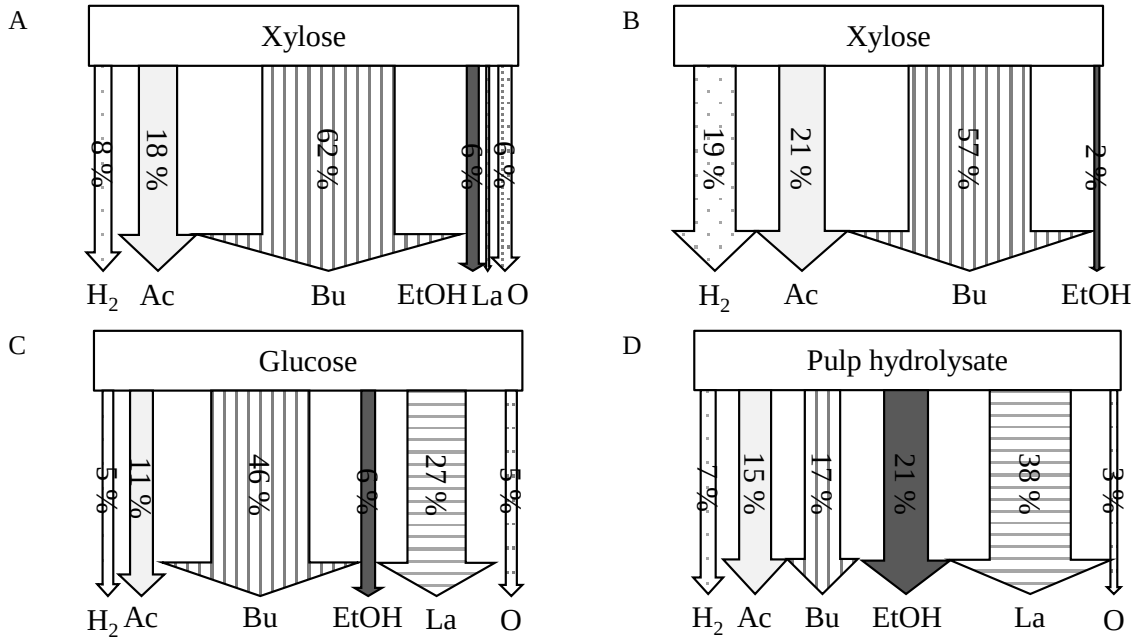


Figure 15. Electron distribution from sugars and pulp hydrolysate. Diagrams are based on electrons present in each metabolic product and are compared to mol substrate utilized. Hot spring culture grown on (A) xylose in batch bottle, (B) xylose in CSTR, (C) glucose in batch bottle (Paper I), or on (D) dry conifer pulp hydrolysate in batch bottle (Paper IV). Ac: acetate, Bu: butyrate, EtOH: ethanol, La: lactate, O: other VFAs (formate, propionate). The width of the arrow is proportional to the electron flux.

10.3 Production of electricity and alcohol(s)

Many exoelectrogenic cultures are able to utilize hexoses and pentoses, the degradation products of cellulosic materials (Rabaey et al. 2003, Catal et al. 2008, Huang and Logan 2008). Microbial community compositions of cultures producing electricity from glucose have been extensively studied (Rabaey et al. 2004, Jung and Regan 2007), whilst there are no studies characterizing exoelectrogenic microbial communities enriched on xylose. In this study, exoelectrogenic and alcohologenic cultures were enriched on xylose in fed-batch two-chamber MFCs from compost and anaerobic digester. The xylose enrichment cultures were characterized for the first time (Paper VI). With compost culture the maximum power density remained the same for the first two enrichment steps, after which it slightly decreased (Table 21). The CE, however, increased from 11 to 24 % during the enrichment. The power density and CE of anaerobic digester culture increased during enrichment from 14 to 54 mW/m^2 and

from 4 to 13 %, respectively. The electricity yields were low due to high internal resistances of 230-530 Ω.

Table 21. Electricity and alcohol(s) yields after enrichments and at higher xylose concentrations.

Culture	Xylose (g/L)	Enr. step	PD (mW/m^2)	CE (%)	EtOH (g/L)	ButOH (g/L)
Compost	1.0	4	25.5	23.9	0.23	0
	4.0	3	0.02	0.0	0.12	13.6
Anaerobic	1.0	3	53.7	12.5	0.30	0
digester	2.5	4	12.8	1.8	0.04	9.78

Enr. step: enrichment step, PD: power density, CE: Coulombic efficiency, EtOH: ethanol, ButOH: butanol

Electricity production with compost and anaerobic digester cultures was accompanied by high ethanol or butanol yields (Table 21). Ethanol and butanol production is beneficial since they are high-energy compounds that can be used as direct replacement or as additives for transportation fuels. In addition, the MFC effluents containing mainly alcohols do not need further treatment, since the alcohol(s) can be separated by distillation (Lee et al. 2008a). This is the first study reporting electricity production from xylose without simultaneous production of volatile fatty acids that would require further treatment. Electron balance was calculated and the electrons in electricity, soluble metabolites and remaining substrate were determined. The electrons that were not recovered from the substrate were considered as losses. During enrichment on 1.0 g/L xylose the compost culture resulted in 65 and 37 % electron recoveries as ethanol in the 2nd and 3rd enrichment steps (Paper VI). With the anaerobic digester enrichment culture 40 % of the electrons were recovered as ethanol. At xylose concentrations of 4 and 2.5 g/L, the electron recoveries as butanol (during the feeding cycle resulting in the highest electricity production) suggested at the highest 80 and 180 % recoveries with compost and anaerobic digester cultures, respectively. Over 100 % electron recovery likely resulted from the accumulation of soluble metabolites from the previous feeding cycles. These soluble metabolites were likely further converted into butanol resulting in high butanol yield. Simultaneous electricity and butanol production was earlier reported by Lakaniemi et al. (2012) from *Chlorella vulgaris* biomass with the highest electricity and butanol yields of 15.0 mW/m^2 and 1.2 g/L, respectively.

In this study, the microbial communities enriched on xylose were characterized for the first time (Paper VI). Both biofilm and solution cultures consisted mainly of *Proteobacteria* and *Bacteroidetes*. In addition, compost enrichment culture contained *Firmicutes* strains (Table 22). The microbial communities in MFCs usually contain *Proteobacteria* and *Firmicutes* (Rismani-Yazdi et al. 2007, Chung and Okabe 2009). However, the bacterial composition depends on the original culture and substrate used for enrichment (Table 22). The results indicate that acetate as substrate enriched mainly *δ*- and/or *β-proteobacteria*, while with glucose the microbial communities had similarities within the cultures obtained from comparable sources; From anaerobic sludge mainly *δ-proteobacteria* were enriched, while previously enriched cultures have contained mostly *Firmicutes*. The microbial communities enriched on more complex substrates do not follow any clear patterns. In addition to *Proteobacteria* and *Firmicutes*, all the enrichment cultures have also contained other bacteria, including *Bacteroidetes* (Paper VI, Jung and Regan 2007) or *Clostridiales* (Rismani-Yazdi et al. 2007). Presence of other bacteria in the anode cultures may enhance fermentation of sugars and more complex substrates (Jung and Regan 2007, Rismani-Yazdi et al. 2007). Furthermore, they may use oxygen leaking from cathode to the anode chamber (Kim et al. 2006b).

Table 22. Characteristics of microbial communities from various MFCs (modified from Kim et al. 2007 and Jong et al. 2011).

		Class (%)[a]						
Culture	**Substrate**	***α-proteobacteria***	***β-proteobacteria***	***γ-proteobacteria***	***δ-proteobacteria***	***Firmicutes***	**Other**	**Reference**
Anaerobic sludge	Acetate	-	71	5	13	3	3	Borole et al. 2009
Anaerobic sludge	Acetate[b]	-	7	7	47	-	40	Jung and Regan 2011
Anaerobic sludge	Acetate	-	25	-	25	-	50	Jung and Regan 2007
Activated sludge	Acetate	24	7	21	21	7	21	Lee et al. 2003
POME	Acetate	-	1	1	89	2	8	Jong et al. 2011
nr	Acetate[b]	-	-	-	50	-	50	Zhang et al. 2011b
nr	Butyrate[b]	-	-	-	50	-	50	Zhang et al. 2011b
Anaerobic sludge	Lactate	-	11	-	56	-	33	Jung and Regan 2007
Anaerobic sludge	Glucose[b]	-	-	-	38	-	46	Jung and Regan 2011
Anaerobic sludge	Glucose	-	-	-	50	-	50	Jung and Regan 2007
Enrichment culture	Glucose	-	9	-	9	55	27	Rabaey et al. 2004
Enrichment culture	Glucose[b]	0.4	3	2	-	68	25	Chung and Okabe 2009
nr	Glucose[b]	33	-	-	-	-	67	Zhang et al. 2011b
Compost	Xylose	11[b]	22[b]	-	-	11[b]	56[b]	Paper VI
		9[c]	18[c]			9[c]	64[c]	
Anaerobic sludge	Xylose	14[b]	29[b]			-	57[b]	
		13[c]	13[c]			-	75[c]	
Anaerobic sludge	Domestic WW[b]	-	-	25	25	25	25	Yu et al. 2012
Activated sludge	Chocolate industry WW[b]	9	51	1	9	5	25	Patil et al. 2009
Cow rumen	Cellulose	1[b]	2[b]	3[b]	1[b]	59[b]	27[b]	Rismani-Yazdi et al.
		-	76[c]	6[c]	-	13[c]	5[c]	2007
POME	POME	1	18	21	9	6	45	Jong et al. 2011
Sludge	Starch WW	24	36	-	-	4	36	Kim et al. 2004
Cattle dung	Cattle dung	12	6	18	-	24	-	Zhao et al. 2012

[a] Occurrence of different classes among the detected bacterial strains (calculated by dividing the number of, e.g., *α-proteobacteria* strains with the total number of bacterial strains detected in DGGE), [b] bacteria in the biofilm, [c] bacteria in the solution, -: not present, nr: not reported, POME: palm oil mill effluent, WW: wastewater

Xylanolytic *Ruminobacillus xylanolyticum* was likely responsible for xylose degradation in this study, while denitrifying bacteria, *Comamonas denitrificans* and *Paracoccus pantotrophus*, produced the electricity (Paper VI). Exoelectrogens can be found from many bacterial groups. Metal-reducing bacteria, such as *G. sulfurreducens* (Bond and Lovley 2003) and *S. putrefaciens* (Kim et al. 1999), sulfate-reducing bacteria, such as *Desulfobulbus* (Holmes et al. 2004), and denitrifying bacteria, e.g. *O. anthropic* (Zuo et al. 2008), are known to transfer electrons to the electrode. Rismani-Yazdi et al. (2007) suggested that a denitrifying *Comamonas* species uses the electrode as electron acceptor instead of nitrate. In addition, Xing et al. (2010) reported electricity production from acetate with a denitrifying bacterium *C. denitrificans*. *C. denitrificans* utilized, e.g. acetate, lactate and arabinose, but the growth on xylose was not studied. Thus, degradation of xylose by *C. denitrificans* in this study cannot be completely excluded. This is the first study reporting a denitrifying bacterium *P. pantotrophus* in the MFC anode.

10.4 Enrichment of microbial communities for production of various energy carriers

Process parameters affect significantly the microbial community compositions of the enriched cultures and the energy carriers produced. A hot spring culture was grown at different process conditions and substrates (Paper I, Karadag and Puhakka 2010a,b). Elevated temperature (60°C) preferred ethanol production, while the highest H_2 yield was obtained at 45°C (Karadag and Puhakka 2010b). Ethanol and hydrogen production at 37°C was pH dependent (Figure 16) directing metabolism towards hydrogen at pH 5.3 and to ethanol at pH above 5.5. Xylose as substrate resulted in higher H_2 yield, 45 % from the theoretical maximum (Paper I), than glucose, 35-43 % from the theoretical maximum (Karadag and Puhakka 2010a,b). The thermophilic cultures contained mainly *Thermoanaerobacteria*, while *Clostridia* dominated at lower temperatures. Culture growth at 50°C or at 37°C and at pH 4.9 was directed towards lactate production and *Bacillus coagulans* was the main bacterium (Figure 16).

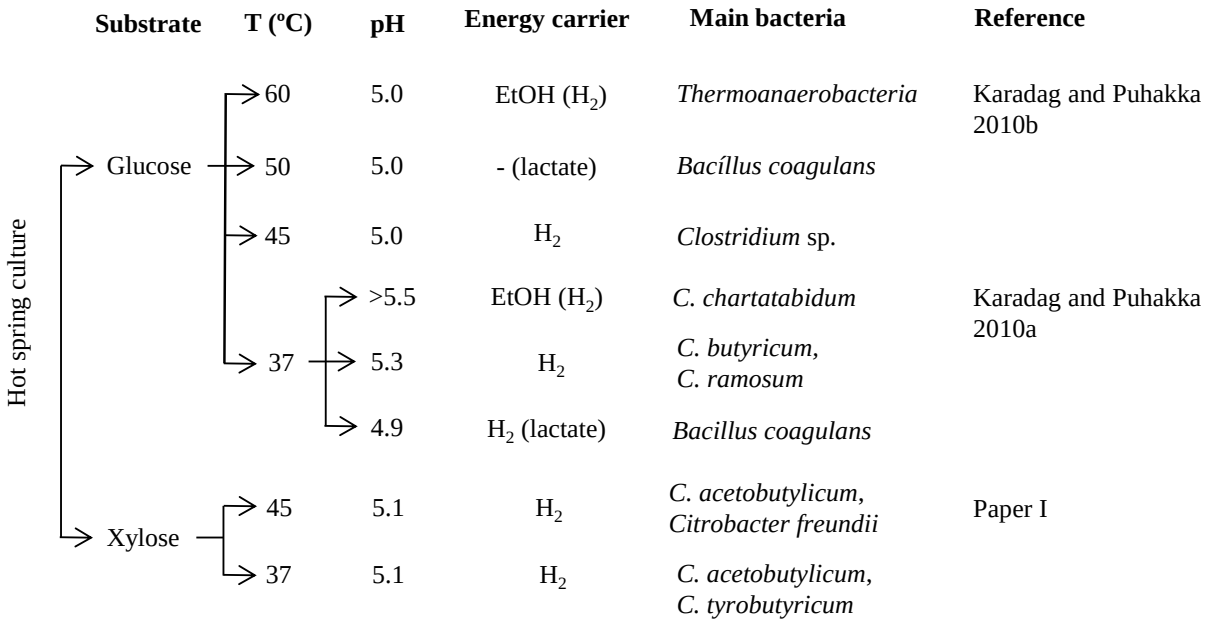


Figure 16. Enrichment of different microorganisms from hot spring culture at different process conditions.

In this study, a culture of compost origin was enriched for the production of hydrogen (Papers II), hydrogen and methane (Paper IV), and electricity and alcohol(s) (Paper VI) at different process conditions (Figure 17). Enrichment of thermophilic hydrogen producers on cellulose resulted in the yields of 1.4 mol H_2/mol hexose (2.4 mol H_2/mol $hexose_{degraded}$) and 0.4 mol EtOH/mol hexose (0.75 mol EtOH/mol $hexose_{degraded}$). Enrichment cultures consisted mainly of two cellulolytic species and a hydrogen producer (Paper II). Pulp materials were used to enrich hydrogenic and methanogenic bacteria at different pH values. The microbial communities differed with pH and the highest hydrogen and methane yields with wet birch and dry conifer pulps were 560 mL H_2/g TS and 4800 mL CH_4/g TS, respectively (Paper IV). Exoelectrogenic and alcohologenic cultures were enriched from compost culture in MFC. The resulting cultures contained mainly a xylose degrader and two denitrifiers responsible for electricity production. Electron recoveries as electricity and ethanol were 16 and 65 %, respectively (Paper VI). The above described results indicate that preferred energy carrier(s) can be produced with a culture of the same origin by changing the process conditions. The results also demonstrate that changes in microbial communities not changes in metabolic pathways are responsible for the changes in fermentation patterns.

	Substrate	T (°C)	pH	Reactor	Energy carrier	Main bacteria	Paper
Compost culture →	Cellulose	52/60	7	Batch bottle	H_2 (EtOH)	*C. cellulosi*, *C. stercorarium*, *T. thermosaccharolyticum*	II
Compost culture →	Pulp	37	6-9	Batch bottle	H_2, CH_4	*Bacteroides* sp., ruminal and clostridial species	IV
Compost culture →	Xylose	37	7	MFC	Electricity, EtOH/ButOH	*C. denitrificans*, *P. pantotrophus*, *R. xylanolyticum*	VI

Figure 17. Enrichment of different microorganisms from compost culture at different process conditions.

10.5 Comparison of different energy carrier production processes

In this study, energy was produced in the forms of hydrogen (Papers I,V), hydrogen and ethanol (Papers II,II), hydrogen and methane (Paper IV), and electricity and ethanol or butanol (Paper VI). The energy yields (kJ/g DM) obtained from these processes were as presented in Table 23. The energy yields of individual energy carriers were calculated based on their lower heating values (Chapter 9.5) and the energy produced in MFCs was calculated by integrating the power over time. When the energy yields of different bioprocesses are compared, it should be taken into consideration that utilizing H_2, CH_4 and alcohols for electricity or heat results in losses that decrease the final energy yields. MFCs, on the other hand, produce directly electricity and thus, the losses are already acknowledged in the energy yields in Table 23.

The highest overall energy yield of 167 kJ/g DM was produced from wet conifer pulp (Table 23) with methane fermentation (4800 mL CH_4/g TS). This methane yield is high compared to methane yields of 356 and 369 mL CH_4/g VS from cellulose (Cho et al. 1995) and office paper (Owens et al. 1993), respectively, or 420 mL/g TS obtained from reed canary grass and

rhubarb (Lehtomäki et al. 2008). The high methane yield resulted from the almost complete conversion of VFAs and alcohols to methane (88 % of the electrons recovered as CH_4) and from the high methane percentage (81 % at the highest) in the gas phase. In addition to efficient conversion, the high methane yield was likely due to the high surface area of the wet pulp and the frequent adjustment of pH with simultaneous purging of the medium with nitrogen. The overall energy obtained from other pulp materials remained lower due to incomplete conversion of soluble metabolites to methane. The energy yields from the fermentation of other pulp materials also consisted mainly from methane and only 1.6-6.0 and 0.9-3.2 kJ/g DM were obtained as hydrogen or ethanol, respectively. Significantly higher energy yields from methane have been reported in sequential H_2 and CH_4 process by Cavinato et al. (2012), who studied pilot-scale process, and by Liu et al. (2006) (Table 23). In addition, two-times higher energy yield was obtained from sequential ethanol and methane than from single-stage methane production process (Dererie et al. 2011). The higher energy yields obtained as methane resulted from high conversion efficiencies. For example, in dark fermentation only 33 % of the substrate can be theoretically converted into hydrogen (Hallenbeck 2009), whilst in practice the H_2 yields are considerably lower (for a review, see Nath and Das 2004). Thus, the dark fermentation effluent contains majority of the substrates energy resulting in higher methane conversion efficiencies. In addition, simultaneous production of H_2 and CH_4 can further decrease electron recoveries as hydrogen, since hydrogenotrophic methanogens may produce methane from H_2 and CO_2 (Chandra et al. 2012).

High overall energy yields were also produced in MFCs at high xylose concentrations, where butanol was produced as the main soluble metabolite (Table 23). The highest butanol yields from xylose in MFCs were 13.6 and 9.8 g/L resulting in energy yields of 113 and 130 kJ/g DM, respectively. The energy yields recovered as electricity in MFCs remained below 0.2 kJ/g DM. Similarly, electrical energy yields obtained from dark fermentation effluents in MFCs have varied from 0.07 to 0.43 kJ/g DM (Nam et al. 2010, Sharma and Li 2010). Energy yields in MFCs are decreased by the internal losses (Logan et al. 2006) that can be as high as 530 Ω (Paper VI). Lakaniemi et al. (2012) reported increased energy yields with MFCs producing butanol simultaneously with electricity. The energy yields in the form of electricity and butanol were 0.01 and 1.4 kJ/g DW from *C. vulgaris* biomass.

Production of alcohol(s) has also increased energy yields of dark fermentation (Paper II, Koskinen et al. 2008a, Zhao et al. 2009). Without simultaneous ethanol production, the highest energy yields from dark fermentative hydrogen production in this study were obtained in CSTR from xylose (3.2 kJ/g DM) and from wet pulp materials (5.3-6.0 kJ/g DM) (Table 23). Similar energy yields of 4.6 kJ/g DM (Gadow et al. 2012) and 2.8 kJ/g DM (Ren et al. 2010) have been obtained from cellulose fermentation to hydrogen. In this study, cellulose fermentation to hydrogen was followed by ethanol production with the highest overall energy yield of 4.9 kJ/g DM (Paper II). Ethanol fermentation may support hydrogen production, if it proceeds through Eq. 7 (Barros and Silva 2012). However, ethanol can also be produced alone (Eq. 9) reducing the amounts of electrons directed to hydrogen fermentation.

In summary, the highest energy yields were obtained in the form of methane, 167 kJ/g DM, from wet conifer pulp digestion (Paper IV). Butanol produced in MFCs simultaneously with electricity at high xylose concentrations also resulted in high energy yields of 113-130 kJ/g DM (Paper VI). In this study, the energy yields as hydrogen remained relatively low but were increased by the simultaneous production of ethanol resulting in the overall energy yield of 4.9 kg/g DM from cellulose (Paper II).

Table 23. Bioenergy yields obtained from different energy carrier(s) production processes.

				kJ/g DM						
Culture	**Substrate**	**T (°C)**	**pH**	**H_2**	**EtOH**	**ButOH**	**CH_4**	**e^-**	**Overall**	**Reference**
Hot spring	Xylose (batch)	37	6.8	1.1	-	-	-	-	1.1	Paper I
	Xylose (CSTR)	45	5.1	3.2					3.2	
T. aciditolerans	Xylose	60	6.0	1.6	9.1	-	-	-	10.7	Koskinen et al. 2008a
	Glucose			1.6	11.0				12.6	
T. thermosaccharolyticum	Xylose	75	7.0	4.5	12.6	-	-	-	17.1	Ngo et al. 2012
E. coli	Xylose	35	6.0	-	12.6	-	-	-	12.6	Wang et al. 2011c
Enrichment culture	Glucose	70	nr	1.0	9.8	-	-	-	10.8	Zhao et al. 2009
C. beijerinckii	Glucose	34	nr	-	-	11.3	-	-	11.3	Qureshi and Blaschek 1999
Hot spring	Pulp hydrolysate	37	6.0	1.0	1.3	-	-	-	2.3	Paper IV
Compost	Cellulose	52	7.0	1.9	3.0	-	-	-	4.9	Paper II
Rumen fluid	Cellulose	60	7.0	0.6	1.4	-	-	-	2.0	Paper III
Cow dung	Cellulose	55	7.6	0.7	6.9	-	-	-	7.6	Lin and Hung 2008
Digested sludge	Cellulose	80	5.7	4.6	-	-	-	-	4.6	Gadow et al. 2012
Cow dung compost	Cellulose	37	6.8	2.8	-	-	-	-	2.8	Ren et al. 2010
Compost	Dry conifer pulp	37	6.0	1.6	1.7	-	-	-	3.3	Paper IV
	Dry birch pulp		6.0	1.6	0.9	-	3.7	-	6.2	
	Wet conifer pulp		7.0	5.3	2.7	-	159.4	-	167.4	
	Wet birch pulp		6.0	6.0	3.2	-	36.0	-	45.2	
Silage	Silage	37	7.0	1.6	-	-	-	-	1.6	Paper V
C. beijerinckii	Cassava flour	40	4.5	-	4.6	17.4	-	-	22.0	Lépiz-Aguilar et al. 2011
nr	Food waste[a]	nr	7.6	0.7	-	-	23.8	-	24.5	Cavinato et al. 2012
Household solid waste	Household solid waste[a]	37	nr	0.4	-	-	16.5	-	16.9	Liu et al. 2006
S. cerevisiae/biogas sludge	Oat straw[a]	30/37	4.5-4.6/ nr	-	4.0	-	7.6	-	11.6	Dererie et al. 2011
Compost	Xylose (1 g/L)	37	7.0	-	6.2	-	-	0.20	6.4	Paper VI
	Xylose (4 g/L)				0.8	112.5		-	113	
Anaerobic sludge	Xylose (1 g/L)	37	7.0	-	8.1	-	-	0.19	8.2	Paper VI
	Xylose (2.5 g/L)				0.4	129.5		0.02	130	
Anaerobic sludge	C. vulgaris biomass	37	7.0	-	-	1.4	-	0.01	1.4	Lakaniemi et al. 2012
Domestic wastewater	BioH_2 effluent[a]	30	nr	3.8	-	-	-	0.43	4.2	Sharma and Li 2010
Activated sludge	BioH_2 effluent[a]	35	7.0	-	-	-	-	0.07	0.07	Nam et al. 2010

[a] sequential process, DM: dry matter, EtOH: ethanol, ButOH: butanol, e^-: electricity, nr: not reported

11. CONCLUSIONS

This study demonstrated the biological production of different energy carriers from cellulosic materials. Continuous hydrogen fermentation from xylose, an important degradation product of lignocellulose, was reported at suboptimal temperature of 45°C. Cellulolytic and hydrogenic cultures were enriched at different process conditions and further characterized. Hydrogen production from hydrolyzed pulp material was optimized and compared to direct pulp fermentation to hydrogen. This study characterized for the first time microbial communities enriched in MFCs on xylose, and reported simultaneous production of electricity and alcohol(s). Based on this study, the following conclusions can be drawn:

- Hydrogen was produced with a hot spring culture from various sugars, important constituents of lignocellulosic materials. The highest hydrogen yield was 0.7 mol H_2/mol xylose and pentoses were favored over hexoses for H_2 fermentation. (Paper I)

- Hydrogen production from xylose in a CSTR resulted in the highest hydrogen yields and production rates of 2.0 mol H_2/mol xylose and 7.3 mmol H_2/L/h, respectively, at suboptimal temperature of 45°C for both mesophiles and thermophiles. *C. acetobutylicum* and *Citrobacter freundii* were dominant species at 45°C. (Paper I)

- Heat treating compost culture at 80°C for 20 min resulted in the highest hydrogen and ethanol yields of 2.4 mol H_2/mol $hexose_{degraded}$ (1.4 mol H_2/mol hexose) and 0.8 mol EtOH/mol $hexose_{degraded}$ (0.4 mol EtOH/mol hexose), respectively, when grown at 52°C (Paper II).

- Heat treating the rumen fluid culture did not enhance hydrogen yields. The highest hydrogen and ethanol yields with rumen fluid culture at 60°C were 1.9 mol H_2/mol $hexose_{degraded}$ (0.4 mol H_2/mol hexose) and 1.0 mol EtOH/mol $hexose_{degraded}$ (0.2 mol EtOH/mol hexose), respectively. (Paper III)

- Cellulose hydrolysis by compost and rumen fluid cultures was at the highest 57 and 21 %, respectively. The incomplete cellulose hydrolysis controlled hydrogen production from cellulose (Papers II,III).

- Heat treatment of rumen fluid culture did not affect the bacterial diversity at the optimal temperature, while the diversity of microbial communities at the optimal temperature decreased when compost culture was heat-treated. The sequential enrichments, on the other hand, decreased the bacterial diversities with both cultures. (Papers II,III).

- Hydrogen and methane production from pulp materials was affected by pH. Methane was not produced at pH 9 and at pH 6 methane was not produced from dry conifer pulp. The highest hydrogen and methane yields were 560 mL H_2/g TS from wet birch pulp at pH 6 and 4800 mL CH_4/g TS from wet conifer pulp at pH 7, respectively. The high methane yield likely resulted from efficient substrate conversion, high surface area of the substrate, and frequent adjustment of pH. (Paper IV)

- Wet and dry pulp materials were hydrolyzed with concentrated sulfuric acid with sugar yields of 33-37 % and 70-84 % after 90 and 180 min treatment, respectively. Hydrogen fermentation from dry conifer pulp hydrolysate resulted in the highest yield of 63 mL H_2/g TS. (Paper IV)

- Comparing hydrogen yields after direct pulp fermentation (120 mL H_2/g TS) and from acid hydrolysates (63 mL H_2/g TS) revealed that higher H_2 yields were produced from directly fermented pulps. However, the hydrogen production from acid hydrolyzed pulp was faster (10 days) that direct pulp fermentation (28 days). (Paper IV)

- Indigenous grass silage bacteria were successfully used to produce hydrogen from silage after neutralizing the pH. The highest hydrogen yield of 163 mL H_2/g TS was obtained with the lowest silage concentration of 25 g/L. Increasing silage concentration decreased hydrogen yields but increased the cumulative hydrogen production. (Paper V)

- Addition of BESA to inhibit methanogens decreased the hydrogen yields from pulp materials with compost culture (Paper IV) and from silage with indigenous silage bacteria (Paper V).

- Exoelectrogenic and alcohologenic cultures were enriched in MFCs on xylose from compost and anaerobic digester samples. The electron recoveries from 1 g/L xylose as electricity and ethanol were 13-24 % and 40-65 %, respectively. With high xylose concentration of 4 g/L, the anaerobic digester culture recovered 33 % of the electrons as butanol and 4 % as electricity. (Paper VI)

- The exoelectrogenic enrichment cultures grown on xylose were characterized for the first time. They consisted mainly of denitrifying bacteria, *Comamonas denitrificans* and *Paracoccus pantotrophus*, and a xylanolytic species, *Ruminobacillus xylanolyticum*. (Paper VI)

- The highest overall energy yields were obtained from simultaneous H_2 and CH_4 fermentation from pulp materials (167 kJ/g TS) and from simultaneous production of electricity and butanol in MFCs (113-130 kJ/g xylose) (Papers IV,VI). The highest energy yield from hydrogen was produced from wet pulps (5.3-6.0 kJ/g TS) and from continuous H_2 production from xylose (3.2 kJ/g xylose) (Papers I,IV). The energy yields from cellulose fermentation to hydrogen were increased by simultaneous production of ethanol, with the highest overall energy yield of 4.9 kJ/g hexose obtained with compost enrichment culture (Paper II).

- Different energy carrier(s) can be produced with cultures from the same origin by controlling the process conditions. The changes in microbial communities, not the changes in metabolic pathways, are responsible for the changes in fermentation patterns. (Papers I,II,IV,VI)

- Cellulosic, hydrogen producing cultures can be successfully enriched from different origins, including compost, rumen fluid and silage (Papers II,III,V).

12. RECOMMENDATIONS FOR FURTHER RESEARCH

Hydrogen was produced continuously from xylose in this study and from glucose by Karadag and Puhakka (2010a,b) with a Hisarkoy hot spring (Turkey) enrichment culture. The same culture was able to produce hydrogen from all the main sugars derived from degradation of cellulosic materials. In addition, the hot spring culture fermented pulp hydrolysate to hydrogen in batch bottle. Thus, the ability of the hot spring culture to continuously ferment pulp hydrolysates into hydrogen should be determined. Hydrolysates may contain compounds that inhibit dark fermentative hydrogen production (Cao et al. 2010, Quéméneur et al. 2011). The potential inhibitors in the pulp hydrolysate should be determined and the effects of the inhibitors for continuous hydrogen fermentation should be evaluated in long-term.

In this study, cellulolytic and hydrogenic cultures were enriched in batch bottles on pure cellulose, cellulosic pulp materials, and on lignocellulosic silage. Based on these studies, the most potential cellulosic hydrogen producing cultures and their optimal growth conditions can be selected. Hydrogen production from cellulose in batch-mode has been studied extensively, but semi-continuous (Ueno et al. 2001) or continuous (Wang et al. 2011b) cellulose fermentation to H_2 is scarce. Semi-continuous (followed by continuous) hydrogen production from cellulose should be studied with the most potential enrichment cultures obtained in this study. Furthermore, the optimal process parameters for semi-continuous/continuous processes should be determined, since they may differ from the optimal conditions deterimened in batch bottles. The direct fermentation of more complex substrates, such as pulp materials and silage, for the continuous H_2 production should also be evaluated. At present, more complex substrates are hydrolyzed before continuous hydrogen fermentation (Kongjan and Angelidaki 2010, Arriaga et al. 2011). Continuous hydrogen production from hydrolysates may proceed faster, but it also increases the complexity and costs of the process. Thus, the necessity of pretreating the complex substrates before continuous H_2 production should be determined by comparing continuous H_2 production from direct pulp fermentation and from pulp hydrolysates.

Cellulolytic hydrogen fermentation was often accompanied with high ethanol yields in this study. Continuous hydrogen and ethanol fermentation from glucose (Koskinen et al. 2008b) and fructose (Wu et al. 2007) has been studied. In addition, the inhibitory effects of ethanol on saccharolytic, hydrogen producing bacteria has been explored (Koskinen et al. 2008a). However, continuous hydrogen and ethanol production from cellulosic substrates has not been reported and should be evaluated with the most potential enrichment cultures obtained in this study. In addition, the inhibitory effects of ethanol on the enrichment cultures should be determined in batch bottles and in continuous reactors.

Dark fermentative hydrogen production results in effluents that still contain high amounts of volatile fatty acids. To be economically feasible, these effluents should be further utilized, for example, for the production of methane (Cavinato et al. 2012), electricity (Mohanakrishna et al. 2010), or hydrogen through photofermentation (Lu et al. 2009, Özgur et al. 2010). In this study, methane was produced simultaneously with hydrogen from pulp materials. The division of these energy carrier processes into two steps for the sequential production of hydrogen and methane should be studied. This would enable the separate collection of H_2 and CH_4 gases. In addition, the processes could be optimized separately. In this study, xylose (and the intermediate products, VFAs) was successfully utilized for electricity and ethanol/butanol

production. The suitability of dark fermentation effluents for the simultaneous production of electricity and alcohol(s) in MFCs should be determined with the enrichment cultures obtained in this study.

Electricity and alcohol(s) were produced simultaneously from xylose in MFCs. The initial xylose concentration had a huge effect on electricity and alcohol(s) yields. The effects of other operational parameters (temperature, pH, external resistance) on the production of electricity and alcohol(s) should be further evaluated. The fundamentals of simultaneous electricity and alcohol(s) production should also be delineated. Furthermore, the substrate utilization efficiency of the two enrichment cultures should be studied with different sugars and fatty acids to discover their potential for utilizing, e.g. dark fermentation effluents. The exoelectrogenic cultures enriched in this study contained many denitrifying bacteria that were mainly responsible for electricity production. One of these denitrifiers, *Comamonas denitrificans*, is known to produce electricity from acetate in MFC (Xing et al. 2010). The ability of the other denitrifiers to produce electricity should be determined, and the utilization of other VFAs and sugars to electricity should be evaluated with all the identified denitrifying bacteria. In addition, the fermentation patterns of these bacteria should be determined to find out, whether some of the denitrifiers produce ethanol or butanol as soluble metabolite in MFCs.

The bacterial communities involved in dark fermentative hydrogen production and in electricity generation were characterized with PCR-DGGE. This enables the monitoring of the changes in the cultures at different process conditions or during the enrichment. If continuous hydrogen production from cellulosic substrates is studied, the changes in the microbial communities should be characterized regularly with PCR-DGGE. However, PCR-DGGE does not differentiate the number of the species or their changes over time (Spiegelmann et al. 2005). Hydrogen producing communities could be quantitatively monitored with quantitative PCR (qPCR) that is more sensitive than PCR (Talbot et al. 2008). Quantitative PCR has been used to analyze the total amount of bacteria (Nadkarni et al. 2002), the amount of specific Clostridium species (Tolvanen et al. 2008), or the changes in the amount of hydrogenase genes (Tolvanen et al. 2010). The different qPCR methods should be tested for monitoring hydrogen producing communities from continuous reactors as well as for monitoring the exoelectrogenic communities in MFCs.

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