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Preface

This thesis is prepared as part of a PhD study during the period April 15, 2006 to April 14, 2010. A PhD research project was carried out at the Department of Environmental Engineering, Technical University of Denmark (DTU). Throughout the period, Professor Irini Angelidaki was the main supervisor. Dr. Booki Min was the co-supervisor in the initial PhD period (Paper I and Paper III).The thesis is categorized into the first part, an introductive review and summary and the second part, the following papers.

I Kongjan P, Min B, Angelidaki I. 2009. Biohydrogen production from xylose at extreme thermophilic temperature (70 ºC) by mixed culture fermentation. Water Research 43 (5), 1414-1424.

II Kaparaju P, Serrano M, Thomsen A B, Kongjan P, Angelidaki I. 2009. Bioethanol, biohydrogen and biogas production from wheat straw in a biorefinery concept. Bioresource Technology. 100 (9), 2562-2568.

III Kongjan P, O-Thong S, Kotay M, Min B, Angelidaki I. 2010. Biohydrogen production from wheat straw hydrolysate by dark fermentation using extreme thermophilic mixed culture. Biotechnology and Bioengineering 105(5), 899-908.

IV Kongjan P, Angelidaki I. 2010. Extreme thermophilic biohydrogen production from wheat straw hydrolysate using mixed culture fermentation: Effect of reactor configuration. Bioresoure Technology. doi: 10.1016/j.biortech.2010.05.024

V Kongjan P, O-Thong S, Angelidaki I. 2010. Performance and microbial community analysis of two-stage process with extreme thermophilic hydrogen and thermophilic methane production from wheat straw hydrolysate. Manuscript.

VI Kongjan P, O-Thong S, Angelidaki I. 2010. Biohydrogen production from desugared molasses (DM) using thermophilic mixed cultures immobilized on heat treated anaerobic sludge granules. Manuscript.

The papers are not included in this www-version, but can be obtained from the Library at DTU Environment. Contact library@env.dtu.dk or Department of Environmental Engineering, Technical University of Denmark, Miljoevej, Building 113, DK-2000 Kgs. Lyngby, Denmark

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Prawit Kongjan

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Furthermore, my sincere thanks go to Dominic Butland for his help in English corrections in the dissertation. A special thank to Kiki L. Larsen, Brian S. Lassen and N'Petch for helping in Dansk Resumé as well.

Last, but definitely not least, I would like to thank my families, Pho(Dad), Mae(Mom), my wife (Joop), my daughter (Moddaeng), and my brother (N'Yut) for all their support and encouragement.

Abstract

Hydrogen and methane generated by microbial conversion of organic wastes /residues have potential to replace fossil fuels as environmentally friendly as well as sustainable and renewable energy carriers. Xylose rich hydrolysate is a liquid phase by-product generated from hydrothermal pretreatment of lignocelluloses and is currently inefficient and uneconomical for bioethanol production. Alternatively, biohydrogen was successfully produced from xylose and hydrolysate in both batch reactor and continuously stirred tank reactor (CSTR) by using mixed culture enriched for hydrogen producing microorganisms. Acetate was found to be the dominant metabolic products during xylose fermentation under extreme thermophilic conditions. Additionally, toxic compounds, furfural and hydroxymethylfurfural (HMF), contained in the hydrolysate were found to be effectively degraded in the CSTR by the H_2 producing bacteria.

Caldanaerobacter subteraneus, *Thermoanaerobacter subteraneus*, and *Thermoanaerobacterium thermosaccharolyticum,* were detected in the mixed culture enriched with xylose rich substrates. In CSTR reactor, the methanogens was suppressed mainly by controlling at a pH of 5-6 and a hydraulic retention time (HRT) of less than 3 days. However, low cell density achieved from operating CSTR with mixed extreme thermophilic fermentation could limit H_2 production at high organic loading rate. It was demonstrated that an up-flow anaerobic sludge blanket (UASB) reactor which contains immobilized biomass, could overcome this limitation and give rather high H_2 yield of 212 ml-H_2/g-sugars, corresponding to a rate of 821 ml-H_2/d·l. The two stage anaerobic process can be sustainable for effective energy recovery and stabilization of hemicelluloses containing hydrolysate.

Combining extreme thermophilic acidogesis and thermophilic methanogesis in UASB reactors connected in series, with total HRT of 4 d (hydrogen, 1 d; methane, 3 d) could dramatically increase the energy conversion efficiency from only 7.5% in the hydrogen stage to 87.5% of the potential energy from hydrolysate, corresponding to total energy of 13.4 kJ/g-VS. Microbial community analysis of the two-stage process confirmed the separation of the processes in the two reactors which had different microbial composition, with hydrogen-producing bacteria of *Thermoanaerobacter wiegelii*,

Caldanaerobacter, subteraneus, and *Caloramator fervidus*. Meanwhile, in the second reactor methanogenesis was taking place with aceticlastic methanogens of *Methanosarcina mazei* and hydrogenotrophic methanogens of *Methanothermobacter defluvii*.

Desugared molasses (DM) is an another potential substrate for H_2 production with a H_2 potential yield of 237 ml-H_2/g-sugar achieved by thermophilc batch fermentation at 55 ºC, with 1.25% (v/v) DM. An UASB reactor with immobilized enriched hydrogen producing mixed culture on its granules, resulted in a satisfactory hydrogen yield of 263 ml-H_2/g-sugar and rate of 4500 ml H_2/d·l by feeding with 10% (v/v) DM at one day HRT. Fluorescent *in situ* hybridization (FISH) analysis of the microbial community of the UASB-granules was dominated by *Thermoanaerobacterium* spp, which are key players in fermentative hydrogen production of DM under thermophilic conditions. Furthermore, the granules in UASB were also containing phylum *Firmecutes* (most *Clotridium*, *Bacillus* and *Desulfobacterium*), which are responsible for the lactate degradation.

Dansk Resumé

Hydrogen og metan produceret ved mikrobiel omdannelse af organisk affald/restprodukter er et miljøvenligt og bæredygtigt alternativ til fossile brændstoffer. Hydrolysat rigt på xylose er et flydende biprodukt, der dannes under hydrotermisk forbehandling af plantematerialet lignocellulose. Produktion af bioethanol fra dette biprodukt er hverken økonomisk eller effektivt. Derimod kan biohydrogen produceres med gode resultater. Ved brug af en blandet mikrobiel kultur beriget med hydrogen producerende mikroorganismer opnås disse resultater både ved brug af batch reaktorer og ved brug af reaktorer med konstant omrørte reaktor-tanke (CSTR). Resultater viste, at under ekstreme termofile forhold var acetat det dominerende metaboliske produkt af xylose-gæringen. Ydermere viste forsøgene, at de toksiske forbindelser furfural og hydroxymethylfurfural (HMF), der findes i hydrolysatet, effektivt bliver nedbrudt af de H_2-producerende bakterier i CSTR reaktorerne.

Caldanaerobacter subteraneus, Thermoanaerobacter subteraneus og *Thermoanaerobacterium thermosaccharolyticum* er de hydrogen producerede bakterier som blev fundet i den blandingskulturen med substrater rige på xylose. I CSTR reaktoren blev methanogenerne holdt under kontrol, ved at opretholde en pH-værdi på fem til seks samt en hydraulisk opholdstid (HRT) på mindre end tre dage. Brugen af CSTR ved ekstrem termofil gæring resulterer i lav celletæthed. En uønsket konsekvens af dette er en begrænset H_2 produktion, hvis tilførslen af det organiske materiale sker for hurtigt. Det blev påvist, at en UASB (Upflow anaerobic sludge blanket) reaktor indeholdende immobiliseret biomasse kan forhindre denne begrænsede produktion. Dette blev vist ved et højt H_2 udbytte på 212 ml H_2/g-sukre, svarende til et udbytte på 821 ml H_2 per dag·per liter). To-trins anaerobe-processer kan derfor anvendes til en effektiv energiudnyttelse og stabilisering af hemicellulosehydrolysat.

Kombinationen af ekstrem termofil acidogese og termofil methanogese i to UASB reaktorer forbundet i serie, kan øge energiomdannelseseffektiviteten markant. Ved en samlet HRT på fire dage (hydrogen en dag; metan tre dage) øgedes den totale potentielle energimængde fra hydrolysatet fra kun 7,5 % i hydrogen-trinnet, til 87,5 % i metantrinnet. Dette svarer til et samlet energiudbytte på 13,4 kJ/gVS (VS: flygtigt fast stof). Den mikrobielle analyse af to-trins processen bekræftede adskillelsen af processerne i de to reaktorer. I den

første reaktor bestod den mikrobielle sammensætning af de hydrogen-producerende bakterier; *Thermoanaerobacter wiegelii*, *Caldanaerobacter subteraneus* og *Caloramator fervidus*, hvorimod metandannelsen fandt sted i den anden reaktor med aceticlastisk methanogens af *Methanosarcina mazei* og hydrogenotrophisk methanogens af *Methanothermobacter defluvii*.

Afsukret melasse (DM) udgør et anden potentielt substrat for H_2-produktion. Ved termofil batch-gæring (ved 55 ºC) og 1,25% (v/v) DM er potentialet for H_2-udbyttet på 237 ml H_2/g-sukker,. En UASB-reaktor med en beriget hydrogen-producerende kultur immobiliseret på reaktorens granulater gav et tilfredsstillende hydrogenudbytte på 263 ml H_2/g sukker samt en hydrogen produktionsrate på 4.500 ml H_2/dag/liter. Dette blev opnået ved indfødning af 10 % (v/v) DM på en dags HRT. Fluorescerende *in situ* hybridiseringsanalyser (FISH) af mikrobielle kulturer i UASB granulater, viste at de var dominerede af *Thermoanaerobacterium* spp, der er centrale aktører i gærende hydrogen produktion af DM under termofile forhold. Derudover indeholdt granulatet i UASB også phylum *Firmecutes* (primært *Clotridium, Bacillus* og *Desulfobacterium*), som resulterer i laktat nedbrydning.

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1 Introduction and aims of study

The world is now facing with both fossil fuel shortage and global climate change. Climate change is widely believed to be linked to the rapid rise in damage caused by natural disasters over the last 30 years and the consequences of global warming, which is caused by the rapidly increasing concentrations of greenhouse gas (CO_2 and others) in the atmosphere, emitted mainly by the combustion of fossil fuels containing carbon like coal, oil, and natural gas. To secure the future supply of energy carriers and help prevent the consequent negative effects of climate change (i.e. changes in precipitation amounts and seasonal patterns in many regions, changes in the intensity and pattern of extreme weather events, sea level rise, and increase in existing risks of species extinction and biodiversity loss), the global community has to change the way it uses and generates energy carries. Less harmful alternatives are urgently needed to replace non-renewable fossil fuels.

Among various options, hydrogen and methane generated from organic wastes and residues using a two stage anaerobic digestion process could play an important role in the future energy economy as environmentally friendly, renewable, sustainable and cheap energy (Antonopoulou et al., 2008; Cooney et al., 2007; Koutrouli et al., 2009; Kyazze et al., 2007; Lee et al., 2010; Liu et al., 2006; Venetsaneas et al., 2009; Zhu et al., 2008). This two-stage anaerobic process is an attractive and promising technology for providing clean energy production whilst treating organic wastes and residues. Additionally, gas mixture blending of hydrogen at 10 – 60% by volume with methane could be considered as an efficient fuel for the vehicles using an internal combustion engine, because hydrogen is a powerful combustion stimulant for accelerating methane combustion. Moreover, a mixture of hydrogen and methane will significantly reduce the emission of CO, CO_2 and NO_x of natural gas powered vehicles (Alavandi and Agrawal, 2008; Porpatham et al., 2007).

The anaerobic two-stage process, in which hydrolysis/acidogenesis and acetogenesis/methanogenesis can take place in separated reactors (because both groups of microorganisms have considerable differences in terms of their physiology, nutritional need, growth kinetics, and sensitivity to environmental conditions), was first proposed by Pohland and Ghosh (1971) in order to enhance the overall process stability and control by optimizing environmental conditions

for each group of microorganisms. The first acidogenic stage of the two-stage anaerobic digestion process has been traditionally employed to enhance production of organic acids for methane production in the second methanogenic stage by using various organic wastes including sewage sludge, mixture of excess sludge and kitchen garbage, dairy wastewater, instant coffee waste, food waste, and agro-industrial wastes as reviewed by Demirel and Yenigun, (2002). However, as previously stated by Liu et al., 2006, the effect of increasing methane production through the two stage system has been argued broadly due to the two main processes of acidogenesis and methanogesis impacts on syntrophic association and prevents interspecies hydrogen transfers. Moreover, the complexity of the two stage anaerobic digestion process could increase the investments and operational costs. Therefore, currently, methane production via a two-stage anaerobic digestion process is only 10% of the full scale plant across Europe (Baere, 2000).

Alternatively, instead of using a precursor or pretreatment for the methanogenic reactor, the first stage anaerobic digestion normally called dark fermentation has become an established and proven technology for biohydrogen production from carbohydrate rich substrates coming mainly from wastewater substrates and biomass substrates (agricultural residues, plant biomass, and industrial effluents (Kapdan and Kargi, 2006; Hawkes et al., 2007; Hallenbeck et al., 2009). Theoretically, degradation of one mole of xylose or glucose can produce 3.33 or 4.0 moles of hydrogen respectively, simultaneously with acetate, or 1.67 or 2.0 moles of hydrogen respectively, simultaneously with butyrate. However, end fermentation products other than acetate and butyrate such as propionate, ethanol, lactate and formate are usually co-produced during fermentative hydrogen production, resulting in a lower hydrogen yield.

Types and proportions of all products during the fermentation are severely depending on the microorganisms in, the environmental factors of (i.e. temperature, pH, and the hydrogen partial pressure), and the oxidation state of the substrate being degraded (Angenent et al., 2004; Levin et al., 2004; Hallenbeck and Ghosh, 2009). As reviewed by Hallenbeck and Ghosh (2009), various techniques such as altering reactor configurations, the use of mixed cultures, metabolic engineering of existing pathways, and modeling and optimization have been tried in order to improve hydrogen production in terms of both technical efficiency (based on hydrogen yield) and economic efficiency

(based on hydrogen production rate), however the single fermentative hydrogen production process cannot be made economical because hydrogen yield are still limited by the above mentioned metabolic pathways. To obtain economical feasibility, the dark fermentation stage has to be coupled with the second stage for sequentially converting fermentation end products to either methane by traditional anaerobic digestion or to hydrogen by photo-fermentation or to electricity by microbial fuel cell system (Hawkes et al., 2007). The combined hydrogen and methane production in a two stage anaerobic digestion process seems like it could be more feasible than the others in the near future because a mixture of hydrogen and methane called hythane has already been demonstrated to work in internal combustion engines (Gattrell et al., 2007) and methane production in the second stage has been established in the full scale plant (Baere, 2000).

When hydrogen is the product of interest in the dark fermentation stage, carbohydrate based substrates are the most preferable substrate because conversion of carbohydrates into hydrogen utilizing many anaerobic bacteria gives high hydrogen yields due to the thermodynamic point of view (de Vrije and Claassen, 2003). Carbohydrates existing in organic wastes and residues are expected to be a potential and major source of sustainable and renewable energy in the world because of their food production decouple and their abundant and relative cheapness (Escobar et al., 2009; Tan et al., 2008). Additionally, using these kinds of wastes and residues as the substrates for bio-fuel production, (now called second generation biofuel technology), not only clean energy can be achieved but also effective waste treatment.

Wheat straw hydrolysate (mainly consisting of hemicellulose sugars (xylose and arabinose) generated during hydrothermal pretreatment, a process applied for releasing cellulosic sugar (glucose) from wheat straw for second generation bio-ethanol production) and desugared molasses soluble (DM) (which is a by-product remaining after sucrose removal from beet molasses) are the initial energy crop residues of wheat and sugar beet, which are mainly produced across Europe, and classed as a process wastewater and by-products respectively (Thomsen et al., 2008; Western sugars, 2006; de Wit and Faaij, 2009). The investigation of these substrates for hydrogen and subsequent methane production is therefore challenging.

The main objective of this PhD. project was to investigate the potential of hydrogen production from sugar rich substrates (D-xylose, wheat straw hydrolysate, and DM) using mixed culture fermentation under moderate and extreme thermophilic temperature. Additionally, the studies of methane production from the hydrogen reactor effluents had been also carried out for increasing the efficiency of energy recovery from the substrates. In order to fulfill this objective, the following work-tasks were addressed.

1 D-xylose fermentation for hydrogen production (*Paper I*)

Adaptation of the original inoculum with D-xylose was investigated in batch reactors. The enriched inoculum was then used to investigate the metabolic pathways and establish kinetic parameters for biohydrogen fermentation at different initial D-xylose concentrations. Furthermore, the enriched mixed culture was sequentially cultivated in the continuously stirred tank reactor (CSTR) for continuous biohydrogen production from D-xylose. Both batch and continuous mode operations were carried out at extreme thermophilic temperature (70ºC).

2 Hydrolysate fermentation for hydrogen production (*Paper II, III*)

The experiments were operated at extreme thermophilic temperature (70ºC). Hydrolysate generated from hydrothermal pretreatment of wheat straw was used as the substrate for hydrogen production in both batch and CSTR reactors. The mixed culture used in this investigation was previously enriched with D-xylose. The bacterial diversity was also identified both for batch cultivation and in continuous fed reactor operation for identifying the main hydrogenogens in the reactors.

3 Optimizing reactor configuration for continuous hydrogen production (*Paper IV*)

Continuous biohydrogen production from hydrolysate was investigated in 3 different types of continuously fed reactors: CSTR), up-flow anaerobic sludge bed (UASB) reactor, and anaerobic filter (AF) reactor using the same microbial inoculum that had been previously enriched with hydrolysate. In all reactors the hydrogen production performance and the effect of changing organic loading rates by operating at extreme thermophilic temperatures (70 $^{\circ}$C) were compared.

4. Hydrogen and methane from hydrolysate in the two-stage anaerobic process (*Paper V*)

Using hydrolysate for potentially combining extreme thermophilic fermentation at 70 °C and thermophilic anaerobic digestion at 55 °C was investigated in the acidogenic UASB and methanogenic UASB reactors that were operated in series. Each reactor was using its own selected mixed cultures. The microbial community in each reactor was also analyzed.

5. Bio-hydrogen production from desugared molasses (DM) by mixed culture fermentation (*Paper VI*)

Hydrogen-producing inoculum, was prepared by exposing digested manure to high DM load (also called shock load), in batch reactors at thermophilic temperature (55ºC). The mixed culture after this treatment was enriched to DM by successive batch cultivations and was further immobilized in an UASB reactor, to evaluate the feasibility of continuous hydrogen production from DM. Additionally, microbial ecology in both batch and UASB reactors was monitored by using the fluorescence *in-situ* hybridization (FISH) method.

2 A two stage anaerobic process

Normally anaerobic digestion is a biological multi-step process involving a large number of micro-organisms working together in the absence of oxygen to degrade, or convert organic matter into the most reduced and oxidized products of methane and carbon dioxide respectively (a mixture commonly called bio-gas). The first step is hydrolysis where hydrolytic and fermentative bacteria excrete enzymes to break down complex organic compounds of carbohydrate, protein, and lipid into single molecules of mono sugar, amino acid, and long chain fatty acids and/or glycerol respectively. Secondly, in the acidogenesis stage, fermentative and acidogenic bacteria convert the hydrolysis products into carbon dioxide, hydrogen, organic acids and alcohols. Thirdly, organic acids and alcohols are then used by the acetogenic bacteria to produce acetic acid along with additional hydrogen and carbon dioxide. In the forth, and final step, methanogens transform these products to methane and additional carbon dioxide (de Mes et al., 2003). Methane released from sources, such as landfill sites, into the atmosphere can cause significant global warming because its radiative forcing power is 23 times higher than that of carbon dioxide (Tilche and Galatola, 2008). As part of an integrated waste management system, treatments of bio-wastes by conventional anaerobic digestion processes are the optimal way to convert organic wastes into renewable energy sources, in the form of bio-gas, helping to replace fossil fuels. Additionally, minerals and nutrients discharged from the anaerobic digestion of organic wastes can be reused as fertilizers in food production (Angelidaki et al., 2003).

So far, a two-stage anaerobic digestion process for hydrogen and methane from carbohydrate rich substrates is still being developed in lab scale reactors. The two-phase digestion process is based on two physiologically different groups of micro organisms. One group of acidogenic bacteria that converts organic matter into hydrogen, carbon dioxide, and soluble organic acids and alcohols, is fast growing, prefers a slightly acidic environment of pH 5.0 to 6.0, and is less sensitive to changes in the incoming feed stream. The other group of methanogenic achaea, which converts soluble matter into biogas, is slow growing, prefers neutral to slightly alkaline environments and is very sensitive to changes. As shown in Figure1 below, the anaerobic process is categorized into two stages based on the products of hydrogen and methane. Hydrogen released from the first stage is called dark hydrogen fermentation, while the soluble end

products generated by this stage are fed into the second stage, a methane phase for further anaerobic methane production by sequential acetogenesis and methanogenesis steps (Liu et al., 2006).

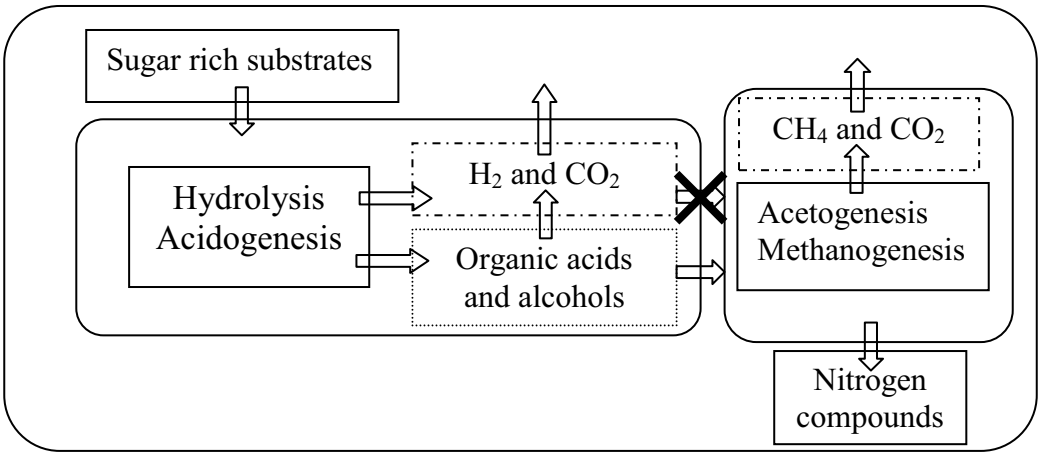

Figure1. Flow diagram of two-stage anaerobic process (Adapted from Liu, 2008)

By obtaining the optimum environmental conditions for each group of organisms, the two-stage anaerobic process provides several advantages over the conventional single stage (Demirel et al., 2010; Bolzonella et al., 2007; Liu et al., 2006), e.g.

- High process and net energy efficiencies, thus better economics.
- More stable digestion, allowing higher throughput.
- Smaller-size tanks (40 – 60% smaller), thus significant capital cost savings.
- Higher methane content in the bio-gas (65– 75% methane vs. 50 – 55% for conventional technologies). Since, carbon oxide in the second stage is mainly generated by aceticlastic methanogenesis and then consumed partly by hydrogenotrophic methanogenesis also existed in the second stage (Paper V) for more methane production, thus resulting in higher methane content. The higher methane content is definitely better fuel value for on-site use.
- Higher digestion efficiency, thus more methane recovered.

2.1 Substrates considerations

The use of pure sugars, such as sucrose, glucose, xylose, arabinose, and lactose are only used for trying to understand the microbial physiology of hydrogen production, and is never intended to be used as a source for hydrogen production on an industrial scale because pure sugar substrates are too expensive. Furthermore, renewable feed stocks need to be utilized to meet the demand for renewable energy as a truly sustainable process (Hawkes et al., 2002). The major criteria for the selection of organic substrates to be used in dark fermentative hydrogen production are cost, availability, carbohydrate content and bio-degradability. It is helpful then to categorize the potential substrates into wastewater and biomass substrates (Hallenbeck et al., 2009; Kapdan and Kargi, 2006; Li and Fang, 2007).

2.1.1 Lignocellulosic residues

Lignocellulosic materials from agricultural and plant biomass generate bioenergy of about 30 EJ/year, which is around 7.5% of the total energy used world wide of over 400 EJ/year (McKendry, 2002). They are also considered to be the largest carbohydrate rich source for the second generation technology for industrial biofuel production, however lignocelluloses do not contain easily fermentable sugars, but complex polymers consisting of tightly bound lignin, cellulose and hemicelluloses. These complex structures are extremely resistant to enzymatic digestion or microbial hydrolysis (Larsen et al., 2008; Levin et al., 2009). Thus, some kind of pretreatment is necessary to disrupt the plant cell wall (lignin) and subsequently liberate cellulose and hemicelluloses to the micro-organisms. Typical hydrothermal pretreatment provides a solid phase containing the main part of cellulose and lignin, and the hydrolysate part containing mainly hemicelluloses (Thomsen et al., 2008). In a bio-refinery concept (Paper II), cellulose is primarily used for bio-ethanol production which is a well established process and efficiently carried out by *Saccharomyces cerevisiae,* with high yield and productivity, meanwhile the harsh hydrolysate is alternatively converted to hydrogen via mixed dark fermentation with a satisfactory yield and rate. Subsequent biogas production from the effluents generated from both the ethanol and hydrogen production process could improve the overall energy yield in this biorefinery system, enabling multiple bio-fuels and increasing the overall economy of the refining process.

2.1.2 Industrial organic residues and wastewater

Organic solid wastes and residues generated by the food oil and sugar industries, domestic households, and wastewater treatment plants could potentially be other abundant substrates for dark fermentative hydrogen production. Apart from containing carbohydrates, such wastes also contain large quantities of proteins and fats as reviewed by Chong et al, (2009), Li and Fang, (2007) and Ntaikou et al, (2010). Generally, carbohydrate based substrates give significantly higher hydrogen yield than fat and protein based substrates (de Vrije et al., 2003). Furthermore, such wastes have quite complex chemical compositions, including different organic and inorganic substances in varying concentrations that may inhibit microorganisms via high organic loading and/or high toxic compounds as the consequences of direct feeding. Thus, dilution of raw wastes has to be performed for almost all of these substrates (Ntaikou et al., 2010).

2.2 Dark fermentative hydrogen production

Carbohydrate rich substrates are the most suitable substrate for fermentative hydrogen production because many fermentative bacteria include the release of hydrogen to obtain the redox balance needed in their metabolic energy (de Vrije and Claassen, 2003). Hexoses and pentoses, which are the first and second most monomeric sugar found in the abundant carbohydrate rich organic wastes/residues can be converted to hydrogen with the maximum theoretical yields of 498 ml-H_2/g-monomeric sugar associated acetate. The lower theoretical yield of 249 ml-H_2/g-monomeric sugar is achieved when butyrate is generated as a fermentation product. The practical yield is even lower when other metabolic compounds such as propionate, ethanol, and lactate are produced as the fermentation products. These metabolic products bypass the major hydrogen producing reaction in carbohydrate fermentation as the consequence of thermodynamic limitations (Angenent et al., 2004).

2.2.1 Thermodynamics

Hydrogen formation during carbohydrate fermentation is mediated by hydrogenase using electrons from reduced ferredoxin (Fd_{red}) and NADH to reduce protons. The proton reducing ability of Fd_{red} and NADH is thermodynamically limited by the maximum hydrogen partial pressures (P_{H2}) of 0.3 and $6x10^{-4}$ atm (60 Pa) respectively. This confers that as long as the P_{H2} is still less than 0.3 atm, hydrogen production can continue with transferring electrons from Fd_{red} which contains electrons from oxidative decarboxylation of

pyruvate by pyruvate:ferredoxin oxidoreductase (PFOR). Meanwhile, the oxidation of NADH by NADH:Fd oxidoreductase (NFOR) can generate Fd_{red} that is subsequently generates additional hydrogen when the P_{H2} is kept less than 60 Pa as shown in Figure2, however, the P_{H2} limited to hydrogen generation via the oxidation of NADH could be increased to 0.1-0.2 atm at a temperature of 70 ºC (van Niel et al., 2003). Therefore, increasing cultivation temperature is necessary to overcome thermodynamic limitation, resulting in a decrease of the Gibbs free energy of conversion according to the second law of thermodynamics ($\Delta G = \Delta H - T \Delta S$) (Stams, 1994).

Thermophilic micro-organisms produce generally higher hydrogen yields than mesophiles because they are thermodynamically favorable (Kengen et al., 2009). High hydrogen yields in a range of 314.0 - 473.0 ml-H_2/g-sugars) have been previously reported by using thermophiles such as, *Clostridium thermocellum* and *Thermoanaerobacterium thermosaccharolyticum* and extreme thermophiles such as, *Thermotoga elfi*, *Caldicellulosiruptor saccharilyticus, Caldanaerobacter subterraneus* (Bothun et al., 2004; Ivanova et al., 2009; O-Thong et al., 2008; van Niel et al., 2002; Yokoyama et al., 2009).

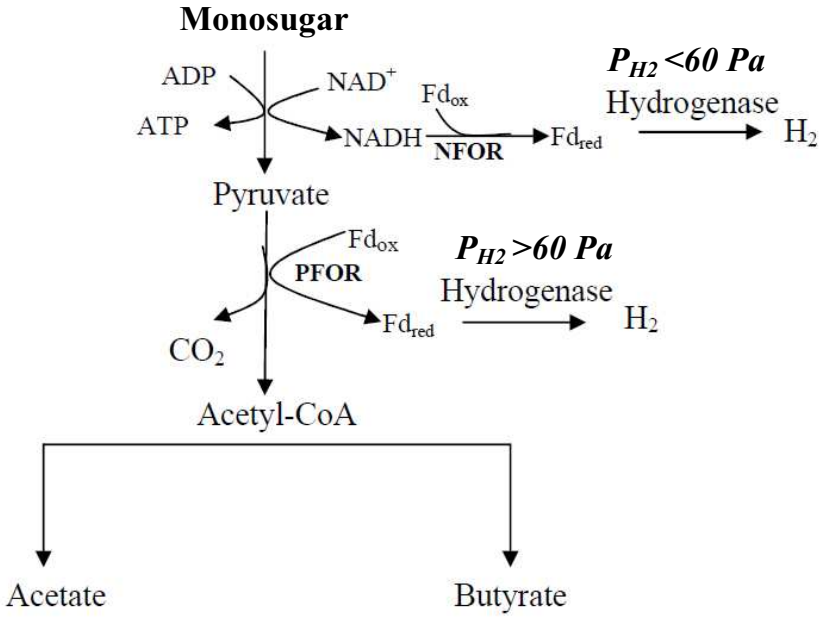

Figure 2 Hydrogen pathways during dark fermentation of sugar (Adapted from Paper I).

2.2.2 Applications of mixed cultures

When the dark fermentation process combines the environmental biotechnology in term of organic wastes or residues treatment with industrial biotechnology that is aiming for hydrogen maximization, mixed culture fermentation could thereby become more attractive than pure culture fermentation, as mixed cultures are applied originally in the waste treatment fields. Compared to pure culture fermentation, mixed culture fermentation has no requirement for sterilization of the media, offers better adaptation capacity due to its high microbial content, and the possibility of mixed substrates co-fermentation, and also allows a continuous fermentation process (Kleerebezem and van Loosdrecht, 2007). Undefined mixed cultures taken from different natural sources need pretreatment or enrichment, by manipulating the operation of the fermentation process and/or by varying the sources of the natural inoculum in order to obtain the required metabolic capacities and the corresponding microbial population for development of the dark fermentation process (Temudo et al., 2008; Ozmihci and Kargi, 2010).

To prepare the inoculum for hydrogen production by fermentation of carbohydrates, the original anaerobic sludge is first pretreated to suppress methanogenic archaea, which consume hydrogen generated and subsequently enrich hydrogen producing bacteria in various reactor configurations (Demirel et al., 2010). Pre-treating anaerobic seed sludge under harsh conditions, spore forming bacteria involved in anaerobic conversion of carbohydrates to hydrogen could have a better chance to survive than non-spore-forming methanogenic archaea. The spores formed can be activated when the required environmental conditions are provided during subsequent enriching for hydrogen production (Li and Fang, 2007). Methods, including heat shock, load shock, acid, base, and chemical pretreatments are usually applied to pre treat anaerobic seed sludge for fermentative hydrogen production

Heat shock

Heat shock has been the most common and effective method for eliminating methanogenic archaea and is achieved by steam heating the seed sludge at 75-121 ºC with an exposure time between 15 and 120 minutes, which is relatively easy and inexpensive (Table 1). The heat shock may also suppress the activity of non-spore-forming propionate producers, but may not effectively deactivate homoacetogens (Hawkes et al., 2007; Arooj et al., 2008). The existence of homoacetogenic bacteria results in a decrease of hydrogen production because

these bacteria further consume hydrogen produced from the fermentation process for the production of acetate (Gavala et al., 2006). Additionally, Duangmanee et al, (2007) have previously observed that inoculum pretreated by heat shock was not stable for hydrogen production in the continuous reactor, and a repeated heat treatment was needed every month to maintain some stability in hydrogen production.

Load shock

During load shock using the pulse load technique in batch and organic fermentation, or hydraulic shock in continuous fermentation, volatile fatty acids (VFAs) tend to accumulate in the fermentative reactor in high concentrations, associated with acidic conditions, they inhibit methanogens (Voolapalli and stuckey, 2001; Kaparaju et al., 2009). Applying a load shock with a pulse load of about 40 – 50 g-sugar/l sucrose, the pretreated anaerobic sludge effectively suppressed methanogenic activity (Luo et al., 2010; O-Thong et al., 2009). Furthermore, O-Thong et al, (2009) has described that load shock pretreated seed sludge could give hydrogen production as high as heat shock pretreated seed sludge, and that load shock would be technically easier to do and more economical than heat shock for implementation on an industrial scale.

Acid and alkali pretreatment

The bio-activity of methanogens during the conventional anaerobic process treatment of organic wastes happens in neutral to slightly alkaline environments (pH 6.8 – 8.0) (Demirel et al., 2010). Limiting methanogenesis can be achieved by adjusting the acidity of the anaerobic sludge substantially away from the preferable range to either pH 3 – 4 or pH 12. The acid or alkali pretreatment is considered to be technically easier than heat shock pretreatment for industrial scale implementation (Hawkes et al., 2007), however, inoculum obtained from an acid or alkali pretreatment requires a much longer acclimatization time of 10 to 30 days to establish hydrogen production (Valdez-Vazquez et al., 2005).

Methanogen inhibitors

2-bromoethanesulfonate acid (BESA), an analog of the coenzyme-M in methanogens, is a chemical that will deactivate methanogens. Using BESA at concentrations of 25 – 100 mM has been found to effectively inhibit the bio-activity of methanogens, however treating anaerobic sludge at these levels would not be cost effective for a commercial scale operation (Li and Fang, 2007).

2.3 Methanogenic anaerobic digestion

The soluble compounds discharged after hydrogen fermentation are mainly dominated by VFAs, like acetate, butyrate, and propionate, which are degraded mainly from carbohydrates (Lee et al., 2009). In the second anaerobic stage, methanogens degrade these soluble products to methane via acetogenesis and methanogenesis. Since these products besides acetate can not be used directly in methanogenesis due to the restricted metabolism of methanogens. Most methanogens are specialized in growth mainly with H_2/CO_2, formate, and acetate. Other organic acids are therefore needed to be firstly oxidized to acetate and H_2/CO_2 in acetogenesis by obligated proton reducing bacteria in syntrophic association with hydrogenotrophic methanogens as low H_2 partial pressure ($<10^{-4}$ atm) is essential for acetogenic reactions to be thermodynamically favorable (Batstone et al., 2002; Stams et al., 2005). Additionally, the degradation of propionate is regarded as the limiting factor in acetogenesis and often accumulates under excessively high organic loading rates because its degradation to acetate and hydrogen and carbon dioxide is highly endergonic (ΔG^o = +76.1kJ/mol) (Tatara et al., 2008). Acetate with hydrogen and carbon dioxide are then converted to methane and carbon dioxide by methanogenic archaea via aceticlastic methanogenesis (Eq.2.1) and hydrogenotrophic methanogenesis (Eq.2.2) respectively (Batstone et al., 2002).

$$CH_3COOH \longrightarrow CH_4 + CO_2 \qquad \Delta G^o = -31.0 \text{ kJ/mol} \qquad 2.1$$

$$4H_2 + CO_2 \longrightarrow CH_4 + 2H_2O \qquad \Delta G^o = -135.0 \text{ kJ/mol} \qquad 2.2$$

Methanosaeta spp.'s are known as exact aceticlastic methanogens as they use only acetate as a substrate. *Methanobrevibacter arboriphilus* are also exact hydrogenotrophic methanogens since they only use hydrogen and carbon dioxide for methane production. Other methanogens are more flexible, *Methanospirillum hungatei* spp and *Methanobacterium formicicum* spp. can use hydrogen or carbon dioxide and formate, and *Methanosarcina* spp can grow on hydrogen or carbon dioxide and acetate (Stams et al., 2005). It was previously noticed that the acetogens and methanogens utilizing mixed VFAs had higher activities than those utilizing a single VFA component (Demirel and Yenigun, 2002).

3 Thermophilic H_2 fermentation using mixed culture

Besides increasing in thermodynamic favorability of hydrogen producing bacteria, resulting in higher hydrogen yields as previous mentioned in Chapter 2, thermophilic hydrogen fermentation has several advantages over mesophilic fermentation, such as;

- Increases in chemical and biological reaction rates, especially hydrolysis, allowing smaller reactors due to shorter HRTs as the consequence of accelerating the conversion (Lu et al., 2008; Ponsa´ et al., 2008).
- The reactor has less risk of contamination by methanogenic Achaea (van Groenestijn et al., 2002)
- Increased liquid solubilization which means it takes less energy to mix (Lee et al., 2009).
- Better destruction of pathogens could result in decreasing retention time required for pathogen reduction (Smith et al., 2005)

The cost of maintaining the temperatures of thermophilic conditions, depend largely on the heat exchange efficiency of the plant, insulation of reactors etc. It has been found that at Danish biogas plants, operating at a thermophilic temperature (55ºC), the energy cost is about 10% of the energy produced at the plant. The extra energy cost for operating at thermophilic compared to mesophilic temperatures is within 1 – 2%. This margin could possibly compensate by feeding hot substrates like wheat straw hydrolysate and desugared molassed which are generated from the hot process. In other situations, thermophilic and extreme thermophilic fermentation can be used for sanitation of manure and other organic wastes, which according to EU regulation require treatment at 70ºC for 1hr (Angelidaki et al., 2003).

3.1 Substrates used

Xylose rich substrates and MDS were alternatively used as the substrates for fermentative hydrogen production as they are not so suitable for bio-ethanol fermentation using *Saccharomyces cerevisiae*, but still contain proper amount of sugars for hydrogen production by dark fermentation (Larsen et al., 2008; Rankovi et al., 2009). Xyloses is the dominant monomeric sugar present in the hemicellulosic part of lignocellulosic materials and contain about 74% of total sugar in hemicelluloses (Xu et al., 2006). DM, a by-product generating during beet molasses has been removed additional sugar, contains sugar approx. 16% (Western sugar Inc., 2006). As significantly distinct characteristic between hydrolysate and DM (Paper II and Paper VI), extreme thermophilic fermentation, in which microorganisms grow optimally at 70 ºC (van Niel et al., 2002) was designed and was carried out with xylose rich substrates (D-xylose and wheat straw hydrolysate). Xylose based substrates are considered to be hard for micro-organism degradation therefore the superior characteristics of extreme thermophilic fermentation could be advantageous (Blumer-Schuette et al., 2008). Moderate thermophilic fermentation (55-60 ºC) was selected for DM because the sugar contained in DM is mainly sucrose, which can be converted to hydrogen with a rather high yield by using a thermophile of *Thermoanaerobacterium thermosaccharolyticum* (O-Thong et al., 2008). Hydrogen yields of 318.4 and 237.2ml-H_2/g-sugar were achieved from batch fermentation with 1.25% (v/v) MDS and 5% (v/v) hydrolysate respectively (Paper III and VI).

3.2 Inoculum preparation

3.2.1 Extreme thermophilic fermentation

The inoculum taken from the CSTR fed with household solid waste at 70 ºC and a HRT of 3 days (Liu et al., 2008) was enriched to xylose 1 g/l by successive batch cultivations at 70 ºC. Hydrogen yield was increased from 0.7 to 1.0 mol-H_2/mol-xylose within 3 times of successive transfers. No further increase of the H_2 yield was observed by additionally successive transfers (Paper I). This result clearly demonstrates that repeated batch cultivation can be use to adapt hydrogen producing microorganisms to new conditions (substrate and medium). Furthermore, this enriched inoculum was then sequentially used in a series of experiments (Paper I – Paper V) dealing with dark fermentation of D-xylose and hemicelluloses hydrolysate at 70 ºC.

3.2.2 Moderate thermophilic fermentation

Hydrogen-producing inoculum, was prepared by exposing digested manure to high DM load (also called shock load), in batch reactors at thermophilic temperature (55ºC) (Paper VI). The load shock pretreatment method is very simple to implement for preparing efficient thermophilic hydrogen producing seed inoculum (Luo et al., 2010; O-Thong et al., 2009). By adding DM at 30% (v/v) which had a sugar concentration of 50.1g/l into digested manure, methanogens could be completely suppressed. Methanogenic activity was limited mainly by the high accumulation of methanogenic substrates of hydrogen, formate, and VFAs during organic load shock (Voolapalli and Stuckey, 2001). The pretreated inoculum was later immobilized on granules in the UASB reactor and gave a very good hydrogen yield by feeding with DM.

3.3 Pathway of H_2 from thermophilic dark fermentation

Mixed extreme thermophiles produced hydrogen from xylose and hydrolysate mainly through acetate, while butyrate was detected in very low concentrations during the xylose fermentation, indicating hydrogen fermentation from xylose based substrates at extreme thermophilic temperatures is acetate type fermentation. (Papers I and III). This result is consistent with the previous investigation of cultivating mixed extreme thermophiles with xylose (Yokoyama et al., 2007). During batch fermentation of sucrose based DM at moderate thermophilic temperatures, hydrogen was produced mainly through butyrate and acetate, which accounted for about 60% (COD based) of the total substrate consumed (Paper VI). O-Thong et al, (2008) reported consistently that butyrate and acetate were the main metabolic products for hydrogen production from sucrose cultivated with *Thermoanaerobacterium thermosaccharolyticum*.

3.4 Reactor operation

3.4.1 Batch reactor

Batch mode operation is a difficult way to enhance hydrogen production efficiency, stability, and sustainability. A batch reactor is usually used to examine characteristics of hydrogen producing bacteria and to optimize culture operating conditions (Show et al., 2008). Inoculum preparation and enhancing hydrogen producing bacteria as described in Chapter 3.2 were carried out by using a batch reactor (Papers I and VI). Potential hydrogen production from all substrates and a kinetic study were also performed in the batch reactor (Papers I,

III, and VI). However, fermentation products, hydrogen, and other toxic compounds can accumulate in high concentrations in the hydrogen batch reactor, causing the inhibition of hydrogenogenic activity. This problem can be simply avoided by applying a continuous flow fermentation process (van Niel et al., 2003).

3.4.2 The suspended continuously stirred tank reactor (CSTR)

Continuous reactors are considered to be practical and economical for industrial hydrogen production, particularly via mixed culture fermentation (van Groenestijn et al., 2002; Hawkes et al., 2007). The two main bio-reactor configurations: suspended and attached, or immobilized, growth types have been applied to optimize mixed culture fermentation process for bio-hydrogen production through advancements in active biomass concentration and substrate conversion efficiency (Gavala et al., 2006; Wu et al., 2008). Most studies on hydrogen production from carbohydrate rich substrates have been conducted in suspended CSTRs, which are simple to construct, easy to regulate both acidity and temperature, and give complete homogeneous mixing for direct contact between the substrate and active biomass (Li and Fang, 2007; Hawkes et al., 2007; Hallenbeck and Ghosh, 2009).

Furthermore, the CSTR is very suitable for substrates with a high suspended solid (SS) content, typically with a volatile solid (VS) content greater than 2% (Liu et al., 2008). However, in this reactor category, HRTs must be greater than the specific growth rate of the micro-organisms in order to control the proper concentration of microbial biomass, but faster dilution rates risk active biomass washout (Hawkes et al., 2007; Hallenbeck and Ghosh, 2009), leading to process failure. In addition, cell density retained in CSTRs is limited, since the active biomass has the same retention time as HRT, resulting in process instability caused by the fluctuation of environmental parameters, including acidity or HRT and then having the consequence of limiting substrate degradation and hydrogen production (Paper IV).

A CSTR operated under extreme thermophilic conditions, with a HRT of 3 days was found to be feasible for hydrogen production by feeding with xylose (1g/l) and hydrolysate (20% (v/v) with hydrogen yields of 169 and 178 ml-H_2/g-sugar (Papers I and III). However, the CSTR reactor reached cell mass washout when it was operated at 70ºC with a HRT of 2.5 days by feeding 25% (v/v) hydrolysate

(Paper III). This was mainly due to the low cell mass generated from operating the CSTR at extreme thermophilic temperature (70 ºC) (Chou et al., 2008; Yokoyama et al., 2009). Techniques where cells are retained in the reactor, such as cell immobilization on granules or carriers, are needed to enable high organic loading (Kotsopoulos et al., 2006; Zheng et al., 2008).

3.4.2 The attached growth reactor

To overcome the above mentioned problem, a new configuration of a continuous flow reactor is required to decouple the cell mass retention from HRT and subsequently retain higher cell densities in the reactor, such as cell immobilization, which can be achieved through granules and bio-film, (Kotsopoulos et al., 2006; O-Thong et al., 2008; Wu et al., 2008; Zhang et al., 2008). Culture immobilization can be employed successfully by using a diluted waste stream with relatively small reactor volumes in CSTRs, AF reactors, fluidized bed reactors, and UASB reactors. However, such a reactor configuration has a poor mass transfer system, which is mainly caused by a lack of mixing; this can lead to gases accumulating in the bio-film or granular sludge that risk losing hydrogen by hydrogen consuming bacteria (Kim et al., 2005; Gavala et al., 2006). Mass transfer can be improved by mechanical stirring or liquid recirculation, depending on the reactor type and configuration. Also, applying proper bio-reactor shapes, and optimizing reactor dimensions such as the height to diameter ratio can help to improve mass transfer efficiency (Kim et al., 2006; Kumar and Das, 2001; Lee et al., 2006; Lo et al., 2009; Zhang et al., 2008).

During continuous hydrolysate fermentation at 70ºC, the attached growth systems of the UASB and AF reactors clearly demonstrated that they can overcome the problems of substrate utilization and hydrogen production at higher organic loading rates better than the CSTR. The UASB reactor gave a higher hydrogen production rate and yield than the AF reactor did (Paper IV). This could possibly be because of the lower surface area available on the carriers in the AF reactor compared to the granules in the UASB reactor, (Kim et al., 2005). Additionally, a rather high hydrogen yield was achieved in the UASB reactor by feeding it with 10% (v/v) DM (v/v) at a HRT of 1 day at 55 ºC, corresponding to 28.4 g-VS/d/l. This could confirm that the UASB reactor is suitable for continuous hydrogen production at high organic loading rates (Paper V; Papers VI). However, attached growth reactors like UASB reactors are limited to

organic substrates with low content of SS (Angenent et al., 2004). Organic substrates with high content of SS are better to be used in CSTR reactors (Liu et al., 2008).

3.5 Factors affecting on the dark fermentation stability

Environmental and physical factors greatly affect dark hydrogen fermentation when using mixed cultures and non-sterile feedstock (Hawkes et al., 2007; Hallenbeck and Ghosh, 2009; Uneo et al., 2006). To stabilize and maximize hydrogen production, it is necessary to direct the metabolic pathway towards acetate and/or butyrate (Fig.2) and also maintain the right hydrogen producing bacteria during operation. The main factors affecting thermophilic dark fermentation are described as follows;

3.5.1 Nutrients and buffers

Carbohydrate based substrates are used as they provide good carbon and energy sources for hydrogen producing bacteria. The fermentation process needs buffering of the growth medium, and to be supplemented with nutrients to enhance the growth of micro-organisms and resist the pH change caused by organic acids produced (O-Thong et al., 2007; van Niel et al., 2002; van Niel et al., 2003). Nutrients used for dark fermentation include mainly macro-nutrients (Nitrogen, Phosphorus, Potassium, Magnesium, and Calcium etc.), growth factor vitamins and micro-nutrients of trace metals (e.g. Iron, Chromium and Copper etc.). A carbonate buffer is normally used for hydrogen dark fermentation.

All nutrients and buffers are generally mixed as basic anaerobic (BA) medium (Angelidaki and Sanders, 2004). In addition to nutrients, yeast extract containing mainly protein provides an important source of organic nitrogen for the growth of thermophiles (Paper I). For laboratory studies, nutrient concentrations were well in excess to ensure optimal conditions for bio-hydrogen production. For industrial applications however, the need for these supplements should be further investigated, in order to reduce the operational cost and increase hydrogen production (Paper III).

3.5.2 Hydrogen concentration (P_{H2})

The P_{H2} in the liquid phase is the major factor affecting hydrogen production as high P_{H2} causes deactivation of hydrogenase, which is involved in proton reduction to form hydrogen as previously mentioned in Chapter 2.2. Decreasing P_{H2} by intermittent nitrogen sparging of batch reactor headspace could enhance hydrogen production during thermophilic fermentation (Valdez-Vazquez et al., 2006; Paper I). In addition to a high P_{H2}, the NADH, which is an electron carrier in the cell, will be oxidized mainly to lactate during extreme thermophilic fermentation with *Caldicellulosiruptor saccharilyticus* (Willquist et al., 2010). The formation of lactate during the overloading or unstable conditions found in Paper IV might be caused by a high P_{H2}.

3.5.3 Process pH and HRT

It's generally well known that the hydrogen producing bacteria is fast growing and prefers slightly acidic pH in the range 5-6 (Hawkes et al., 2007; Kyazze et al., 2007). By applying this principle, Liu et al, (2008) have produced hydrogen free of methane, continuously using an extreme thermophilic CSTR fed with household solid waste at acidic pH range of 5.0 – 5.5 and a short HRT of 3 days without any pretreatment to inhibit methanogens contained in the initial thermophilic digested manure. HRT, the main optimization parameter of the continuous reactors, is inversely related to the organic loading rate (OLR) and the bacterial growth rate. In the CSTRs, short HRTs or high dilution (D) rates can be used to eliminate methanogens which have significant low growth rate (Hawkes et al., 2007; Kyazze et al., 2007), however HRT is needed to be maintained in a proper level that still give a D value less than specific growth rate of hydrogen producing bacteria. Otherwise, the CSTR reaches washout conditions as the sludge retention time (SRT) of microorganisms is equal to HRT for the CSTR (Batstone et al., 2002).

In paper IV, operating CSTR at a 2.5-day HRT caused cell mass washout. In contrast to the CSTR, the immobilized-growth reactor system allows lower HRTs or higher OLRs due to SRT microorganisms is much higher than HRT, resulting in high cell density retained in the immobilized-growth reactor. On the other hand, too short HRT (high OLR) can cause overloading and subsequently reduce the hydrogen production metabolism through increase of fermentation products (P_{H2} and soluble products). pH regulation is needed to keep the process stable as accumulation of soluble end products can result in a decrease of pH below the

favorable range of 5.0 – 6.0 for hydrogen producing microorganisms. Bicarbonate buffers contained in the BA medium, as previously mentioned, can be used to effectively regulate acidity (Paper I – Paper VI)

3.5.4 Toxic compounds

Hydrolysate produced by the hydrothermal pretreatment of wheat straw does not contain only sugars, but also toxic compounds which are derived from sugar and lignin degradation. These compounds, including acetic acid from the hydrolysis of acetyl groups contained in hemicelluloses, phenolics dominated by vanillin, 2-furoic, coumaric acid and ferulic acid from lignin decomposition, and HMF and furfural from sugar conversion are microbial toxicants, inhibiting the dark fermentation process (Papers II – Paper V). DM contains significant amounts of lactic acid, acetic acid, and sulfate, which are generated during beet sugar processing and can also inhibit hydrogen production (Paper VI). However, there were some micro-organisms contained in the mixed culture, especially sulfate reducing bacteria, like the *Desulfovibrio* species, that are able to degrade these toxic compounds as reported in Papers V and VI.

4 Thermophilic methane production from H_2 reactor effluent

As previously mentioned in Chapter 3, C-5 sugar rich substrates of hemicelluloses hydrolysate have potential for fermentative hydrogen production using enriched mixed culture under extreme thermophilic temperature. However, in the first stage of acidogenesis, the organic matter removal of volatile solids (VS) is around 10-20%, which is distributed mainly to hydrogen production and cell mass growth, while the rest was still presented in soluble forms, with VFAs consisting mainly acetate and butyrate being the main product of fermentation (Paper V). Butyrate and acetate contained in acidogenic effluent stream are the most suitable to be converted to methane by the sequential anaerobic steps (Tatara et al., 2005).

The anaerobic conversion of VFAs to methane is mainly associated with sequential stages of acetogenesis and methanogesis as aforementioned in Chapter 2.3. A two stage process technique, combining acidogenesis and methanogesis appears to give more efficient waste treatment and energy recovery than a single methanogenic process (Hawkes et al., 2007). As the results reported by Kongjan et al., 2010 (Figure 3), mixed gas of CH_4, CO_2, and H_2 with the volumetric content of 44.8 %, 38.7%, and 16.5%, respectively, containing approx. 10% H_2 on energy basis could be achieved. This specification was found to be most suitable for burning directly in the internal combustion engines (Porpatham et al., 2007) and could be biohythane. In addition to economical concern, the two-stage thermophilic anaerobic process has been previously evaluated that the pay-back time is around 2 – 6 years, depending on the disposal costs of organic wastes/residues (Bolzonella et al., 2007).

For the high rate anaerobic reactor of a UASB reactor, it was previously reported by Lepistö and Rintala, (1999) that operating at moderate thermophilic temperature (55 ºC) could provide better VFAs degradation than that at mesophilic temperature (35 ºC) when the OLR of the reactors was doubly increased. This is mainly attributed to the increase of chemical and biological reaction rates for operating temperature of thermophilic condition and the organic acid oxidation reactions become more energetic at higher temperature (Batstone et al., 2002; van Lier, 1996).

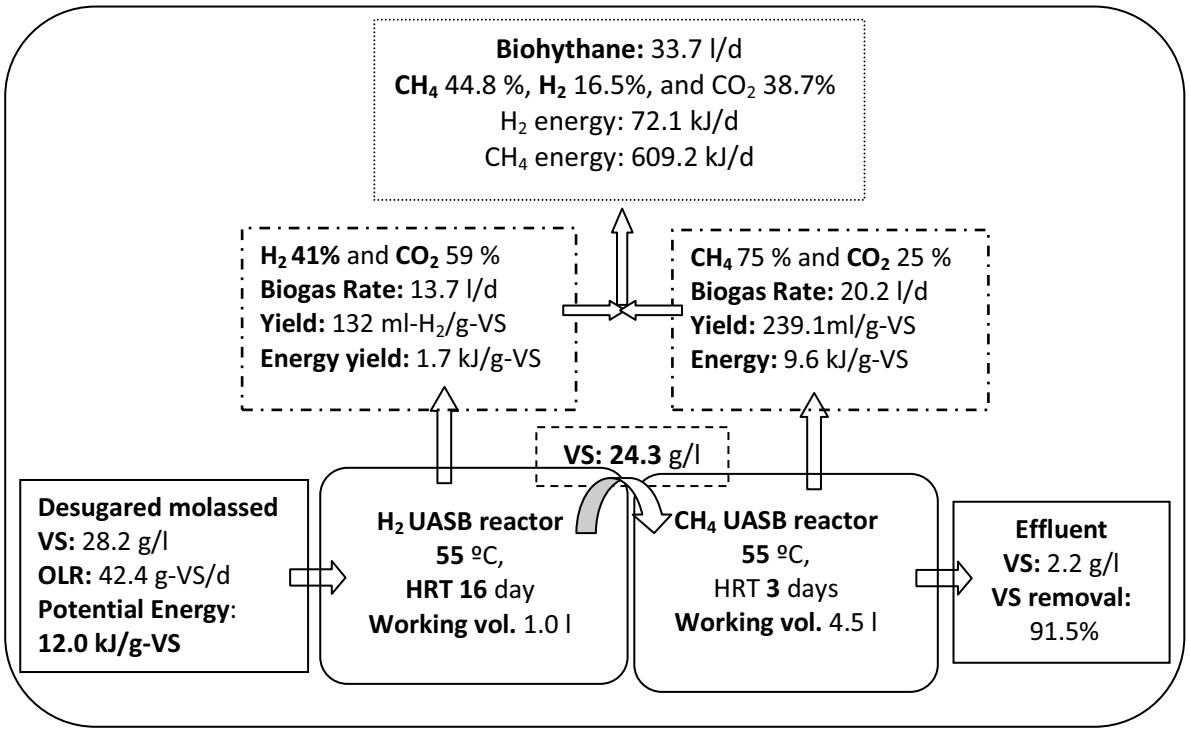

Figure 3 Feasible flow diagram of mass and energy balance in the two stage anaerobic process for biohythane production for desugared molasses (Adapted from Kongjan et al, (2010)).

4.1 Reactor configuration

Because the hydrogen reactor effluents are in soluble form of organic matters as the consequence of hydrolysis and acidogenesis in the first stage, the reactor type used to convert these soluble organic matters to methane in the second stage are based on high rate bio-film systems as reviewed by Demirel et al, (2010). Cell mass is retained well in the bio-film/granular aggregates in bio-film systems, leading to have much higher sludge retention time (SRT) compared to (HRT), which provides the advantage that the reactor can run at higher flow rate and can tolerate higher toxic concentrations (Saravanan and Sreekrisnan, 2006). Various types of high rate bio-film systems like UASB reactors, AF reactors, and down-flow anaerobic packed-bed reactors (DAPR) can be operated by continuous feeding with the hydrogen reactor effluent, with HRTs of less than 5 days (Paper V; Kongjan et al., 2010; Lepistö and Rintala, 1999; Tatara et al., 2005). Among the high rate reactor types, the UASB is the most popular for anaerobic treatment of soluble organic matters due to the large surface area of granular sludge, which provides fast bio-film development and improves methanogenesis. Also clogging

and channeling occur less in the UASB reactor than other biofilm systems (Parawira et al., 2006).

4.2 Process optimization

When optimizing a methanogenic process using VFA rich, soluble organic matters, the goal is to maximize both methane production and VFA degradation, whilst keeping the reactor stable (Demirel and Yenigun, 2002). As previously mentioned in Chapter 2.3, the actogenesis is limited mainly by VFA degradation, especially propionate which is the rate limiting factor in the second stage anaerobic process. The investigation into optimizing the methanogenic reactor is mostly carried out by varying OLRs via increasing the substrate concentration or decreasing the HRTs to obtain satisfactory performance (Paper V; Cavinato et al., 2010; Lee et al., 2010).

The main signs of methanogenic reactor instability or overloading are decreasing pH and increasing amounts of VFAs (Parawira et al., 2006). As a drop of pH actually corresponds to VFA accumulation and pH below 6.3 has an impact on enzyme activity in the microorganisms involved in the second stage anaerobic digestion. Methanogenic archea can function properly in a pH range between 6.5 and 7.8 (Lay et al., 1997). Thus a buffering solution is needed in order to resist a pH drop from VFA accumulation in the methanogenic process, maintain stability. The main buffer in the anaerobic digester is bicarbonate (HCO_3^-), which is usually added to carbohydrate rich substrates before feeding them to the first stage of hydrogen fermentation because the first stage needs to be control the pH favorable range of 5-6 for H_2 producing bacteria (Paper V; Forbes et al., 2010; Venetsaneas et al., 2009).

Lee et al, (2010) found the pH drop below 6.4 was caused by the accumulation of 122 mM VFAs in the attached growth reactor operated at 55 ºC and fed 11.0 g-VS/d·l (5.13 d HRT) of the effluent from food waste fermentation. The 6.4 pH could inhibit the bioactivity of methanogenesis. Meanwhile, the maximum methane production rate of 2100 ml-CH_4/d·l with a CH_4 content of 65% was obtained at pH around 7.5, where the reactor was operated at a 7.7 day HRT (7.9 g-VS/d·l OLR) and almost VFA degradation was achieved. Using effluent from extreme thermophilic fermentation fed with hydrolysate (Paper V), the CH_4 UASB reactor operated at 55 ºC and a 1 day HRT (8.2 g/d·l OLR) gave a maximum methane production rate of 2088 ml-CH_4/d·l (70% CH_4 content) and

maintained very low level of VFA (8 mM) at pH around 7.0. The process failure was found beyond the OLR of 15.7 g-VS/d·l, or a HRT of 0.5 day with sharp increase of VFA concentrations (43 mM) and drop of the pH to 5.85.

5 Microbial communities and activities

In this study, the microbial communities were analyzed for the most dominant organisms in the process for better understanding on process behavior. The monitoring of microbial communities is based on the identification of organisms, relating to environmental conditions and performance. Studying microbial community and function was done by using the genetic fingerprint techniques of denaturing gradient gel electrophoresis (DGGE) (Paper III; Paper V) and fluorescence *in-situ* hybridization (FISH) (Paper VI). DGGE is one of the first techniques used to describe mixed culture involved in dark hydrogen fermentation. It is a rapid and simple method providing characteristic band patterns for different samples. This molecular method allows quick sample profiling, while retaining the possibility of a more thorough genetic analysis by the sequencing of particular bands.

DGGE provides information about the structure of microbial communities, and can relatively quantify species abundance through DNA band intensities (Stamper et al., 2003). By using DGGE method in Paper (III), phylogenetic analysis of the mixed culture revealed that members involved hydrogen producers in both batch and CSTR reactors were related to the *Caldanaerobacter subteraneus*, *Thermoanaerobacter subteraneus*, and *Thermoanaerobacterium thermosaccharolyticum*. Extreme thermophiles, *Caldanaerobacter subteraneus* and *Thermoanaerobacter subteraneus,* can produce hydrogen along with acetate as the major soluble products during carbohydrate fermentation (Yokoyama et al., 2007).

Thermoanaerobacterium thermosaccharolyticum has optimal growth at moderate thermophilic temperature (60 ºC) and can convert carbohydrate to hydrogen via butyrate and acetate type fermentation (O-Thong et al., 2008). Its presence in the reactors operated at extreme thermophilic temperature (70 ºC) indicates that it can tolerate a higher temperature than its optimal growth temperature and was able to compete with other extreme thermophiles.

The fluorescence *in-situ* hybridization (FISH) analysis, an easy and fast method, can give direct analysis and quantification. This molecular analysis method was applied to monitor the spatial distribution of hydrogen producing bacteria in sludge and granules from hydrogen fermentative reactors (O-Thong et al, 2008).

The results obtained in thermophilic fermentation using DM as the substrate (Paper VI) demonstrated by FISH analysis of load shock seed sludge obtained from batch DM fermentation with a concentration of 1.25% (v/v) were mainly dominated by the hydrogen producing bacteria of *Thermoanaerobacterium* spp. Meanwhile, the microbial community structure in a thermophilic UASB reactor fed with 10% (v/v) DM, comprised of 36% *Thermoanaerobacterium* spp., 10% *T. thermosaccharolyticum*, 27% of phylum *Firmicutes* (mostly *Clotridium*, *Bacillus* and *Desulfobacterium).*

Thermoanaerobacterium species are well known as good hydrogen producing bacteria (O-Thong et al, 2008; Zhang et al., 2003). The presence of *Clotridium*, *Bacillus* and *Desulfobacterium* is in accordance with the significant removal of lactate in the UASB reactor since *Clostridium* and *Desulfobacterium* spp. are able to degrade lactate to acetate and/or hydrogen (Zellner et al., 1994).

The dominant microorganisms detected by the DGGE method in the acidogenesis and methanogenesis stages using hydrolysate) as the substrate (Paper V) are presented in Table 1. It's clear that methanogenens were suppressed in the acidogenic reactor operated at 70 ºC by feeding with hydrolysate 30% (v/v). The fact there were no methanogens detected was mainly attributed to the effectiveness of the operating conditions used (Lee et al., 2009; Lee et al., 2010). The dominant microorganisms found in the extreme thermophilic acidogenesis are *Thermoanaerobacter weigelii, Caloramator fervidus*, *Thermoanaerobacterium* sp., and *Caldanaerobacter subteraneus.* These microorganisms are capable of degrading carbohydrates to give hydrogen with mainly acetate as soluble product (O-Thong et al., 2008; Yokoyama et al., 2007).

In the thermophilic methanogenesis stage, fed with the effluent stream from hydrolysate degradation, microbial communities were comprised of both archaea *(Methanothermobacter thermautotrophicus*, *Methanothermobacter defluvii* and *Methanosarcina mazei)* and bacteria (*Thermoanaerobacterium* sp*., Clostridium roseum*, *Clostridium isatidis*, *Thermodesulfovibrio isladicus*). The *Methanosarcina* spicies dominant at high acetate concentration primarily utilize acetate as substrate (Karakashev et al., 2005). They are also capable of utilizing other substrates such as H_2/CO_2, methanol, or methylamines (Raskin et al., 1994; Ohba et al., 2006).

Meanwhile, some acidogenic bacteria, *Thermoanaerobacterium* sp.*, Clostridium roseum*, and *Clostridium isatidis,* which are H_2 producers (Chang et al., 2006; Compton et al., 2000; Yokoyama et al., 2007*)* were also detected, confirming that some H_2 and CO_2 were also produced. However, the presence of the hydrogenotrophic methanogens of *Methanothermobacter defluvii* and *Methanothermobacter thermautotrophicus* could possibly consume H_2, thus no hydrogen could be detected when the methanogenic reactor reached stable conditions. *Methanothermobacter defluvii* and *Methanothermobacter thermautotrophicus* can reduce CO_2 to CH_4 with H_2, formate or acetate as terminal electron acceptors (Weiss et al., 2008). *Desulfovibrio* species detected in both the acidogenesis and methaonogenesis stages are non spore forming sulfate reducing bacteria and able to convert furfural effectively to acetate (Boopathy, 2002; Brune et al., 1983). The significant removal of furfural and other toxic compounds found in hydrolysate was mainly due to the degradation activity of the *Thermodesulfovibrio* species.

Table 1 Dominated microorganisms involved in hydrogen and methane production process using the 2 stage anaerobic digestion fed with hydrolysate 30% (v/v) in the UASB reactors

H_2 Reactor (70ºC)	**CH_4 reactor (55ºC)**	
Bacteria	**Bacteria**	**Archea**
Thermoanaerobacterium sp.	*Thermoanaerobacterium* sp.	*Methanothermobacter thermautotrophicus*
Thermoanaerobacter weigelii	*Clostridium roseum*	*Methanothermobacter defluvii*
Caloramator fervidus	*Clostridium isatidis*	*Methanosarcina mazei*
Caldanaerobacter subteraneus	*Desulfomicrobium* sp.	
Thermodesulfovibrio isladicus		

6 Conclusions

This thesis has mainly focused on hydrogen production from sugar rich substrates including xylose, hemicelluloses hydrolysate, and desugared molasses by fermentation with enriching mixed cultures. The potential of using effluent stream produced by the hydrolysate fermentation process for anaerobic methane production was also investigated. The major contributions of this thesis work are summarized as follow.

- Using D-xylose as the substrate for extreme thermophilic hydrogen production, mixed hydrogen producing inoculum obtained from a lab scale CSTR reactor fed with household solid wastes at 70 ºC could be adapted by repeated batch fermentations to hydrogen production from xylose at 70 ºC. 1 g/l yeast extract amended in BA medium could enhance hydrogen yield during the batch xylose fermentation. Xylose fermentation for hydrogen production could be successfully achieved in CSTR operated at 70 ºC with a 3 day HRT, and influent xylose concentration of 1 g/l. Under steady state conditions, the hydrogen yield and the production rate were 1.36 mol-H_2/mol-xylose and 62 ml/d·l, respectively. The main reduced products from the reactor were acetate followed by formate and ethanol respectively.

- Using hemicellulose hydrolysate for hydrogen production and subsequent biogas production from its effluent could improve the overall energy yield of wheat straw based bio-refinery system, in which cellulose is primarily used for ethanol production and fermentation effluent is subsequently converted to biogas as well. Hydrogen containing biogas, free of methane, could be successfully produced along with mainly acetate by extreme thermophilic fermentation of hemicelluloses rich hydrolysate in both batch and continuous mode operations by using mixed cultures. A stable hydrogen production rate of 184 ml-H_2/d·l was achieved in the CSTR reactor operated at a HRT of 3 days and 20 % (v/v) hydrolysate fed. The extreme thermophiles of *Thermoanaerobacter subteraneus* dominated in the extreme thermophilic fermentation fed with xylose rich substrates.

- Different reactor configurations i.e. CSTR, UASB, and AF reactor systems resulted in different hydrogen production efficiencies, from 25%

(v/v) hydrolysate due to the varying amounts and forms of hydrogenogenic bacteria. The highest rate and yield of 821 ml-H_2/d·l and 212 ml-H_2/g-sugars, respectively achieved during the steady state condition of the UASB reactor operated at a HRT of 1day and 70 ºC. From the experimental results, it has been shown that hydrogen production from hydrolysate is technically feasible by using a UASB reactor.

- Applying a two stage anaerobic process using hydrolysate as the substrate was shown to be feasible, with hydrogen and methane yields of 89 ml-H_2/g-VS and 307 ml-CH_4/g-VS respectively, corresponding to 87.5% of hydrolysate potential energy. In extreme thermophilic acidogenesis stage, the hydrogen-producing bacteria of *Thermoanaerobacter wiegelii*, *Caldanaerobacter subteraneus* and *Caloramator fervidus* were dominated. Meanwhile, *Methanosarcina mazei* and *Methanothermobacter* spp. were the dominant methanogens in the thermophilic methanogenesis stage. Combining the extreme thermophilic acidogenic and thermophilic methanogenic processes enhanced substrate degradation efficiency (81% VS removal) from hydrolysate along with generation of both hydrogen and methane, indicating sustainability of the process.

- The load shock of anaerobic digested manure is intesting method to suppress methanogens. No methane could be detected in the mixed gas produced. The highest yield of 237 ml-H_2/g-sugar was obtained during the DM batch fermentation at 1.25% (v/v). The bacterial community analyzed by FISH method was dominated by *Thermoanaerobacterium* spp. Further immobilization of the enriched inoculum in the UASB reactor could achieve high and stable hydrogen production. The UASB reactor showed good sugar degradation and hydrogen production. By continuous feeding DM with an OLR of 16.7 g/d·l at a 24-hr HRT in the UASB reactor, a hydrogen production rate and yield of 4500 ml-H_2/d·l and 263 ml-H_2/g-sugar, respectively was achieved.

7 References

Ahring, B. 1994. Status on science and application of thermophilic anaerobic digestion. Water Science and Technology, 30(12), 241-250.

Alavandi S, Agrawal A. 2008. Experimental study of combustion of hydrogen–syngas/methane fuel mixtures in a porous burner. International Journal of Hydrogen Energy, 33(4), 1407-1415.

Angelidaki I, Ellegaard L, Ahring B. 2003. Applications of the anaerobic digestion process. Advances in Biochemical Engineering Biotechnology, 82, 1-34.

Angelidaki I, Sanders W. 2004. Assessment of the anaerobic biodegradability of macropollutants. Reviews in Environmental Science and Biotechnology 3(2):117-129.

Angenent L, Karim K, Al-Dahhan M, Wrenn B, Domíguez-Espinosa R. 2004. Production of bioenergy and biochemicals from industrial and agricultural wastewater. Trends in Biotechnology, 22(9), 477-485.

Antonopoulou G, Gavala H, Skiadas I, Angelopoulos K, Lyberatos G, 2008. Biofuels generation from sweet sorghum: Fermentative hydrogen production and anaerobic digestion of the remaining biomass. Bioresource Technology, 99(1), 110-119.

Arooj M, Han S, Kim S, Kim D, Shin H. 2008. Continuous biohydrogen production in a CSTR using starch as a substrate. International Journal of Hydrogen Energy, 33(13), 3289-3294.

Baere L. 2000. Anaerobic digestion of solid waste: state of the art. Water Science and Technology, 41(3), 283-290.

Batstone, D., Keller, J., Angelidaki, I., Kalyuzhny, S., Pavlostathis, S., Rozzi,A., Sanders, W., Siegrist, H., Vavilin, V. 2002. Anaerobic digestion model no. 1 (ADM1), IWA publishing, London, UK.

Blumer-Schuette, S., Kataeva, I., Westpheling, J., Adams, M., Kelly, R. 2008. Extremely thermophilic microorganisms for biomass conversion: status and prospects. Current Opinion in Biotechnology, 19(3), 210-217.

Bolzonella D, Pavan P, Zanette M, Cecchi F. 2007. Two-phase anaerobic digestion of waste activated sludge: Effect of an extreme thermophilic prefermentation. Industrial and Engineering Chemistry Research, 46(21), 6650-6655.

Boopathy R. 2002. Methanogenesis from furfural by defined mixed cultures. Current Microbiology, 44(6), 406-410.

Bothun, G., Knutson, B., Berberich, J., Strobel, H., Nokes, S. 2004. Metabolic selectivity and growth of Clostridium thermocellum in continuous culture under elevated hydrostatic pressure. Applied Microbiology and Biotechnology, 65(2), 149-157.

Brune, G., Schoberth, S.M., Sahm, H., 1983. Growth of a strictly anaerobic bacterium on furfural (2-furaldehyde). Applied microbiology and biotechnology, 46(5), 1187-1192.

Cavinato C, Bolzonella D, Eusebi A, Pavan P. 2009. Bio-hythane production by thermophilic two-phase anaerobic digestion of organic fraction of municipal solid waste. Preliminary results. Chemical Engineering Transactions, 17, 269-274.

Chang J, Chen W, Shih S, Yu S, Lay J, Wen F, Huang C. 2006. Molecular detection of the clostridia in an anaerobic biohydrogen fermentation system by hydrogenase mRNA-targeted reverse transcription-PCR. Applied Microbiology and Biotechnology, 70(5), 598-604.

Chou, C., Jenney, F., Adams, M., Kelly, R., 2008. Hydrogenesis in hyperthermophilic microorganisms: Implications for biofuels. Metabolic Engineering, 10(6), 394-404.

Chong M, Sabaratnam V, Shirai Y, Hassan M. 2009. Biohydrogen production from biomass and industrial wastes by dark fermentation. International Journal of Hydrogen Energy, 34(8), 3277-3287.

Compton R, Perkin S, Gamblin D, Davis J, Marken F, Padden A, John P. 2000. Clostridium isatidis colonised carbon electrodes: voltammetric evidence for direct solid state redox processes. New Journal of Chemistry, 24(3), 179-181.

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

de Mes TZD, Stams AJM, Reith JH, Zeeman G. 2003. Methane production by anaerobic digestion of wastewater and solid wastes. In: Bio-methane & Bio-hydrogen: Status and perspectives of biological methane and hydrogen production. Ed: Reith JH, Wiffels RH, Barten H. Dutch Biological hydrogen Foundation, The Netherlands, 58-102. Available on the Internet: http://www.biohydrogen.nl/publicfiles/16_20804_2_Bio_methane_and_Bio_hydrogen_2003.pdf (Accessed 11.06.2003).

Demirel B, Scherer P, Yenigun O, Onay T. 2010. Production of Methane and Hydrogen from Biomass through Conventional and High-Rate Anaerobic Digestion Processes. Critical Reviews in Environmental Science and Technology, 40(2), 116-146.

Demirel, B., Yenigun, O. 2002. Two-phase anaerobic digestion processes: a review. Journal of Chemical Technology and Biotechnology, 77(7), 743-755.

de Vrije T, Claassen PAM. 2003. Dark hydrogen fermentations. In: Bio-methane & Bio-hydrogen: Status and perspectives of biological methane and hydrogen production. Ed: Reith JH, Wiffels RH, Barten H. Dutch Biological hydrogen Foundation, The Netherlands, 103-123 p. Available on the Internet: http://www.biohydrogen.nl/publicfiles/16_20804_2_Bio_methane_and_Bio_hydrogen_2003.pdf (Accessed 11.06.2003).

de Wit M, Faaij A. 2009. European biomass resource potential and costs. Biomass and Bioenergy. doi:10.1016/j.biombioe.2009.07.011.

Duangmanee T, Padmasiri S, Simmons J, Raskin L, Sung S. 2007. Hydrogen production by anaerobic microbial communities exposed to repeated heat treatments. Water Environment Research, 79, 975-983.

Escobar J, Lora E, Venturini O, Yáñez E, Castillo E, Almazan O. 2009. Biofuels: Environment, technology and food security. Renewable and Sustainable Energy Reviews, 13(6-7), 1275-1287.

Forbes C, Hughes D, Fox J, Ryan P, Colleran E. 2010. High-rate anaerobic degradation of 5 and 6 carbon sugars under thermophilic and mesophilic conditions, Bioresource Technology, 101(11), 3925-3930.

Gattrell M, Gupta N, Co A. 2007. Electrochemical reduction of CO_2 to hydrocarbons to store renewable electrical energy and upgrade biogas. Energy Conversion and Management, 48(4), 1255-1265.

Gavala H, Skiadas I, Ahring B. 2006. Biological hydrogen production in suspended and attached growth anaerobic reactor systems. International Journal of Hydrogen Energy, 31(9), 1164-1175.

Hallenbeck P, Ghosh D. 2009. Advances in fermentative biohydrogen production: the way forward? Trends in Biotechnology. 27(5), 287-297.

Hallenbeck P, Ghosh D, Skonieczny M, Yargeau V. 2009. Microbiological and engineering aspects of biohydrogen production. Indian Journal of Microbiology, 49(1), 48-59.

Hawkes F, Dinsdale R, Hawkes D, Hussy I. 2002. Sustainable fermentative hydrogen production: challenges for process optimisation. International Journal of Hydrogen Energy, 27(11-12), 1339-1347.

Hawkes F, Hussy I, Kyazze G, Dinsdale R, Hawkes D, 2007. Continuous dark fermentative hydrogen production by mesophilic microflora: Principles and progress. International Journal of Hydrogen Energy, 32(2), 172-184.

Ivanova G, Rákhely G, Kovács K. 2009. Thermophilic biohydrogen production from energy plants by *Caldicellulosiruptor saccharolyticus* and comparison with related studies. International Journal of Hydrogen Energy 34(9): 3659-3670.

Kaparaju P, Serrano M, Angelidaki I. 2009. Effect of reactor configuration on biogas production from wheat straw hydrolysate. Bioresoure Technology., 100, 6317-6323.

Kapdi S, Vijay V, Rajesh S, Prasad R. 2005. Biogas scrubbing, compression and storage: perspective and prospectus in Indian context. Renewable energy, 30(8), 1195-1202.

Kapdan I, Kargi F. 2006. Biohydrogen production from waste materials. Enzyme and Microbial Technology, 38(5), 569-582.

Karakashev D, Batstone D, Angelidaki I. 2005. Influence of environmental conditions on methanogenic compositions in anaerobic biogas reactors. Applied and Environmental Microbiology, 71(1), 331-338.

Kengen S, Goorissen H, Verhaart M, Stams A, van Niel E, Claassen P. 2009. Biological hydrogen production by anaerobic microorganisms. Biofuels, 197–221.

Kim J, Kim Y, Yeom S, Song B, Kim I. 2006. Enhancing continuous hydrogen gas production by the addition of nitrate into an anaerobic reactor. Process Biochemistry, 41(5), 1208-1212.

Kim J, Kim Y, Ryu J, Song B, Kim I, Yeom S. 2005. Immobilization methods for continuous hydrogen gas production biofilm formation versus granulation. Process Biochemistry, 40(3), 1331-1337.

Kleerebezem R, van Loosdrecht M. 2007. Mixed culture biotechnology for bioenergy production. Current Opinion in Biotechnology, 18(3), 207-212.

Kongjan P, O-Thong S, Angelidaki I. 2010. Biohythane production from desugarized molasses (DM) using two-stage thermophilic anaerobic process. Manuscript.

Kotsopoulos T, Zeng R, Angelidaki, I. 2006. Biohydrogen Production in granular up-flow anaerobic sludge blanket (UASB) reactors with mixed cultures under hyper-thermophilic temperature (70ºC). Biotechnology and Bioengineering, 4(2): 296-302.

Koutrouli E, Kalfas H, Gavala H, Skiadas I, Stamatelatou K, Lyberatos G. 2009. Hydrogen and methane production through two-stage mesophilic anaerobic digestion of olive pulp. Bioresource Technology, 100(15), 3718-3723.

Kumar N, Das D. 2001. Continuous hydrogen production by immobilized Enterobacter cloacae IIT-BT 08 using lignocellulosic materials as solid matrices. Enzyme and Microbial Technology, 29(4-5), 280-287.

Kyazze G, Dinsdale R, Guwy A, Hawkes F, Premier G, Hawkes D. 2007. Performance characteristics of a two-stage dark fermentative system producing hydrogen and methane continuously. Biotechnology and Bioengineering, 97(4), 759-770.

Larsen J, Petersen ØM, Thirup L, Li WH, Iversen FK. 2008. The IBUS process-lignocellulosic bioethanol close to a commercial reality. Chemical Engineering and Technology; 31(5): 765-772.

Lay J, Li Y, Noike T, Endo J, Ishimoto S. 1997. Analysis of environmental factors affecting methane production from high-solids organic waste. Water Science and Technology, 36(6-7), 493-500.

Lee M, Hidaka T, Hagiwara W, Tsuno H. 2009. Comparative performance and microbial diversity of hyperthermophilic and thermophilic co-digestion of kitchen garbage and excess sludge. Bioresource Technology, 100(2), 578-585.

Lee D, Ebie Y, Xu K, Li Y, Inamori Y. 2010. Continuous H_2 and CH_4 production from high-solid food waste in the two-stage thermophilic fermentation process with the recirculation of digester sludge. Bioresource Technology. 101(1S), 42-47.

Lee K, Lo Y, Lin P, Chang J. 2006. Improving biohydrogen production in a carrier-induced granular sludge bed by altering physical configuration and agitation pattern of the bioreactor. International Journal of Hydrogen Energy, 31(12), 1648-1657.

Lepistö R, Rintala J. 1999. Extreme thermophilic (70 ºC), VFA-fed UASB reactor: performance, temperature response, load potential and comparison with 35 and 55 ºC UASB reactors. Water Research, 33(14), 3162-3170.

Levin D, Pitt L, Love M. 2004. Biohydrogen Production: Prospects and Limitations to Practical Application. International Journal of Hydrogen Energy. 29(13), 1425-1426.

Li C, Fang H. 2007. Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. Critical Reviews in Environmental Science and Technology, 37(1), 1-39.

Liu D. 2008. Bio-hydrogen production by dark fermentation from organic wastes and residues. PhD. dissertation, Department of environmental Engineering, Technical University of Denmark, Denmark.

Liu D, Liu D, Zeng R, Angelidaki I. 2006. Hydrogen and methane production from household solid waste in the two-stage fermentation process. Water Research; 40(11): 2230-2236.

Liu D, Zeng R, Angelidaki I. 2008. Effects of pH and hydraulic retention time on hydrogen production versus methanogenesis during anaerobic fermentation of organic household solid waste under extreme-thermophilic temperature (70 ºC). Biotechnology and Bioengineering, 100(6), 1108-1114.

Lepistö R, Rintala J. 1999. Extreme thermophilic (70 ºC), VFA-fed UASB reactor: performance, temperature response, load potential and comparison with 35 and 55 ºC UASB reactors. Water Research, 33(14), 3162-3170.

Lo Y, Lee K, Lin P, Chang, J. 2009. Bioreactors configured with distributors and carriers enhance the performance of continuous dark hydrogen fermentation. Bioresource Technology, 100, 4381-4387.

Lu J, Gavala H, Skiadas I, Mladenovska Z, Ahring B. 2008. Improving anaerobic sewage sludge digestion by implementation of a hyper-thermophilic prehydrolysis step. J Environ Manag 88 (4): 881-889.

Luo G, Xie L, Zou Z, Wang W, Zhou Q. 2010. Evaluation of pretreatment methods on mixed inoculum for both batch and continuous thermophilic biohydrogen production from cassava stillage. Bioresource Technology. 101, 959-964.

McKendry P 2002. Energy production from biomass (part 1): overview of biomass. Bioresource Technology. 83(1), 37-46.

Ntaikou I, Antonopoulou G, Lyberatos G. Biohydrogen Production from Biomass and Wastes via Dark Fermentation: A Review. Waste and Biomass Valorization. 1: 21-39.

Ohba M, Li YY, Noike T. 2006. Analysis of microbial community in two-phase circulating process for hydrogen and methane fermentation. Journal of Japan Society on Water Environment, 29(7), 399–406.

O-Thong S, Prasertsan P, Birkeland N. 2009. Evaluation of methods for preparing hydrogen-producing seed inocula under thermophilic condition by process performance and microbial community analysis. Bioresource Technology, 100(2), 909-918.

O-Thong S, Prasertsan P, Karakashev D, Angelidaki I. 2008. Thermophilic fermentative hydrogen production by the newly isolated *Thermoanaerobacterium thermosaccharolyticum* PSU-2. International Journal of Hydrogen Energy, 33(4), 1204-1214.

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

Ozmihci S, Kargi F. 2010. Comparison of different mixed cultures for bio-hydrogen production from ground wheat starch by combined dark and light fermentation. Journal of Industrial Microbiology and Biotechnology. 37:341-347.

Parawira W, Murto M, Zvauya R, Mattiasson B. 2006. Comparative performance of a UASB reactor and an anaerobic packed-bed reactor when treating potato waste leachate. Renewable Energy, 31(6), 893-903.

Pohland F, Ghosh S. 1971. Developments in anaerobic stabilization of organic wastes: the two-phase concept. Environmental letters, 1(4), 255-266.

Porpatham, E., Ramesh, A., and Nagalingam, B. (2007). Effect of hydrogen addition on the performance of a biogas fuelled spark ignition engine. International Journal of Hydrogen Energy, 32(12), 2057-2065.

Ponsá S, Ferrer I, Vázquez F, Font X. 2008. Optimization of the hydrolytic–acidogenic anaerobic digestion stage (55°C) of sewage sludge: Influence of pH and solid content. Water Research, 42(14), 3972-3980.

Rankovi J, Dodi J, Dodi, S, Popov S. (2009). Bioethanol production from intermediate products of sugar beet processing with different types of *Saccharomyces cerevisiae*. Chemical Industry and Chemical Engineering Quarterly,15 (1), 13-16.

Raskin L, Stromiey JM, Rittmann BE, Stahl DA. 1994. Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. Applied and Environmental Microbiology, 60(4), 1232–40.

Show K, Zhang Z, Lee D. Design of bioreactors for biohydrogen production. Journal of Scientific&Industrial Research, 67. 941-949.

Saravanan V, Sreekrishnan T. 2006. Modelling anaerobic biofilm reactors-A review. Journal of Environmental Management, 81(1), 1-18.

Smith S, Lang N, Cheung K, Spanoudaki K. 2005. Factors controlling pathogen destruction during anaerobic digestion of biowastes. Waste Management, 25(4), 417-425.

Stamper D, Walch M, Jacobs R. 2003. Bacterial population changes in a membrane bioreactor for graywater treatment monitored by denaturing gradient gel electrophoretic analysis of 16S rRNA gene fragments. Applied and Environmental Microbiology, 69(2), 852.

Stams A. 1994. Metabolic interactions between anaerobic bacteria in methanogenic environments. Antonie van Leeuwenhoek, 66(1), 271-294.

Stams A, Plugge C, de Bok F, Van Houten B, Lens P, Dijkman H, Weijma J. 2005. Metabolic interactions in methanogenic and sulfate-reducing bioreactors. Water Science and Technology, 52(1-2), 13.

Tan K, Lee K, Mohamed A. 2008. Role of energy policy in renewable energy accomplishment: The case of second-generation bioethanol. Energy Policy. 36(9):3360-3365.

Tatara M, Yamazawa A, Ueno Y, Fukui H, Goto M, Sode K. 2005. High-rate thermophilic methane fermentation on short-chain fatty acids in a down-flow anaerobic packed-bed reactor. Bioprocess and Biosystems Engineering, 27(2), 105-113.

Tatara M, Makiuchi T, Ueno Y, Goto M, Sode K. 2008. Methanogenesis from acetate and propionate by thermophilic down-flow anaerobic packed-bed reactor. Bioresource Technology, 99(11), 4786-4795.

Temudo M, Muyzer G, Kleerebezem R, van Loosdrecht M. 2008. Diversity of microbial communities in open mixed culture fermentations: impact of the pH and carbon source. Applied Microbiology and Biotechnology, 80(6), 1121-1130.

Thomsen M, Thygesen A, Thomsen A. 2008. Hydrothermal treatment of wheat straw at pilot plant scale using a three-step reactor system aiming at high hemicellulose recovery, high cellulose digestibility and low lignin hydrolysis. Bioresource Technology, 99(10), 4221-4228.

Tilche A, Galatola M 2008. The potential of bio-methane as bio-fuel/bio-energy for reducing greenhouse gas emissions: a qualitative assessment for Europe in a life cycle perspective. Water Science Technology, 57(11), 1683-1692.

Ueno Y, Sasaki D, Fukui H, Haruta S, Ishii M, Igarashi Y. 2006. Changes in bacterial community during fermentative hydrogen and acid production from organic waste by thermophilic anaerobic microflora. Journal of Applied Microbiology, 101(2), 331-343.

Valdez-Vazquez, I., Rios-Leal, E., Carmona-Martinez, A., Munoz-Paez, K., Poggi-Varald,H., 2006. Improvement of biohydrogen production from solid wastes by intermittent venting and gas flushing of batch reactors headspace. Environmental Science and Technology, 40(10), 3409-3415.

van Groenestijn J, Hazewinkel J, Nienoord M, Bussmann P. 2002. Energy aspects of biological hydrogen production in high rate bioreactors operated in the thermophilic temperature range. International Journal of Hydrogen Energy, 27(11-12), 1141-1147.

van Lier, J. 1996. Limitations of thermophilic anaerobic wastewater treatment and the consequences for process design. Antonie van Leeuwenhoek, 69(1), 1-14.

van Niel E, Claassen P, Stams A. 2003. Substrate and product inhibition of hydrogen production by the extreme thermophile, *Caldicellulosiruptor saccharolyticus*. Biotechnology and Bioengineering, 81(3), 255-262.

van Niel E, Budde M, De Haas G, van der Wal F, Claassen P, Stams A. 2002. Distinctive properties of high hydrogen producing extreme thermophiles, *Caldicellulosiruptor saccharolyticus and Thermotoga elfii*. International of Jounal Hydrogen Energy 27(11-12): 1391-1398.

Venetsaneas N, Antonopoulou G, Stamatelatou K, Kornaros M, Lyberatos G. 2009. Using cheese whey for hydrogen and methane generation in a two-stage continuous process with alternative pH controlling approaches. Bioresoure Technology, 100(15), 3713-3717.

Voolapalli R, Stuckey D. 2001. Hydrogen production in anaerobic reactors during shock loads-influence of formate production and H_2 kinetics. Water Research, 35(7), 1831-1841.

Willquist K, Zeidan A, Pawar S, van Niel E. 2010. Hydrogen tolerance of the extreme thermophilic *Caldicellulosiruptor saccharolyticus.* Submitted manuscript.

Weiss, A., Jerome, V., Freitag, R., Mayer, H.K. 2008. Diversity of the resident microbiota in a thermophilic municipal biogas palnt. Applied Microbiology and Biotechnology, 81, 163-173.

Wu SY, Lin CY, Lee KS, Hung CH, Chang JS, Lin PJ, Chang FY. 2008. Dark fermentative hydrogen production from xylose in different bioreactors using sewage sludge microflora. Energy and Fuels, 22, 113-119.

Western sugar cooperative Inc. 2006. Molasses desugarized solubles. http://www.westernsugar.com/MDS.aspx

Xu F, Liu C, Geng Z, Sun J, Sun R, Hei B, Lin L, Wu S, Je J. 2006. Characterisation of degraded organosolv hemicelluloses from wheat straw. Polymer Degradation and Stability; 91(8): 1880-1886.

Yokoyama H, Moriya N, Ohmori H, Waki M, Ogino A, Tanaka Y. 2007. Community analysis of hydrogen-producing extreme thermophilic anaerobic microflora enriched from cow manure with five substrates. Applied Microbiology and Biotechnology, 77(1), 213-222.

Yokoyama H, Ohmori H, Waki M, Ogino A, Tanaka Y. 2009. Continuous hydrogen production from glucose by using extreme thermophilic anaerobic microflora. Journal of Bioscience and Bioengineering, 107(1), 64-66.

Zhang T, Liu H, Fang H.2003. Biohydrogen production from starch in wastewater under thermophilic condition. Journal of Environmental Management, 69: 149-156.

Zhang Z, Show K, Tay J, Liang D, Lee D. 2008. Enhanced continuous biohydrogen production by immobilized anaerobic microflora. Energy Fuels, 22(1), 87-92.

Zellner G, Neudörfer F, Diekmann H. 1994. Degradation of lactate by an anaerobic mixed culture in a fluidized-bed reactor. Water Research, 28(6): 1337-1340.

Zheng, H., Zeng, R., Angelidaki, I., 2008. Biohydrogen production from glucose in upflow biofilm reactors with plastic carriers under extreme thermophilic conditions (70°C). Biotechnology and Bioengineering. 100(5), 1034-1038.

Zhu H, Stadnyk A, Beland M, Seto P. 2008. Co-production of hydrogen and methane from potato waste using a two-stage anaerobic digestion process. Bioresource Technology, 99(11), 5078-5084.

8 Appendices

I Kongjan P, Min B, Angelidaki I. 2009. Biohydrogen production from xylose at extreme thermophilic temperature (70 ºC) by mixed culture fermentation, Water Research 43 (5), 1414-1424.

II Kaparaju P, Serrano M, Thomsen A B, Kongjan P, Angelidaki I. 2009. Bioethanol, biohydrogen and biogas production from wheat straw in a biorefinery concept. Bioresource Technology, 100 (9), 2562-2568.

III Kongjan P, O-Thong S, Kotay M, Min B, Angelidaki I. 2010. Biohydrogen production from wheat straw hydrolysate by dark fermentation using extreme thermophilic mixed culture. Biotechnology and Bioengineering 105(5), 899-908.

IV Kongjan P, Angelidaki I. 2010. Extreme thermophilic biohydrogen production from wheat straw hydrolysate using mixed culture fermentation; Effect of reactor configuration. Bioresoure Technology. doi: 10.1016/j.biortech.2010.05.024.

V Kongjan P, O-Thong S, Angelidaki I. 2010. Performance and microbial community analysis of two-stage process with extreme thermophilic hydrogen and thermophilic methane production from wheat straw hydrolysate. Manuscript.

VI Kongjan P, O-Thong S, Angelidaki I. 2010. Biohydrogen production from desugared molasses using thermophilic mixed cultures immobilized on heat treated anaerobic sludge granules. Manuscript

The papers are not included in this www-version, but can be obtained from the Library at DTU Environment. Contact library@env.dtu.dk or Department of Environmental Engineering, Technical University of Denmark, Miljoevej, Building 113, DK-2000 Kgs. Lyngby, Denmark

The Department of Environmental Engineering (DTU Environment) conducts science-based engineering research within four themes:
Water Resource Engineering, Urban Water Engineering,
Residual Resource Engineering and Environmental Chemistry & Microbiology.
Each theme hosts two to five research groups.

The department dates back to 1865, when Ludvig August Colding, the founder of the department, gave the first lecture on sanitary engineering as response to the cholera epidemics in Copenhagen in the late 1800s.

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