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Ethanol and Hydrogen Production from Lignocellulosic Biomass with Thermophilic Bacteria

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School of Business and Science
Faculty of Natural Resource Sciences
University of Akureyri
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Útdráttur

Rannsókn þessi var tvíþætt. Í fyrsta lagi var skrifuð yfirlitsgrein um etanól- og vetnisframleiðslu úr sykrum og flóknum lífmassa með hitakærum bakteríum. Öflugustu vetnisframleiðandi bakteríur eru aðallega innan tveggja ættkvísla, *Thermotoga* og *Caldicellulosiruptor*. Ástæðu þess má rekja til þess að tegundir innan þeirra ættkvísla lifa við mjög hátt hitastig sem gerir það að verkum að hærri nýtni vetnis fæst vegna orkufræðilegra ástæðna. Þær bakteríur sem framleiða hinsvegar mest etanól koma frá ættkvíslunum *Thermoanaerobacter*, *Thermoanaerobacterium* og *Clostridium*. Í ljós hefur komið að þar eru mun fleiri þættir en hitastig sem hafa áhrif á etanólframleiðslu og má þá helst nefna hlutfall gass og vökva í ræktun og upphafsstyrk sykra. Hægt er að stýra þessum umhverfisþáttum til að hámarka etanólframleiðslu stofnanna.

Í öðru lagi voru gerðar tilraunir á sjö stofnum hitakærra baktería sem voru einangraðar úr íslenskum hverum með megin áherslu á etanólframleiðslu úr mismunandi hýdrólýsötum sem gerð voru úr flóknum lífmassa. Niðurstöður raðgreininga leiddu í ljós að fjórir stofnar tilheyra ættkvísl *Thermoanaerobacterium,* tveir tilheyra *Thermoanaerobacter* og einn *Paenibacillus*. Vaxtartilraunir leiddu í ljós skýran mun á kjörvaxtarhitastigi á milli ættkvísla. Etanólþol allra stofnanna var kannað og sýndu niðurstöður lágt þol (MIC = 1.6% v/v) hjá *Thermoanaerobacter* en mun hærra (MIC = 3.2% v/v) fyrir *Thermoanaerobacterium* og *Paenibacillus* stofnana. Sex af sjö stofnum voru með etanól nýtni á milli 1.0 til 1.5 mól-EtOH $mól^{-1}$ glúkósa og 0.4 til 1.3 mól-EtOH $mól^{-1}$ xýlósa en einn stofn framleiddi mun minna etanól. Besta nýtnin var hjá stofni AK_{17} á glúkósa, eða 1.5 mól-EtOH/ mól glúkósa. Vöxtur var einnig athugaður í 0.75% (w/v) "hýdrólýsötum" sem voru gerð úr sellulósa (Whatman pappír), ólituðum pappír, lituðum pappír, glanspappír, viðarsagi og vallarfoxgrasi (*Phleum pratense*). Flestir stofnarnir gáfu ágæta etanólframleiðslu. Stofn AK_{17} framleiddi 43.4 mM etanól úr selluósa, 21.2 mM úr vallarfoxgrasi, á milli 14.4 og 23.3 mM úr hinum þremur gerðum af pappír og 3.2 mM úr viðarsaginu. Aðrir stofnar framleiddu minna af etanóli en framleiðslan var í réttu hlutfalli við etanólframleiðslu þeirra á glúkósa og xýlósa. Aðrar afurðir úr hýdrólýsötum voru ediksýra og vetni líkt og á einsykrum.

Lykilorð: Loftfirrtar hitakærar bakteríur, vetni, etanól, flókinn lífmassi, formeðhöndlun

Abstract

This investigation was twofold. Firstly, an overview article about ethanol and hydrogen production from sugars and lignocellulosic biomass with thermophilic bacteria was written and published. The main findings were that the most efficient hydrogen producing bacteria belong to two genera, *Thermotoga* and *Caldicellulosiruptor.* The reason for such high yields is that these bacteria grow at very high temperature which makes hydrogen production thermodynamically favourable. The best ethanol producing bacteria however belong to *Thermoanaerobacter*, *Thermoanaerobacterium* and *Clostridium.* Other environmental factors besides from temperature affect ethanol yields. The ratio of gas and liquid and initial substrate concentration are the most critical factors which can be controlled to mamixmize ethanol production.

Secondly, experiments on seven strains of thermophilic bacteria isolated from Icelandic hot springs were performed with the main focus on ethanol production from different lignocellulosic hydrolysates. Phylogenetic studies revealed that four of the strains belong to the genus *Thermoanaerobacterium*, two belong to *Thermoanaerobacter* and on to *Paenibacillus*. Physiological experiments showed a distinct difference in temperature optima between genera. Ethanol tolerance was low (MIC = 1.6% v/v) for *Thermoanaerobacter* to moderatly high (MIC = 3.2% v/v) for *Thermoanaerobacterium* and *Paenibacillus*. Six of seven strains showed ethanol yields between 1.0 and 1.5 mol-EtOH mol^{-1} glucose and 0.4 to 1.3 mol- EtOH mol^{-1} xylose, but one strain produced significantly less than the others. AK_{17} gave the best yields on glucose (1.5 mol-EtOH mol^{-1} glucose). Growth on 0.75 % (w/v) hydrolysates made from cellulose (Whatman paper), non inked paper, inked paper, glossy paper, sawdust and grass (*Phleum pratense*) resulted in good ethanol production yields for most of the strains. Strain AK_{17} produced 43.4 mM of ethanol from cellulose, 21.2 mM from grass, between 14.4 to 23.3 mM from the three types of paper hydrolysates and 3.2 mM from sawdust. Other strains produced less ethanol from biomass hydrolysates but its production was in correlation to lower ethanol production yields from monosugars fermentation.

Keywords: Anaerobic thermophilic bacteria, hydrogen, bioethanol, lignocellulosic biomass, pretreatment.

”If we knew what it was we were doing, it would not be called research, would it?”

Albert Einstein

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Abbreviations

EtOH	Ethanol
H_2	Hydrogen
CO_2	Carbon dioxide
CH_4	Methane
HL	Hydrolysate
SSF	Simultaneous saccharification and fermentation
SSCF	Simultaneous saccharification and co-fermentation
SHF	Separate hydrolysis and fermentation
CBP	Consolidated bioprocessing
PV	Photovoltaics
CSP	Concentrated solar power
C_5 sugars	Sugars with 5 carbon molecules (pentoses)
C_6 sugars	Sugars with 6 carbon molecules (hexoses)
ATP	Adenosine-5´-triphosphate
NADH	Nicotineamide adenine denucleotide reduced
NAD^+	Nicotineamide adenine denucleotide oxidized
NAD(P)H	Nicotineamide adenine denucleotide phosphate reduced
PFOR	Pyruvate ferredoxin oxidoreductase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
PDC	Pyruvate decarboxylase
ADH	Alcohol dehydrogenase
ML	Million liters

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1 Background and research objectives

The objective of this research was twofold. Firstly, investigation of ethanol and hydrogen production with thermophilic bacteria from pure sugars and lignocellulosic biomass was conducted. A literature investigation led to the publication of an overview chapter published in the book *Progress in Biomass and Bioenergy production* (chapter 19) by InTech Publication in 2011. That is presented as Manuscript I in this thesis. The main findings were that thermophilic bacteria are capable of producing both hydrogen and ethanol with high yields from sugars and variety of pretreated lignocellulosic biomass. The best ethanol producing strains belong to three genera: *Thermoanaerobacter*, *Thermoanaerobacterium* and *Clostridium* and the best hydrogen producing strains belong to *Caldicellulosiruptor* and *Thermotoga* and thrive at very high temperatures.

Secondly, the objective was to investigate ethanol production from monosugars and lignocellulosic biomass with thermophilic bacteria isolated from hot springs in Iceland. Seven strains of thermophilic bacteria isolated on various carbohydrates from Grensdalur (SW Iceland) and Viti (NE Iceland) were chosen. Phylogenetic studies revealed that four of the strains belong to the genus *Thermoanaerobacterium*, two belong to *Thermoanaerobacter* and one to *Paenibacillus*. Physiological experiments were conducted for all strains as well as experiments concerning ethanol tolerance and ethanol production capacities on monosugars and several different lignocellulosic hydrolysates. The results have already been published in the journal *Icelandic Agricultural Sciences* and are presented in this thesis as Manuscript II.

2 Introduction

The world´s population continues to increase. In July 2011, the world´s total population was estimated to be around 6.97 billions and it is expected to reach 9.3 billions in 2050 (United Nations, 2011). With the expansion of human population and industrialization of developing countries, the global energy consumption is likely to increase as well. Fossil fuel sources of energy such as oil, coal and natural gas have been the world´s primary energy source for the last 200 years, resulting in high levels of carbon dioxide (CO_2) and other greenhouse gases in the atmosphere that are a major factor in anthropogenic climate change (Demirbas, 2009). It is expected that climate change will have great social and ecological impacts in the coming century. It affects the world in many ways, e.g. by increased frequency of heat waves, increased floods and other climate related disasters as well as malnutrition and changes in the distribution of diseases (Tingem & Rivington, 2009).

It is a fact that the increasing concentration of greenhouse gases in the atmosphere is raising the earth´s temperature by trapping heat that radiates from the earth´s surface. The levels of atmospheric CO_2 have been increasing rapidly over the past decades resulting in an increased temperature on global scale (Dincer, 1999; Mauna Loa Observatory, 2012). Mauna Loa Observatory located in Hawaii monitores atmospheric CO_2 and gives a monthly update of CO_2 levels. Figure 1 shows the concentration of atmospheric CO_2 (ppm) from June 1958 until June 2012. The annual increase for the decade 1992-2001 was 1.6 ppm per year but the increase for the past decade, 2002-2011, was 2.07 ppm (Mauna Loa Observatory, 2012).

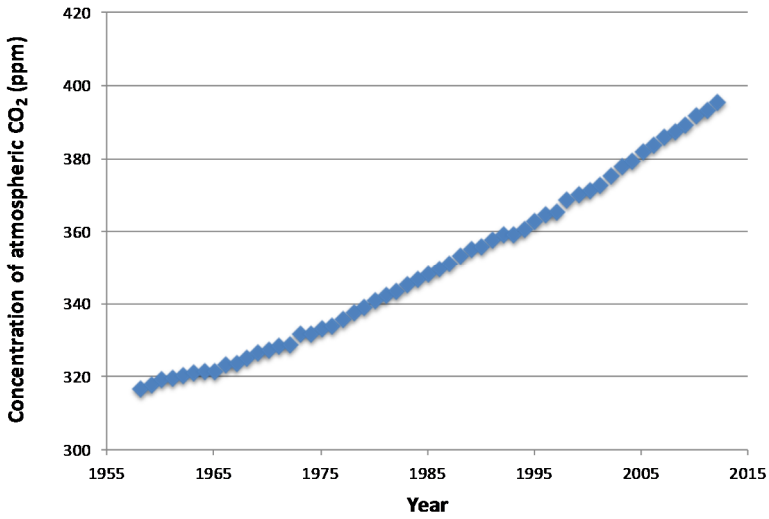

Figure 1. The concentration of atmospheric CO_2 from 1958-2012 (Data from Mauna Loa Observatory, 2012).

The greenhouse gas reduction targets that were set for most countries with the Kyoto protocol will expire in 2012. The European Commission (EC) has already proposed new targets for 2020 which will replace the Kyoto targets (European Commission Energy Efficiency Economy, 2008). Precise estimates of time frames for fossil fuel depletion are difficult. Some analysts have suggested that the world peak in oil production occured in 2007-2008, but a modal estimate assumes that the maximum of world oil production will be achieved sometime in 2020-2030 and at that time the production will be around 4.2-4.7 billion tons a year (Kontorovich, 2009). Figure 2 shows the world´s total oil production until 2009 and then three different probabilistic estimates of the global conventional recoverable oil resource based on sedimentary investigations.

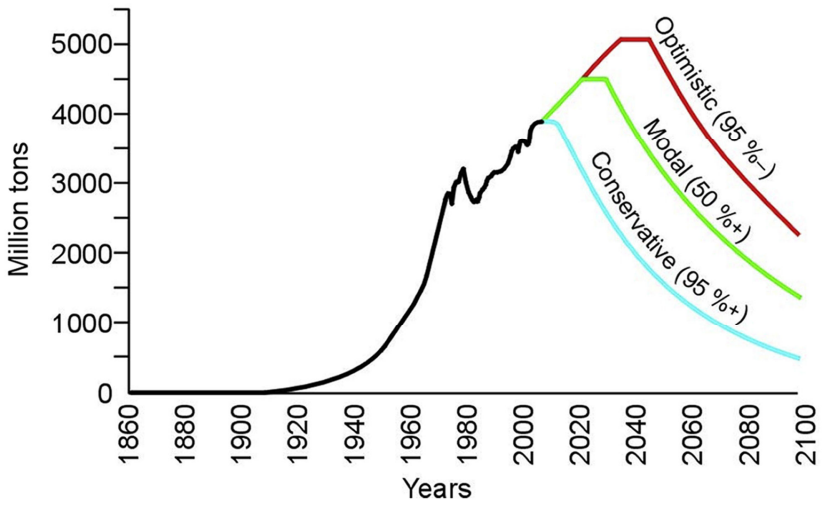

Figure 2. A probabilistic estimate of the world's oil production until 2100. (Modified from Kontorovich, 2009).

In response to fossil fuel depletion and increasing greenhouse gas emissions, continuous efforts have been made in exploration of alternative energy sources which are cheap, renewable and clean. There are several types of renewable energy sources available today and will be further discussed in next chapter.

2.1 Renewable energy

Energy resources can be split into three categories; fossil fuels, renewable resources and nuclear resources. Renewable energy sources (e.g. wind, water, solar, biomass, geothermal) have the potential to provide energy with low or no emissions of both air pollutants and greenhouse gases and thus meet future energy demand (Saxena et al., 2009). The production of renewable energy sources has been growing rapidly in the last few years and will, most probably continue to grow even faster in the near future. Many countries have started programs regarding renewable energy production and signed initiatives with the goal to decrease the dependence on fossil fuel and increase the production of renewable energy sources (Taylor *et al*., 2009). In 2010 it was estimated that renewable energy sources supplied 16.7% of the total energy demand. Of this total, 8.2% came from ‘‘modern’’ renewables (hydropower, wind, solar, geothermal, biofuels and biomass). ‘‘Traditional’’ biomass is still widely used and accounts for 8.5% of total final energy (Renewable Energy Policy Network for the 21st Century (REN21), 2012). Figure 3 shows the total world energy consumption by energy source as well as the share of each renewable source.

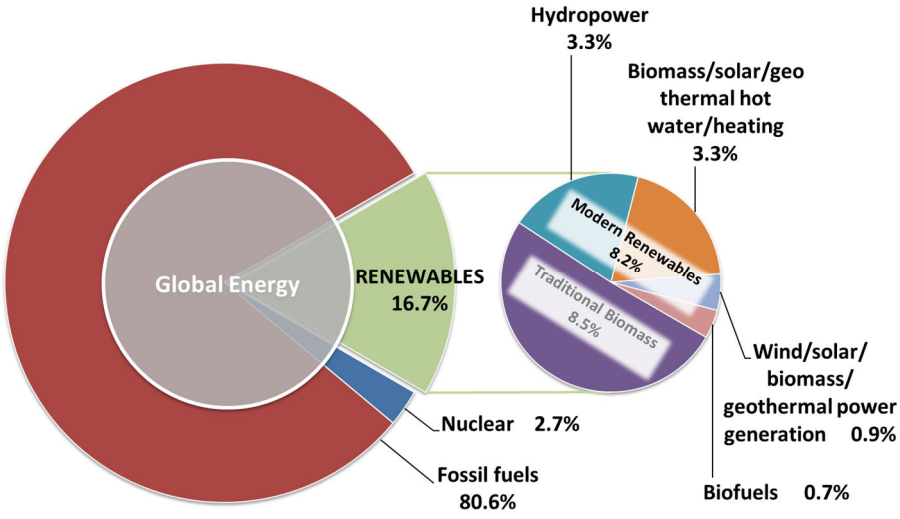

Figure 3. Renewable energy share of global final energy consumption in 2010 (REN21, 2012).

It is unlikely that a single energy source is going to replace fossil fuels in the coming decades. To find solutions to energy problems the proponents for the renewable energy sources need to work together and find a way to make use of the best local and imported energy in individual countries and regions (Fridleifsson, 2001).

Solar power

Solar power is energy derived from the sun through radiation and is the most abundant renewable energy source available. There are two main kinds of solar energy; Solar photovoltaic (PV) and concentrated solar power (CSP). PV cells are used to convert energy from the sun (light) directly into electricity. CSP devices however transform heat from the sun´s rays indirectly into mechanical energy and then into electricity (solar thermal electricity) (Barlev *et al.*, 2011). Solar technologies can be characterized as either passive or active solar energy forms, depending on the way they capture, convert and distribute the energy. The use of PV panels and solar thermal collectors to harness the energy are an example of active solar technology while passive solar techniques relate to the design of buildings collecting and transforming solar energy using it for passive heating (Philibert, 2005).

The solar PV market has been growing for the last 5 years. In 2007, the world´s total solar PV capacity was 9.4 GW but in 2011 it had reached 70 GW. Germany and Italy produce more than 53% of world´s total PV operating capacity (REN21, 2012).

Geothermal energy

Geothermal energy is energy that is generated and stored as heat in the earth, originating from the earth´s core. The heat conducts to surrounding rocks and with pressure causes rocks to melt, making magma. Magma then flows upward making a thermal gradient (30°C/km) towards the surface and heats up the rocks present (Barbier, 2001). Water that sinks below the earth´s surface is heated by the rocks and accumulates in aquifers that are an essential part of geothermal fields. That water can be exploited for both hot tap water and high-temperature steam generation. In most cases though, the reservoir is covered with impermeable rocks preventing the water from reaching the surface. Drilling down to those reservoirs makes industrial production of electricity from the extremely hot water or steam available (Barbier, 2001).

In 2010, the International Geothermal Association (IGA) reported 10.715 MW of installed generating capacity worldwide, expecting to

generate 67.246 GWh of electricity (International Geothermal Association, 2012). Today, geothermal heat and electricity accounts for only about 0.2% of world´s total energy supply but that number is expected to grow in the future. In 2010, the United States led the world in geothermal electricity production with 77 power plants. The Philippines and Indonesia are the second and third largest producers of geothermal energy while Iceland was the seventh largest producing country with the highest percentage of total national electricity production, or 30% (International Geothermal Association, 2012).

Wind power

Wind is created when air moves across the surface of the earth from high to low pressure areas. The sun´s rays heat up earth surface and warm air rises above cool air forming high pressure areas. That combines with the rotation of the earth causing wind patterns across the earth (RenewableUK, 2012). The power of the wind can be harnessed and converted into mechanical or electrical power via turbines, windmills or wind pumps. Wind power is renewable, widely distributed and the energy created is clean without any greenhouse gas emissions during operation. Windfarms are often located offshore to harness more powerful winds than are available on land (Panwar et al., 2010).

The world´s total wind power capacity has been increasing rapidly over the last six years. From 2006 to 2011 the world's production has almost increased by a factor of five, or from 76 GW to 238 GW. The five largest wind power producing countries in the world are China, USA, Germany, Spain and India (REN21, 2012).

Hydroelectric power

Hydroelectric power is energy that comes from the force of moving water. Like windpower, the origin is the heat from the sun that causes water (rivers and oceans) to evaporate. Then the water vapor cools down, condenses and forms clouds. The moisture eventually falls back down as rain or snow, making the water cycle complete. Gravity then drives water from high ground to lower areas and the force of the water can be harnessed by several different technologies to make hydroelectric power (International Energy Agency, 2012). Hydroelectric power has several benefits including high availability, simple harnessing technology, and low levels of greenhouse gas emission (Environmental Literacy Council, 2008). It has been used for over 2000 years when the Greeks used waterwheels to grind grains into flour. The production of electricity with

hydropower started before the 1900´s. Modern technology mostly relies on hydroelectric dams (Environmental Literacy Council, 2008).

The leading countries for hydropower capacity are China, Brazil, USA, Canada, and Russia, which together account for 51% of world´s total installed capacity (970 GW) (REN21, 2012). The total installed hydropower capacity of Iceland in 2010 was 1.883 MW with the total of 12.592 GWh in produced electricity (Orkustofnun, 2011).

Bioenergy from biomass

Bioenergy is a form of renewable energy derived from biomass which is produced by plants and microorganisms by photosynthesis. When burned, the chemical energy in the biomass is released as heat (Biomass Energy Center, 2012). Biomass can be characterized as ‘‘modern’’ or ‘‘traditional’’. Modern biomass is produced in a sustainable way and examples include electricity generation, heat production and transportation fuels production from agricultural and forest residues as well as solid waste. Traditional biomass, however, is used as a non-commercial way for cooking and heating in many rural areas and developing countries. It is non-sustainable but it is included as a renewable source (Goldemberg & Coelho, 2004).

Biofuels

Biomass can be converted into a number of useful energy carriers such as ethanol, methanol, butanol, biodiesel, hydrogen and methane (Nigam & Singh, 2011).

Biodiesel is a liquid biofuel which is a promising alternative energy source candidate. Biodiesel is a monoalkyl ester of fatty acids present in oil rich plants, fat rich waste and algae. It is renewable, biodegradable and non-toxic. The production of biodiesel is carried out by catalytic transesterification with petrochemically derived methanol (alcoholysis) (Saka & Kusdiana, 2001). However, the main drawback of using vegetable oil is the high viscosity and low volatility which causes poor combustion in diesel engines (Doan et al., 2011). Instead of using vegetable oil, microalgae can be grown in photobioreactors or open ponds and used for the production. The advantage of using microalgae for the production is that they do not require a large area of land for growing unlike plants and they are not affecting the supply of food and other crop related products (Doan et al., 2011). The biodiesel market has been expanding over the past years. The United States became the biggest

producer in the world in 2011 after a record production growth in one year (159%) (REN21, 2012).

Biomethane is a renewable natural gas made from organic sources such as agricultural residues and wastes, energy crops and manure (Kurchania et al., 2010). Biomethane can be used to generate heat and electricity or it can be separated from the other gases and used as transport fuel or injected into the gas grid. Biomethane production is a complex process that can be divided into a three stage process called anaerobic digestion (hydrolysis, acidogenesis and methanogenesis). Each step involves a different set of anaerobic and facultative anaerobic microorganisms. In the first step, polysaccharides, proteins and fats are hydrolyzed into oligosaccharides, sugars, amino acids, fatty acids and glycerol. Fermentation of these products into organic acids, aromatic compounds, alcohols, CO_2 and H_2 is the next step. Finally, CH_4 is formed along with CO_2 and by-products (NH_3 and H_2S), usually by slow growing archaea, the methanogens (Antoni et al., 2007). Thus, biogas is the final product and is a combination of CH_4 (40-70%), CO_2 (30-60%) and other gases (1-5%) (Kurchania et al., 2010).

The biogas industry has been growing, particularly in Europe, i.e. in Denmark and Germany (REN21, 2012). Bioethanol and biohydrogen can be produced by fermentation and will be discussed in detail in later chapters.

2.2 Biomass

Biomass is an organic matter that grows by the photosynthetic conversion of solar energy and carbon dioxide to more complex carbon compounds. It is the only renewable energy resource that contains carbon that can be converted into solid, liquid or gaseous products that can then further be converted into heat, electricity and transport fuels. Biomass is currently the fourth largest energy source after fossil fuel derived energy (coal, oil and natural gas) (Biomass Energy Center, 2012; REN21, 2012). The biomass used as a feedstock for both EtOH and H_2 production can be classified as either simple or complex biomass. Simple biomass includes starch and sucrose, which is derived from corn and sugarcane, and has been used for bioethanol production for decades. Complex biomass (lignocellulose), or "plant biomass", is composed of three different biopolymers (cellulose, hemicellulose, and lignin) that are strongly bound together by noncovalent forces as well as covalent cross-linkages (Glazer & Nikado, 2007).

Starch

Starch is an important storage polysaccharide in plant cells. It occurs intracellularly as large clusters or granules. Starch molecules are heavily hydrated due to the number of exposed hydroxyl groups available to hydrogen bond with water. There are two types of glucose polymers that form starch: amylose (Figure 4) and amylopectin (Figure 5). Both are homopolymers of D-glucose units, however, the former is an unbranched chain where the units are connected with (α-1→4) linkages while the latter is highly branched with (α-1→6) linkages at the branch points (Nelson & Cox, 2008).

Starch has been used for decades in bioethanol production, especially in the United States. Among agricultural biomass that is high in starch is corn (maize), wheat, oats, rice, potatoes and cassava. In order to make the carbohydrates in starchy biomass fermentable by both yeast and bacteria, the polymers have to be hydrolyzed and degraded with enzymes (Nigam & Singh, 1995). The enzymes that hydrolyze starch into the constituent sugars are known as amylases. A number of fungi, bacteria and plants produce amylases. The two main types of amylases are α- and β- amylases. α-amylase acts randomly along the starch chain and cleaves long-chain carbohydrates into smaller oligosaccharides, ultimately yielding maltose and maltotriose. β-amylase acts more slowly because it works from the non-reducing end, cleaving off two glucose units at a time (maltose) (Nelson & Cox, 2008; Nigam & Singh, 1995).

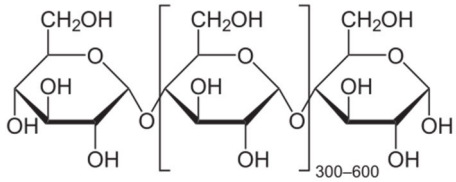

Figure 4. Structure of an amylose molecule.

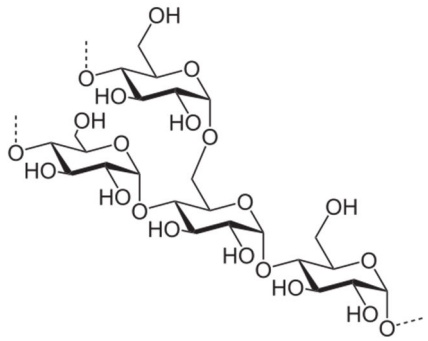

Figure 5. Structure of an amylopectin molecule.

Lignocellulose

The primary cell wall is composed of approximately 10% proteins and 90% polysaccharides which can be divided into three groups: cellulose, hemicelluloses and pectin. The composition of each polymer varies between species but the average is around 30% cellulose, 30% hemicellulose and 35% pectin (Cosgrove, 1997; McNeil *et al*., 1984). Figure 6 demonstrates the structure of a plant cell wall. Cellulosic microfibrils are linked with hemicellulosic tethers to form a network which is embedded in the pectin matrix.

Some plants have a secondary cell wall as well, located between the primary cell wall and the plasma membrane. The major polymers that make up that cell wall are cellulose and xylan, surrounded by lignin that penetrates the spaces between, strengthening the wall (Bacic *et al*., 1988; Cosgrove, 2005). The composition of these three polymers is also very different from one plant species to another (cellulose, 36-61%; hemicellulose, 13-39%; lignin 6-29%) (Olsson & Hahn-Hagerdal, 1996). Among popular lignocellulosic feedstocks used today for EtOH and H_2 production are wheat straw, napier grass, switchgrass, miscantus, corn stover and sorgum (Hattori & Morita, 2010).

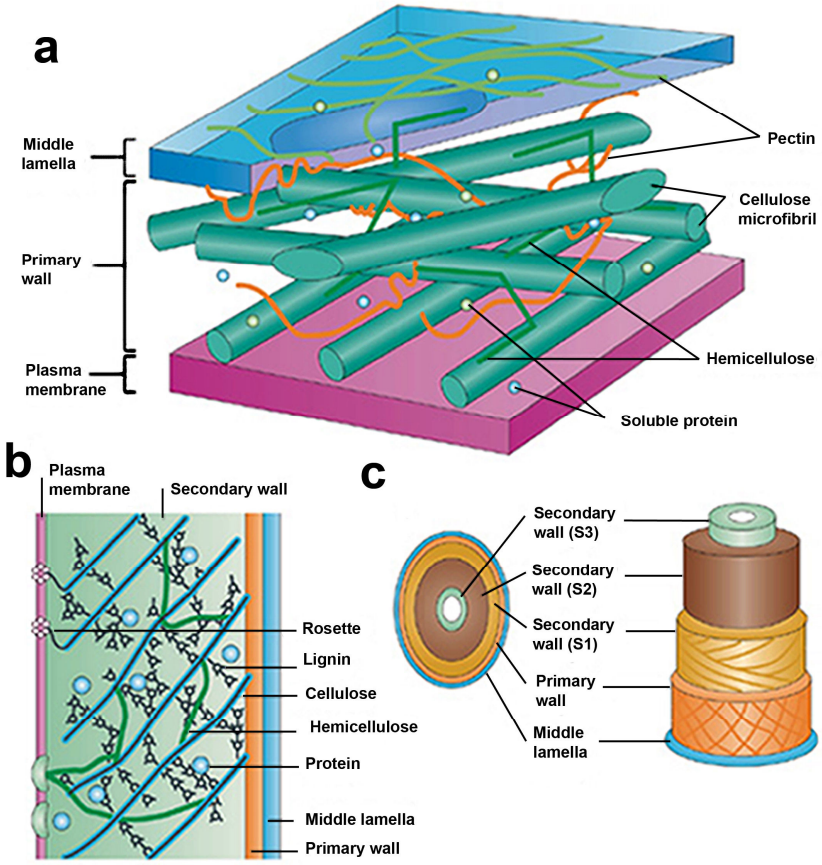

Figure 6. a: Cell wall containing cellulose microfibrils, hemicellulose, pectin, lignin and soluble proteins. b: Cellulose synthase enzymes are in the form of rosette complexes, which float in the plasma membrane. c: Lignification occurs in the S1, S2 and S3 layers of the cell wall (Sticklen, 2008).

Cellulose

Cellulose is the most abundant organic polymer on earth. In one year the worldwide production of this biopolymer is estimated to be between 10^{10} and 10^{11} tons (Lavoine *et al*., 2012). Cellulose is a tensile, water-insoluble homopolysaccharide that consists of 10.000-15.000 linear D-glucose molecules (Figure 7). Unlike amylose in starch, the glucose residues in cellulose are linked by β-1,4 glycosidic bonds which give those two polymers very different structural and physical properties (Nelson & Cox, 2008). The basic chemical structure is a cellobiose dimer that rotates

through 180° along the chain axis and causes the polymer to look ribbon like. Each linear cellulose unit connects to other units with hydrogen bonds resulting in very long crystalline microfibrils which then form larger fibrils (Bayer *et al*., 1998; Lavoine *et al*., 2012).

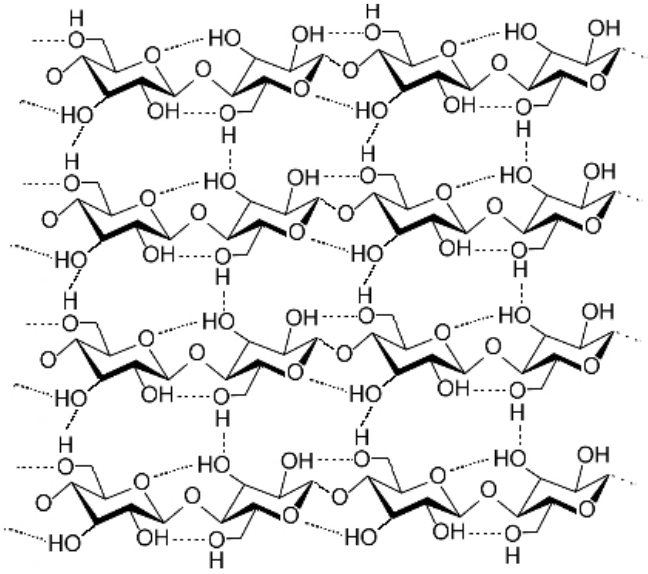

Figure 7. Cellulose; glucose units linked by β-1,4 and with hydrogen bonds within and between cellulose molecules.

Cellulases are a group of enzymes that can break down cellulose. They are divided into three groups: endoglucanases, cellobiohydrolases and β-glucosidases and will be discussed further in chapter 2.3 (enzymatic hydrolysis) (Lynd *et al*., 2002). A number of bacteria and fungi can degrade amorphous cellulose but relatively few have the complete enzyme system to break down crystalline cellulose. In the early 1980´s the cellulosome in *Clostridium thermocellum* was discovered (Bayer & Lamed, 2006); the cellulosome is a multifunctional protein complex that is a scaffolding unit which includes a series of nine highly similar binding modules called domains that interact together in the complete breakdown of crystalline cellulose (Shimon *et al*., 1997; Bayer *et al*., 1998).

Hemicellulose

Hemicellulose is the non-cellulose cell-wall polysaccharide of plants that represents an immense renewable resource of biopolymers. Hemicellulose can be divided into four general groups: xylans, mannans, mixed linkage β-glucans, and xyloglucans (Ebringerová *et al*., 2005). The components of

hemicellulose are complex polysaccharides that are structurally homologous to cellulose because they have a backbone made of 1,4-linked β-D-pyranosyl units (Glazer & Nikado, 2007). Hemicellulose represents in general 15-35% of plant biomass, it is highly branched and contains both pentoses (β-D-xylose, α-L-arabinose), hexoses (*β*-D-mannose, *β*-D-glucose, *α*-D-galactose), and uronic acids (*α*-D-glucuronic, α-D-4-*O*-methylgalacturonic, and *α*-D-galacturonic acids). Other sugars can be present in small amounts such as *α*-L-rhamnose and *α*-L-fucose (Gírio *et al.*, 2010). The most relevant hemicelluloses are xylans and glucomannans. Xylan is most abundant as it is a major structural polysaccharide in plant cells, and the second most abundant polysaccharide in nature, accounting for over 30% of all renewable organic carbon on earth. Xylan (Figure 8) is believed to play a crucial role in fiber cohesion and plant cell wall integrity as it is found at the interface between lignin and cellulose (Collins *et al.*, 2005).

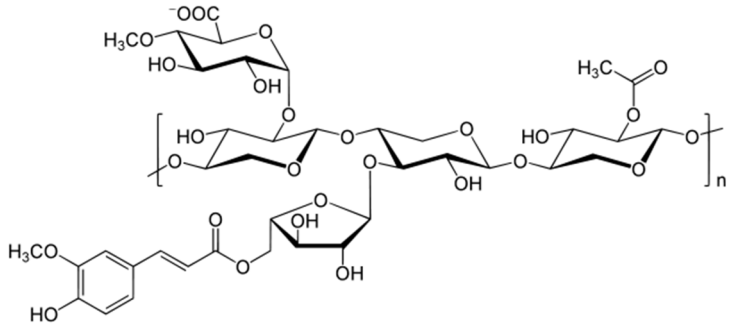

Figure 8. The structure of xylan.

Lignin

Lignin is a non-fermentable part of lignocellulose. It is the third most abundant biopolymer on earth, after cellulose and hemicellulose, but the most abundant aromatic compound. It is found in the cell wall of higher plants as structural and support material allowing huge trees to grow and remain upright, even over 100 meters high. Lignin is randomly constructed heteropolymer of phenylpropane (C_9) units and has three alcohol derived precursors: conyferyl, sinapyl and *p*-coumaryl alcohols (Figure 9). Lignin is often referred to as softwood lignin, hardwood lignin and grass lignin, depending on the amount of each building block in biomass (Glazer & Nikado, 2007).

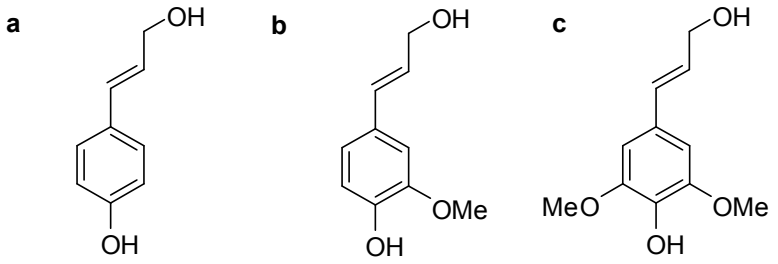

Figure 9. Lignin precursors: (a) p-coumaryl alcohol, (b) coniferyl alcohol and (c) sinapyl alcohol.

Lignin protects cell wall polysaccharides from microbial degradation and is thus an inhibiting factor in the conversion of plant biomass to biofuels. The process of lignin removal is a costly process and has been target of increased research focus in the past years on e.g. designing plants that either deposit less lignin or produce lignin that are more amenable to chemical degradation (Vanholme *et al*., 2010). Wood-decaying fungi are the primary contributors of wood degradation in nature. They produce and secrete enzymes that can degrade the polymers present in wood. Over 90% of all known wood decaying fungi are white rot fungi. The rest is classified as brown rot fungi and soft rot fungi. White rot fungi are able to degrade cellulose, hemicellulose, and lignin although they have different degradation rates for each polymer Lignin degradation is a very complex process where a variety of enzymes are needed for the degradation. Lignin peroxidase, Mn-peroxidase and laccase are the main enzymes in lignin degradation and environmental conditions like moisture, temperature, pH and levels of nitrogen and oxygen at the locations of enzyme action are critical factors (Glazer & Nikado, 2007; Tuor *et al*., 1995).

2.3 Pretreatment of biomass

Pretreatment of biomass has been regarded of importance in biofuel production from complex biomass; it alters the structure of lignocellulosic biomass in order to make it more accessible to the enzymes that convert the polymeric carbohydrates into sugars before fermentation. One of the reasons why biofuel production from lignocellulosic biomass has not been competitive to fossil fuel is the high cost and challenges of the pretreatment processes. Although major progress has been made in the past few years, minimizing energy consumption to achieve maximum sugar and ethanol yield remains a challenge (Mosier *et al*., 2005; Zhu *et al*., 2010).

There are not many reports on bacteria degrading untreated plant biomass although the anaerobic thermophile *Anaerocellum thermophilum* has though recently been showed to do so. This bacterium can degrade various types of untreated biomass, even with high lignin content and cellulose crystallinity (Chang & Yao, 2011; Yang *et al*., 2009). However, when using unpretreated biomass, the degradation and fermentation process is much slower and the yields are usually lower than compared to using biomass that has been pretreated.

There are many different pretreatment options available and no single method is regarded as the "best one"; all methods have their advantages and disadvantages. The most common ways to pretreat biomass are chemical, physical, physio-chemical, biological and mechanical methods. An effective pretreatment method needs to meet certain requirements to be economically feasible, e.g. it has to avoid size reduction, preserve hemicellulose fractions, limit the formation of inhibitory compounds, minimize energy input and be cost effective (Banjaree *et al*., 2010). Below, the main pretreatment methods are discussed but for more comprehensive details, recent review articles should be addressed (Chiaramonti *et al*., 2012; Menon & Rao, 2012; Talebnia *et al*., 2010).

Physical pretreatment

Physical pretreatment of biomass is mainly used to reduce cellulose crystallinity. Most lignocellulosic biomass requires some mechanical processing such as milling, chipping, or grinding to improve hydrolysis. Physical pretreatment is often used for woody biomass (Zhu *et al*., 2010; Mosier *et al*., 2005). Pyrolysis is also a type of physical pretreatment method that has been used where the biomass is heated up to temperatures greater than 300°C causing a rapid hydrolysis of cellulose (Sun & Cheng,

2002). The main disadvantage of physical pretreatment methods is high energy cost and in most cases the cost is even higher than the theoretical energy content available in the biomass used (Menon & Rao, 2012; Sánchez & Cardona, 2008).

Chemical pretreatment

Acid pretreatment methods

Chemical pretreatment is a very common method and widely used when dealing with lignocellulosic biomass. Both concentrated and diluted inorganic acids such as hydrochloric acid (HCl) and sulfuric acid (H_2SO_4) have been used because they are powerful agents in the hydrolysis of cellulose. Acid treatments can both be performed with high and low temperatures. High temperature along with the acid treatment is favorable to attain better yields of glucose release from cellulose (Guo *et al*., 2008; Talebnia *et al*., 2010). Acid causes hydrolysis of both cellulose and hemicellulose as well as alteration of lignin structure in the biomass. Also, the sugar yields are satisfactory. The method is suitable for pilot scale and the acid can be reused. The disadvantages of the method are the formation of inhibitory by-products, the equipment cost and energy consumption as well as corrosion of pipes and containers used in the process (Hsu *et al*., 2010; Sun & Cheng, 2002; Talebnia *et al*., 2010).

Alkaline pretreatment methods

Alkaline methods involving sodium-, calcium- and ammonium hydroxide have also been used for pretreatment of biomass. These methods are especially powerful in the separation of lignin and the effectiveness therefore depends on the amount of lignin in the biomass. Alkaline disrupt the ester bonds crosslinking xylan and other compounds like lignin resulting in hemicellulose and cellulose rich fractions. Alkali also causes a partial decrystallization of cellulose (Yang & Whyman, 2007).

The process of alkaline pretreatment methods usually involves soaking the biomass in alkaline solutions at a certain temperatures for a period of time followed by a neutralizing step for the removal of lignin and inhibitory compounds. Thereafter an enzymatic pretreatment is performed (Menon & Rao, 2012). Alkaline pretreatments are usually performed at lower temperatures and pressures compared to other methods. When compared to acid pretreatment, alkaline processing leads to less sugar degradation but the formation of irrecoverable inhibiting salts may occur (Mosier *et al*., 2005). Alkaline pretreatment methods have been used with success for common feedstocks like wheat straw, soybean

straw, cornstalk, switchgrass, hardwood and more (McIntosh & Vancov, 2010; Wan et al., 2011; Zhao *et al*., 2008).

Other chemical pretreatment methods

Other chemical pretreatment methods have been developed and used like organosolv, ozonolysis, and methods involving ionic liquids. In the organosolv process, organic solvents such as methanol, ethanol, acetone, and ethylene glycol are used along with acid catalysts to break the bonds between lignin and hemicellulose. Ionic liquid based methods are a relatively new method based on using ionic liquids that consists of salt ions that are efficient for dissolution of lignocellulosic materials (Menon & Rao, 2012; Sun & Cheng, 2002; Talebnia *et al*., 2010).

Physio-chemical pretreatment

Methods that combine both chemical and physical processes are referred to as physio-chemical processes.

Steam explosion

Steam explosion is the most commonly used physio-chemical method for the pretreatment of lignocellulosic biomass. High pressure and saturated steam is used to treat the biomass. The temperature is usually between 160-260°C and pressure from 0.69 to 4.83 MPa. After a certain amount of time the biomass is exposed to atmospheric pressure (Talebnia *et al*., 2010). The main advantage of this process is that less hazardous chemicals are used making it more environmental friendly than some of the other pretreatment methods. However, degradation of sugars and lignin causes the formation of inhibitory compounds which can inhibit microbial fermentation later in the process (Datar *et al*., 2007; Martín-Sampedro *et al*., 2011).

Ammonia fiber explosion (AFEX)

This method is similar to steam explosion except that is uses liquid ammonia at high temperatures and pressure followed by a rapid pressure reduction. This can significantly improve saccharification rates and has been used for various types of lignocellulosic biomass (Vlasenko *et al*., 1997). The main disadvantage of this method is that it is not particularly efficient for biomass with a high lignin content. The advantages are that the production of inhibitory products is minimal and sugar yield is relatively high (Mosier *et al*., 2005; Sun & Cheng, 2002).

Liquid-hot water

Unlike steam explosion and AFEX processes, the liquid-hot water method utilizes increased pressure so water stays in liquid state at elevated temperatures. The main goal is to solubilize hemicellulose to make cellulose more accessible and also to minimize the formation of inhibitors. In order to achieve this, the pH has to be between 4 and 7 (Hendriks & Zeeman, 2009; Mosier *et al*., 2005). This pretreatment method has several advantages: it is a chemical free process so there is no need for the neutralization of liquid streams and chemical conditioning and biomass size reduction is not needed and less amount of inhibitors are formed (Wan *et al*., 2011). There are three different types of reactors configurations that can be used when applying this method; flow-through, co-current and countercurrent. Research has shown that flow-through systems can remove more lignin and hemicellulose from some types of biomass as compared to batch systems (Hendriks & Zeeman, 2009).

Other physio-chemical pretreatment methods

Microwave-chemical pretreatment is a method which has proven to enhance enzymatic digestability of the biomass. The method has been used on rice-straw and is more effective than conventional heating (Ma et al., 2009). Other physio-chemical method is SO_2 added steam explosion of biomass which improves enzymatic hydrolysis of the solid fraction and increases recovery of hemicellulose sugars. Finally, CO_2 explosion of biomass increases the accessible surface area of substrate by an explosive release of CO_2 pressure (Chiaramonti *et al*., 2012).

Enzymatic hydrolysis

After the various pretreatment methods have been used, the biomass is usually separated to its three major components: lignin, cellulose, and hemicellulose. In most cases the lignin is removed but the two other fractions need to be hydrolyzed completely to their building blocks, mainly sugars. Therefore, enzymatic hydrolysis is usually performed after pretreatment of the biomass, a critical component of the lignocellulosic bioconversion process. Huge research interest has been on various enzyme systems in the past decades.

Cellulases and hemicellulases are important enzymes in this process. There are three types of cellulases as mentioned in chapter 2.2 (cellulose) and they all have to work together for a complete degradation

of cellulose. Each one functions differently. Endo-glucanases attack the middle of a low crystalline cellulose chain while exo-glucanases work from the end to create a cellobiose disaccharide. Finally, *β*-Glucosidases hydrolyze cellobiose in two free glucose molecules (Bayer *et al*., 1998).

Hemicellulases is another group of enzymes that degrade xylan and other parts of hemicellulose. Since hemicellulose is a much more complex polymer than cellulose the efficient degradation of the polymer requires the action of many enzymes working together synergistically (Shallom & Shoham, 2003). Hemicellulases are either glycoside hydrolases or carbohydrate esterases. The main enzyme groups are: Xylanases, β-mannanases, *α*-L-Arabinofuranosidases, *α*-D-Glucuronidases, *β*-Xylosidases, and hemicellulolytic Esterases. The enzymes belonging to each group then hydrolyze different parts of hemicellulose (Shallom & Shoham, 2003). Figures 10 and 11 demonstrate where different enzymes perform the cleavage.

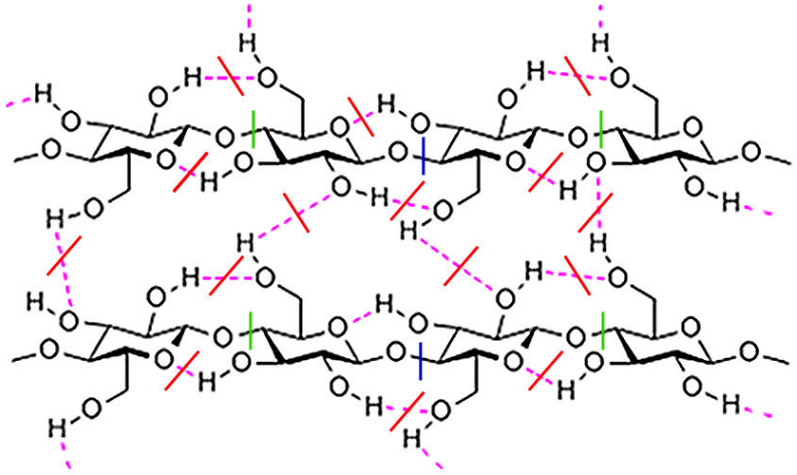

Figure 10. Demonstration of cellulose degradation with cellulases. Pink dashes represent hydrogen bonds. The colored lines indicate where different enzymes perform the cleavage. Red: Endo-gluconases; Blue: Exo-gluconases; Green: β-glucosidases.

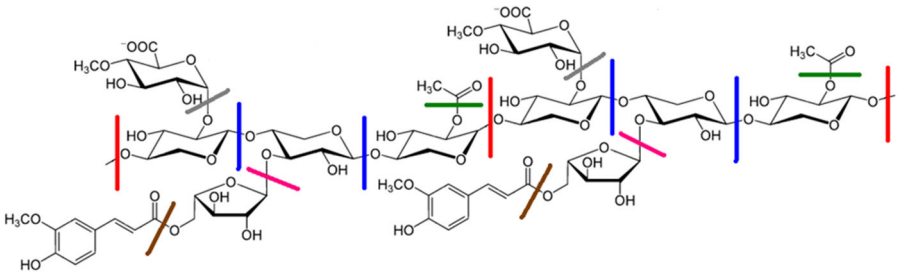

Figure 11. Demonstration of xylan degredation with hemicellulases. The colored lines indicate where different enzymes perform the cleavage. Red: Endo-β-1,4-xylanase; Grey: α-D-Glucuronidase; Pink: α-L-Arabinofuranosidase; Green: Acetyl-xylan-esterase; Blue: β-xylosidase.

Since enzymatic pretreatment is an expensive part of bioethanol production from lignocellulosic biomass, it is important to optimize the hydrolysis process to improve yield and hydrolysis rate. Both yield and rate of hydrolysis are mainly affected by substrate concentration. Recent experiments have focused on using enzymes from different sources and mixing them in appropriate proportions to optimize hydrolysis (Jin *et al*., 2009).

Biological pretreatment

Biological pretreatment is another option that utilizes wood degrading organisms like fungi (white-, brown- and soft-rot) and bacteria in order to change the structure of lignocellulosic biomass so that it becomes more accessible to enzymes. Cellulases are required for the degradation of cellulose and they are produced by fungi such as *Trichoderma reesei*, *Aspergillus niger* and *Clostridium thermocellum* as previously mentioned (Talebnia *et al.,* 2010). The fact that biological pretreatment is a chemical free method, and therefore an environmentally friendly, makes it a desirable pretreatment option. The energy input is also very low but the obvious drawback is that it is a very time consuming process and it requires a lot of space and control of growth conditions (Menon & Rao, 2012).

Inhibitory effects of pretreatment

The pretreatment of lignocellulosic biomass does not solely have positive effects on the fermentation process. An inevitable part of pretreatment is the formation of inhibitory compounds. These inhibitors may have negative effect on the growth of the fermenting microbes and tend to decrease ethanol and hydrogen productivity and yields, respectively (Palmqvist & Hahn-Hägerdal, 2000). The amount and type of toxic compounds formed depend on what kind of feedstock is used as well as operational conditions employed for hydrolysis (temperature, time, pressure and pH) (Taherzadeh *et al*., 2000). These toxic compounds that are formed during lignocellulosic pretreatment can be divided into four groups: sugar degradation products, lignin degradation products, compounds derived from lignocellulose structure, and heavy metal ions (Olsson & Hahn-Hägerdal, 1996).

When glucose and xylose are placed under high pressure and elevated temperatures they can be degraded into furfural and hydroxymethylfurfural compounds that can further be degraded into other toxic compounds. These two compounds negatively affect the cells specific growth rate and cell-mass yield. During lignocellulosic hydrolysis, a large variety of aromatic and phenolic compounds are released due to lignin degradation. Even in very low concentration, these compounds are very toxic; they affect the cells membranes and interrupt their enzyme matrices (Palmqvist & Hahn-Hägerdal, 2000). Other inhibitory compounds formed during pretreatment are acetic acid from acetyl groups present in hemicellulose and heavy metal ions that can originate from corrosion of hydrolysis equipment. The heavy metal ions affect various metabolic pathways (Olsson & Hahn-Hägerdal, 1996).

It is important to remove these inhibitory compounds or preventing them from forming to improve the efficiency of the fermentative process. There are four different ways to minimize the presence of these inhibitors in hydrolysates: avoiding the formation of inhibitors during pretreatment, detoxifying the hydrolysate before fermentation, developing inhibitor resistant microbes, or converting inhibitors into non-hibiting compounds (Taherzadeh *et al*., 2000). Every hydrolysate is different from others when it comes to raw materials and hydrolysis conditions. The composition has to be considered before choosing a detoxifying method.

2.4 Fermentation

There are three ways for organisms to obtain energy: photosynthesis, heterotrophy (from organic compounds), or lithotrophy (from inorganic chemicals). Chemically based energy is obtained by oxidizing compounds and it is conserved in the cell as the high-energy-compound ATP (Madigan *et al.*, 2003). Fermentation is a process where organic compounds such as carbohydrates are oxidized in the absence of oxygen and any other external electron acceptor and energy is derived from substrate level phosphorylation. Organic compounds such as alcohols and acids are formed as major end products. Fermentation starts with glycolysis (the Embden-Meyerhof pathway), which is a major biochemical pathway of glucose metabolism in both aerobic and anaerobic microorganisms (Perry *et al.*, 2002). Glycolysis can be divided into two main stages. Stage I involves no oxidation or reduction and is often called the preparation stage. The input of energy in the form of two ATPs is needed for molecule rearrangements. In stage II, the oxidation part of the pathway, six reactions lead to the formation of two molecules of pyruvate, 2 NADHs and 4 ATPs.

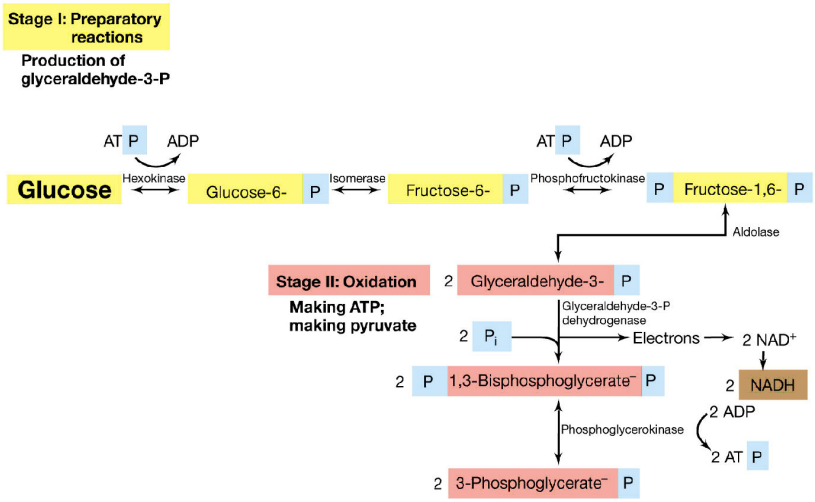

Figure 12. Stage I and II in glycolysis; Preparation and oxidation (Madigan et al., 2003).

After glycolysis, under anaerobic conditions, pyruvate can be reduced to several end products (stage III), like organic acids or alcohols along with CO_2 and H_2. The main reason for this reduction is the regeneration of NAD^+ from NADH. The oxidation state of the substrates and the end products will remain the same. Compared to respiration, fermentation

only yields 2 ATP molecules while respiration yields 38 ATP molecules (Madigan *et al*., 2003; Nelson & Cox, 2005).

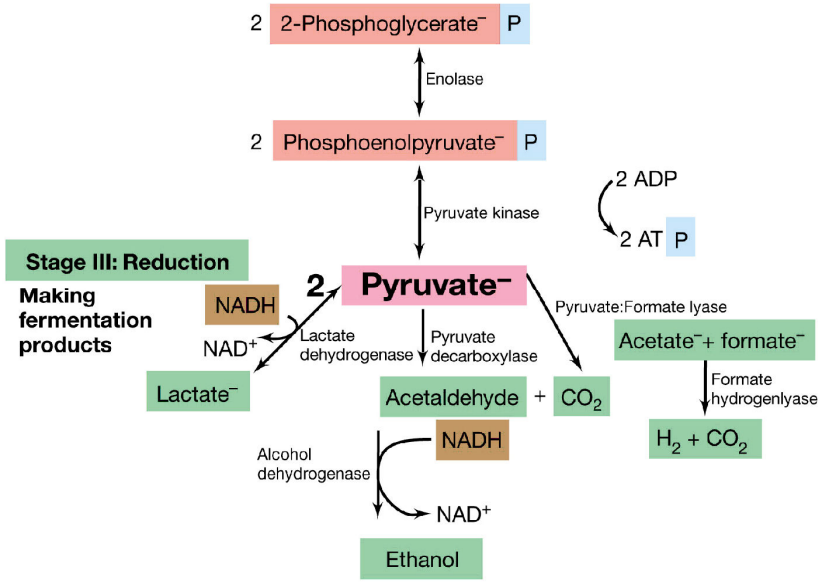

Figure 13. Stage III; Reduction. The formation of EtOH, H_2, CO_2 and organic acids.

There are a few bacteria that substitute classic glycolysis with the Entner-Doudoroff pathway, which is an alternative pathway that catabolizes glucose in pyruvate with different sets of enzymes. This pathway yields fewer energy carriers than glycolysis, or only 1 ATP, 1 NADH and 1 NAD^+ for every glucose molecule processed. The powerful EtOH producer *Zymomonas mobilis* is among bacteria using this metabolic pathway (Baratti & Bu'lock, 1986).

2.5 Production of bioethanol and biohydrogen

Bioethanol

Bioethanol is a renewable liquid fuel that has been used for decades and is the most common biofuel in the world. What makes EtOH different from traditional gasoline derived from fossil fuels is that it reduces hydrocarbon and carbon monoxide emissions due to improved combustion. It has a high octane number and vaporization temperature which makes it ideal to blend with regular gasoline (MacLean & Lave, 2003). The most common mixture is 10% ethanol and 90% gasoline (E10) but blends with higher EtOH concentration such as E25 and E85 are also used, e.g. for flexible-fuel vehicles that have specially designed engines (American Coalition for Ethanol, 2012). EtOH was first used in the 1880´s when the first ignition engine was invented by Nikolaus August Otto it wasn´t until 1925 that EtOH was first produced from biomass (i.e. bioethanol) and used as a fuel, mixed with gasoline in Brazil. After the World War II, gasoline prices dropped substantially and outcompeted bioethanol (Goettemoeller & Goettemoeller, 2007). The oil crisis along with low prices of sugar in the 1970´s pushed the Brazilian government for new solutions. The result was a policy to substitute gasoline with sugarcane alcohol which allowed the country to become the world´s largest producer. Today, Brazil is the number one exporter of bioethanol but US has emerged as the largest producer. This was the start of the National Program of Alcohol (PróAlcool) in 1975 where the main priority was the energy supply of the country (Soccol *et al*., 2010).

Throughout the years, the technology used for the production of bioethanol has relied on fermentation of sugars derived from feedstocks like corn (glucose) and sugarcane (sucrose) with traditional yeast (*Saccharomyces cerevisae*). That technology can be referred to as "first-generation bioethanol production". Second generation bioethanol production however relies on lignocellulosic agricultural biomass which are non-edible such as residues of food crop production and whole plants (Sánchez & Cardona, 2008).

The production of first generation bioethanol has been strongly criticized because of conflicts with food and feed supplies. People have been debating whether food crops such as corn, sugarcane, soybeans and palms should be used to make biofuel because agricultural land and water is limited. This debate is known as "food vs. fuel" debate (Nordhoff, 2007). Another debate concerning economic sustainability and energy efficiency of second generation bioethanol production has led to increased

interest in cyanobacteria and microalgae. These microorganisms have a number of advantages and can be utilized for bioethanol production. They grow to high cell densities and have high per-acre productivity. They also use non-productive land and can utilize a wide variety of water like seawater and wastewater (Asha *et al*., 2011).

The majority of the production in both Brazil and United States is first generation bioethanol, however, bioethanol production from lignocellulosic EtOH is increasing rapidly. Dozens of new companies are using new technologies to produce EtOH from waste products and agricultural residues and there are already a few commercial-scale facilities under construction (Renewable Fuels Association, 2012a). Figure 14 shows the five biggest producers in the world and their total production.

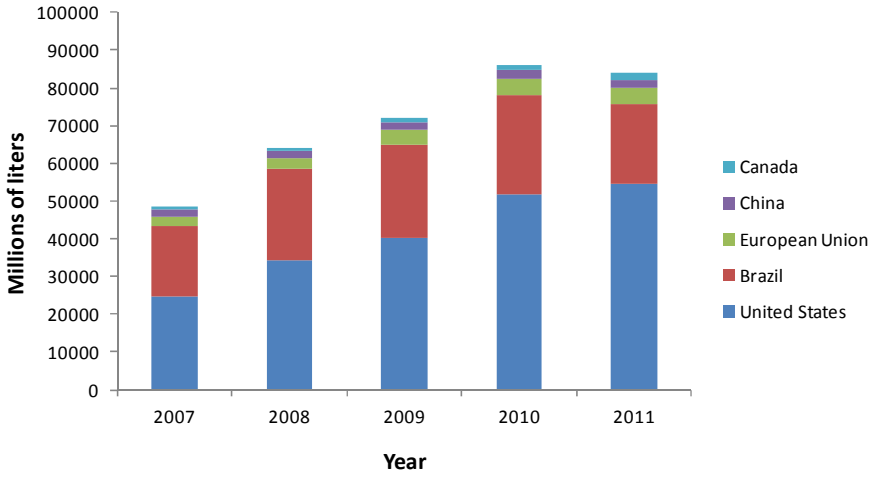

Figure 14. World's largest bioethanol producers from 2007 to 2011 (Data from Renewable Fuels Association, 2012b)

Status of second generation ethanol production world wide

The demand for cellulosic EtOH is increasing every year. United States Environmental Protection Agency (EPA) is responsible for developing regulations regarding transportation fuel that is sold in US. The Renewable Fuel Standard (RFS) program set an annual goal of 136 billion liters of renewable fuel EtOH by 2022. Estimation suggests that only 45-57 billion liters can be produced from simple biomass, thus the remaining 79 billion liters will need to be met by cellulosic ethanol (United States Environmental Protection Agency, 2012). To meet this requirement, it is

estimated that as many as 350 to 400 bio-refineries will have to be in operation by 2022 (POET, 2012b).

Today there are several pilot and demonstration cellulosic EtOH plants already operating. Most of these plants have been operating as research facilities in close cooperation with governmental or educational institutions are now expanding to a commercial level. Table 1 shows most of the facilities operating today as well as many of the commercial plants that are and/or have been under construction but will start production in the near term. Most of the production values are obtained from Menon & Rao (2012), European Biofuels Technology Platform (2012) as well as and from homepages of the companies.

Table 1. Demonstration, pilot and commercial facilities developed and under construction for lignocellulosic EtOH production world wide.

Company	**Location**	**EtOH year^{-1} (ML)**	**Status**	**Feedstock**	**Pretreatment technology**	**Process**
Iogen	Canada	2	Demo in 2004	Straw (Wheat, barley, oat)	Modified steam explosion	SHF
SEKAB	Sweden		Demo in 2004	Wood chips or sugarcane	Physio-chemical	SHF w/ yeast
POET	USA	208	Pilot in 2008 (commercial in 2013)	Corn stover and cob		
TMO Renewables	UK		Demo in 2008	Cassava stalk and more		Three stage conv. w/ thermophilic organism
Abengoa	Spain	5	Demo in 2009	Wheat straw	Physio-chemical (acid + steam explosion)	SSF w/ yeast
BioGasol	Denmark		Demo in 2004	Wheat straw and other agricultural residues	Wet explosion	SSCF with yeast + thermophilic anaerobic bacterium
Blue Sugars	USA		Demo in 2009	Various agricultural wastes	Thermal-mechanical	SSCF
Inbicon	Denmark	5.5	Demo in 2009	Wheat straw	Liquid hot water	SHF w/ yeast
Chempolis	Finland		Pilot in 2010	Straw, bagasse and more	Selective fractionation	SHF w/ yeast
Weyland	Norway	0.2	Pilot in 2010	Various agricultural wastes	Conc. acid	SHF
DuPont	USA	102	Commercial in 2012	Corn stover and cob, switchgrass and more	Mechanical/ thermochemical	SHF w/ *Z. mobilis*
Ineos	USA	30	Commercial in 2012	Vegetative waste	Physio-chemical	Gasification Fermentation
M&G	Italy	49	Commercial in 2012	Wheat straw	Steam and water	SSF
M&G	Brazil		Commercial in 2012	Sugarcane straw and bagasse	Steam and water	SSF w. yeast
Mascoma	USA	76	Commercial in 2012-2013	Hardwood		CBP w. yeast and bacteria
Abengoa	USA	94	Commercial in 2013-2014	Sugar cane straw and bagasse		SSF w/ yeast
Futurol	France	0.18	Future commercial scale	Various agricultural wastes		

The biorefining company Poet in the USA began producing cellulosic EtOH in 2008 at pilot scale at their research center. They are now in partnership with Royal DSM (The Netherlands) opening a commercial scale EtOH plant in Iowa that will start operating in late 2013. The feedstock will be corn residues (cob, leaves, husk and stalk) (POET, 2012a). The UK based biofuel production company TMO Renewables also started a pilot plant operation in 2008 producing bioethanol from a wide range of lignocellulosic biomass. Their newest addition is cassava stalk originating from China. Their aim is to produce 15 ML in 2015 (TMO Renewables, 2012). Their technology has attracted Chinese investors to develop their first commercial scale cellulosic EtOH which is expected to have the capacity of 38 ML. Biogasol in Denmark has been operating a demo plant since 2004. Their process features a thermochemcial pretreatment and a unique fermentation process with a patented thermophilic anaerobic bacterium which converts both C_5 and C_6 sugars to EtOH. Biogasol recently partnered with two companies, Sweetwater Energy Inc. for a new pretreatment technology, and Pacific Ethanol Inc. to build the West Coast Biorefinery located in Oregon, USA. The expected annual production is 10ML of EtOH (European Biofuels Technology Platform, 2012).

Chempolis in Finland produces EtOH from straw, bagasse and other agricultural feedstocks. They claim to have a unique pretreatment method that fractionates all the main components present in lignocellulose enabling them to be converted into high yields of EtOH. Their processes are designed to be self-sufficient and low in water consumption (Chempolis, 2012). DuPont have been operating a demo facility since 2008 in Tennessee (USA) but a new biorefinery in Iowa (USA) has been under construction and is estimated to produce more than 102 ML of EtOH annually. The production will start this year (2012) and a wide range of feedstock will be used (POET-DSM, 2012). The company Ineos has been operating a pilot plant since 2008. They have a unique pretreatment and fermentation technology based on a strain of bacteria isolated from compost heaps. Unlike most other cellulosic bioethanol facilites, they produce EtOH from syngas (CO and H_2) produced from organic matter by gasification with high heat and oxygen. The feedstock includes primarily vegetative waste (citrus and agricultural wastes) as well as yard and wood waste. The company is currently constructing a commercial biorefinery capable of producing 30 ML EtOH per year (European Biofuels Technology Platform, 2012). There are only two companies (Mascoma and Qteros) known to operate commercial scale plants of which EtOH is produced in a single step, consolidated bioprocessing (CBP) (see chapter 2.6). This process reduces cost dramatically (European Biofuels Technology Platform, 2012).

Among other companies (not listed in table 1) is Enerkem in Canada which currently has three commercial scale plants under construction, two in Canada and one in the United States. The feedstock that will be used is post-sorted municipal solid waste and sorted industrial, commercial and institutional waste. Each facility is expected to produce of 144 ML per year. Operations in at least one of those three plants are scheduled to start in early 2013 (Enerkem, 2012). Edeniq is another company that is launcing a new commercial biorefinery producing bioethanol from corn stover, switchgrass and woodchips in 2012 and Süd-Chemie AG in Germany is constructing the largest cellulosic bioethanol plant in the country with the estimated annual production of 127.000 L EtOH (European Biofuels Technology Platform, 2012).

Biohydrogen

Hydrogen is the most abundant and lightest element in the universe. It is found on earth in combinations with other molecules such as carbon, oxygen and nitrogen. H_2 is regarded as an ideal transportation fuel for the future because it is non-pollutant, eco-friendly, efficient and it has a high energy density (Pallavi & Anjana, 2011). There are a few different ways to produce H_2. The first commercial technology was based upon the electrolysis of water to produce pure H_2. It is not an ideal process since it requires high capital costs (International Energy Agency, 2012) and the input of electricity. H_2 can also be produced from fossil fuel resources; gas, coals and oil. Finally, H_2 can be produced biologically via photolysis, photofermentation, and dark fermentation (U.S. Department of Energy, 2011). Photolysis can either be indirect or direct and involves light-driven decomposition of water and micro-algae or cyanobacteria. Another possibility for H_2 production is the direct splitting of water by solar radiation (Nath & Das, 2004). There are two main advantages that biological H_2 production has over other processes. Firstly, renewable sources are utilized and secondly, very little or even no CO_2 is formed since most of it is fixed by autotrophic cell growth and energy metabolisms. Biological processes also require a lot less energy than chemical and electrochemical processes (Das *et al*., 2008; Nath & Das, 2004). This chapter only focuses on the production of H_2 with dark fermentation.

Dark fermentation

Hydrogen is a key compound in the metabolism of many anaerobic microorganisms. In the absence of external electron acceptors excess electrons generated can be disposed by reducing protons to H_2 by hydrogenase enzymes (Hallenbeck, 2005). Dark fermentation is a conversion of organic substrate to biohydrogen with fermentation. The name "dark" indicates that it occurs without the presence of light, unlike photofermentation. The process is complex and is carried out by a diverse group of bacteria. Dark fermentation is a promising method for H_2 production for a number of reasons. The production rate of H_2 is for example high compared to biophotolysis and photofermentation and a wide range of substrates can be used such as cheap agricultural residues and other waste products (Hallenback, 2008).

When glucose is degraded, strict anaerobes produce H_2 from two major pathways: from NAD(P)H by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and also from pyruvate ferredoxin oxidoreductase (PFOR). Hydrogen yields depend on the by-products formed in the fermentation process and also on the type of microorganism. Most mesophilic bacteria generate only 1 to 2 moles H_2 per mole glucose compared to a maximum yield of 4 moles H_2 per mole glucose with extremophilic bacteria. The reason for the low yields in mesophilic bacteria is that it is thermodynamically unfavorable to re-oxidize NADH to NAD^+ (Verhaart *et al*., 2010).

When glucose is degraded to acetate and CO_2, a maximum yield of H_2 of 4 moles H_2 per mole glucose can be obtained (Levin *et al*., 2004):

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COO^- + 4H_2 + 2HCO_3^- + 4H^+$$

If butyrate is the only end product, the theoretical yield of H_2 is only 2 moles H_2 per mole of glucose (Levin *et al.,* 2004):

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2CH_2COO^- + 2H_2 + 2HCO_3^- + 3H^+$$

If EtOH and lactate are the final fermentation products, no H_2 is generated at all (Koskinen *et al*., 2008b). Figure 15 shows a simplified breakdown of glucose in *Clostridium acetobutylicum*. The arrows indicate the reactions in each pathway.

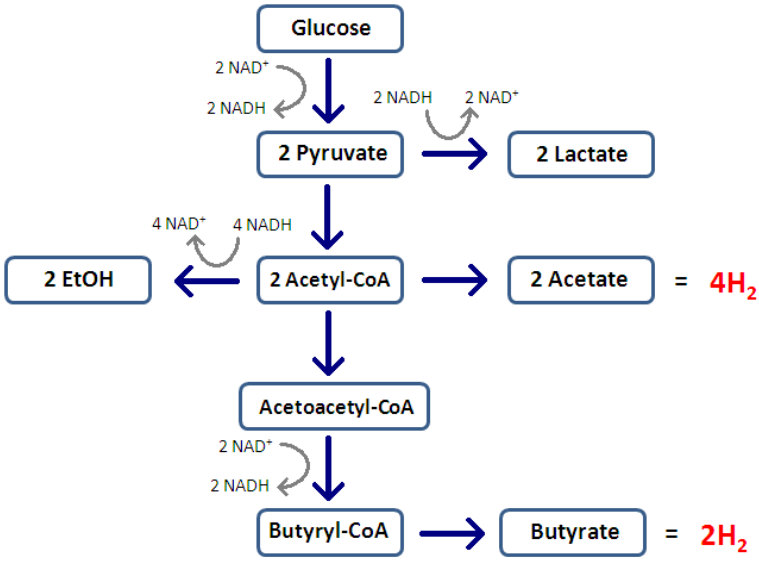

Figure 15. Anaerobic fermentation in Clostridium. The amount of H_2 produced along with each end product (adapted from Mathews & Wang, 2009).

Various aspects of H_2 production involving the partial pressure of H_2 and related thermodynamic aspects will be dicussed in detail in Manuscript I.

2.6 Bioethanol processes

There are mainly four different ways to convert lignocellulosic biomass into second generation biofuel products after pretreatment. The process configurations are referred to as separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF) and consolidated bioprocessing (CBP). These processes involve several different transformation steps; the production of saccharolytic enzymes, carbohydrate hydrolysis and the fermentation of both C_5 and C_6 sugars (Lynd *et al*., 2005). The difference between the processes is the combination of hydrolysis and fermentation. Figure 16 shows the integration of process steps in lignocellulosic EtOH production. Each process will be addressed in more detail in the following sub-chapters.

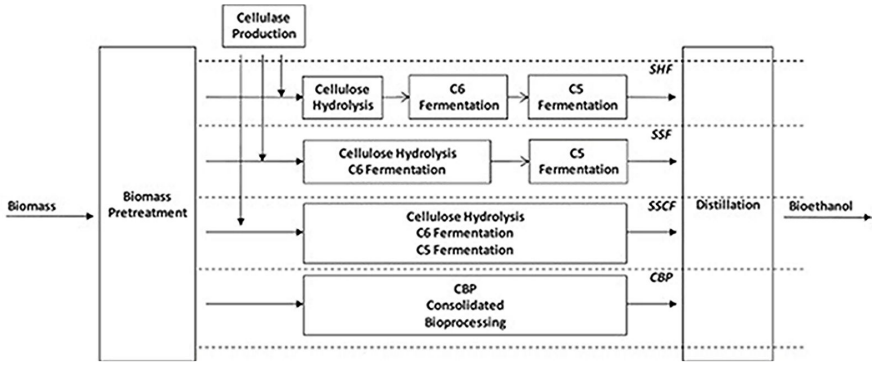

Figure 16. Integration of process steps in lignocellulosic ethanol production, (Chiaramonti et al., 2012).

SSF and SSCF

SSF is a process where enzymatic saccharification and fermentation take place in the same vessel. One reason why this process has been used is the avoidance of cellulase inhibition by glucose which can inhibit saccharification rate end EtOH yield. The challenge of this process however is the compatibility of the optimal conditions for the enzymatic activities and the microorganisms used for fermentation (pH, temperature and substrate concentration). Saccharification with cellulolytic enzymes is usually performed around 50°C, while most common bacteria and yeast used for the fermentation have optimal growth temperature between 28-37°C. That results in lower efficiency and lower product yield (Babiker *et*

al., 2010; Rao *et al*., 2012). This is one of the reasons why thermophilic bacteria have been proposed as interesting candidates for this production as previously mentioned. Studies on SSF with *S. cerevisae* have shown that the saccharification rate slows down at 35°C but the EtOH yield is higher at that temperature. Maximum saccharification was reached at 45°C but the rate of EtOH production was low at that temperature. Therefore, 40°C are sometimes chosen as an optimum in SSF when conventional yeast is used (Krishna *et al*., 1997). Due to the optimization problem, research has been aimed towards the development of thermotolerant yeast. Some thermotolerant strains have both been isolated and modified for the production of EtOH from biomass that can grow and produce EtOH at temperatures up to 48°C (Hasunuma & Kondo, 2012).

SSCF is a process very similar to SSF configurations except that it also includes pentose fermentation which is an essential part of efficient EtOH production from lignocellulosic biomass. This process along with SSF has been reported to obtain higher productivity, EtOH titer and yield when compared to SHF (Chang & Yao, 2011). Thermophilic bacteria are known for their ability to utilize C_5 sugars and would therefore make good candidates for SSCF process (Ahring *et al*., 1996). An example of SSCF utilizing both yeast and thermophilic bacterium is the Biogasol process mentioned earlier. There are also several natural xylose fermenting yeasts, such as *Pichia stipitis*, *Candida shehatae*, and *Pachysolen tannophilus* which may be of interest in the fermentation of hemicellulosic materials (Shupe & Liu, 2012).

SHF

SHF is a conventional two step process where in the first step enzymes are used to hydrolyze lignocellulose into sugars and in the second step the sugars are fermented to EtOH in another fermentor. The main advantage of this method is the fact that both hydrolysis and fermentation are performed at their optimum conditions. This could be crucial for the use of. thermophilic bacteria that have higher optimum temperature than what is needed for the enzymes in the hydrolysis step. The main limitation however is the accumulation of glucose during enzymatic hydrolysis that can strongly inhibit further cellulase activity (Chang & Yao, 2011).

A comparison of EtOH yields from wheat straw biomass using SSF and SHF with *Saccharomyces cerevisae* was done using steam explosion pretreatment. The results showed that by using SHF the EtOH yields were 81% of the theoretical yields but when using SSF that the yields of 68% of theoretical yields were achieved but the process time was much shorter (Alfani *et al*., 2000). This is opposite to the findings of Chang and Yao (2011) concerning the use of SSCF and it is clear that the complexicity of

the different types of biomass may be of huge importance and further experiments are needed to fully exploit both methods.

CBP

CBP is different from the above mentioned processes because it integrates enzyme production, saccharification and fermentation in a single process. The main idea behind the method is to lower cost and attain higher efficiency at the same time. The savings of using CBP can be huge since the enzyme cost in the other three processes can be very high (Olson *et al*., 2012). Since this process takes place in a single step, the choice of microorganism used has to be very carefully made. There are several requirements that this particular organism has to fulfill. It must be able to produce the enzymes needed for the degradation of both hemicellulose and cellulose as well as produce EtOH at high yield under industrial conditions (Lynd *et al*., 2005).

Since no wild type microbes with all the desired abilities have been found so far in nature, CBP requires highly engineered microbial strains that are capable of both hydrolyzation and high-titer EtOH production. There are two main strategies to produce that microbe, category I and II CBP. The CBP I pathway is a strategy where a microbe that has the native ability to degrade cellulosic biomass is used and then further engineered to become a powerful ethanol producer. The CBP category II however, is aimed at genetical engineering of a microbe that is a good EtOH producer but does not have the ability to produce the enzymes needed for the biomass breakdown (Amore & Faraco, 2012).

The candidates for category I CBP are fungi, free-enzyme bacteria or cellulosome-forming bacteria (Amore & Faraco, 2012; Olson *et al*., 2012). The thermophilic bacterium, *Clostridium thermocellum* has been looked at as a good category I CBP candidate due to its multienzyme complex cellulosome. However, this bacterium has several drawbacks such as the production of by-products like acetate and lactate and the lack of being able to degrade some of the C_5 sugars from hemicellulose like xylose. However, genetic manipulation has been successful. Argyros and co-workers were able to delete the genes encoding for lactate dehydrogenase (Ldh) and phosphotransacetylase (Pta) in *Clostridium thermocellum* that increased the EtOH titer and in co-culture with an organic-acid deficient strain of *Thermoanaerobacterium saccharolyticum* a fermentation of 92 g/L Avicel resulted in 38 g/L of EtOH (0.41 g/g avicel). This is the highest EtOH titer known so far produced by a cellulolytic thermophilic culture (Argyros *et al*., 2011).

Several different species of fungi have been proposed as possible CBP I candidates because of their ability to produce extracellular

cellulases in significant amounts. Fungal cellulases (endo-, exo- and β-glucosidases) work together synergistically for a complete cellulose degradation in the same way as bacterial cellulases. Filamentous fungi such as *Trichoderma*, *Aspergillus*, *Rhizobus,* and *Fusarium* have the ability to directly ferment cellulose to ethanol using two different metabolic routes. The first route involves the aerobic production of cellulases followed by an anaerobic fermentation where EtOH and other by-products such as acetate are produced (Amore & Faraco, 2012; Kumar *et al*., 2008).

Zymomonas mobilis, *Escherichia coli,* and *Klebsiella oxytoca* are among bacteria that have been proposed as good CBP II candidates. They are all good EtOH producers and have been genetically modified in attempt to express heterologous genes for enzyme production. However genetic engineering has shown to be a challenging process and some expression difficulties have occurred (laGrange *et al*., 2010). *Saccharomyces cerevisae* has a long history in first generation EtOH production and is an attractive CBP II candidate due to many advantages such as high EtOH tolerance and yield. The main disadvantage of this organism is the lack of enzymes systems needed for lignocellulosic biomass utilization. However, recent efforts to express heterologous cellulase genes have been pursued with success (see Hasunuma & Kondo, 2012, and references therein). *S. cerevisae* strain with two heterologous fungi cellulase genes has shown efficient hydrolysis of cellulosic materials with EtOH yields of over 80% of theoretical (Jeon *et al*., 2009). Another recombinant *S. cerevisae* strain produced EtOH from pretreated corn stover without the addition of exogenously produced enzymes (Khramtsov *et al*., 2011).

Table 2 shows the comparison of four possible CBP candidates; *S. cerevisae*, *Z. mobilis*, *C. thermocellum* and *T. reesei*. Two are naturally ethanologenic and the other two are naturally cellulolytic. The table summarizes the desired properties that a good CBP microorganism should have, and the qualities and the drawbacks of each candidate.

Table 2. Comparison of four possible CBP candidates (modified from Xu et al., 2009).

	Naturally ethanologenic		**Naturally cellulolytic**	
Candidate	**Yeast (*S. cerevisiae*)**	**Bacteria (*Z. mobilis*)**	**Bacteria (*C. thermocellum*)**	**Fungi (*T. reesei*)**
Cellulase genes	Heterologous expression of two fungi cellulases	Unknown	Naturally express cellulases in cellulosomes	Naturally produce several cellulases
Cellulase production	Heterologous expression of two fungi cellulases	Unknown	Produce a few grams per liter	Produce more than 100 g/L
Ethanol production	Up to 160 g/L of EtOH	Up to 130 g/L EtOH	Very slow rate and low yield	Very slow rate and low yield
Ethanol tolerance	Very high	High	Very low	Low
Multi-sugar usage in native strains	No	No	Do not utilize xylose	Yes
Resistance to inhibitors from HLs	High	High	Low	Very high
Amenability to genetic manipulation	Excellent	Good	Very poor	Good
Commercial acceptance	Very high	Acceptable	Unknown	Very high

CBP has great potential in reducing cost of lignocellulosic bioethanol production significantly. Some progress has been made in CBP microorganism development (both categories). It seems as though that the second category is a more difficult task than category I CBP because of enzyme system genetic engineering problems that still remain a challenge. That gives *C. thermocellum* an advantage because of its cellulosome which is crucial in CBP. If *C. thermocellum* is chosen as a CBP microorganism there are environmental factors that have to be considered that affect cellulosome activity. Xu and co-workers recently identified optimal enzymatic conditions for isolated cellulosome (from *C. thermocellum*) and also investigated cellulosome resistance towards inhibitors generated during biomass pretreatment. The results showed that the cellulosome activity was actually promoted by acetate, formate and lactate and also that the cellulosome has a higher EtOH tolerance and thermostability than a conventional *T. reesei* cellulase. Finally, cellulosomes showed resistance towards furfural (5 mM) and a few other known inhibitors present in hydrolysates (Xu *et al*., 2010).

2.7 Thermophiles

Microorganisms can be defined by their temperature optima and thus be divided into four groups; psycrophiles, mesophiles, thermophiles and hyperthermophiles. Thermophiles grow at temperatures between 45-80°C (Madigan *et al*., 2003) and have been isolated from different natural thermal environments such as geothermal areas (hot springs, solfatara fields, and acidic mud pots), hydrothermal vents and sub-surface environments. Thermophiles have also been isolated from different anthropogenic environments like factory effluents and in compost waste management (Wagner & Wiegel, 2008).

The reason for thermophiles being able to thrive at such high temperature areas are their thermostable proteins, enzymes and membranes. The cell membrane in thermophiles contains more saturated fatty acids than in mesophiles and psycrophiles. Enzymes in mesophilic organisms usually start to denature around 40°C and are completely inactive at 50-60°C. That makes thermophiles very interesting from an industrial perspective due to their potential application (Holst *et al*., 1997). One of the most used enzymes in biotechnology today is a thermostable DNA polymerase from the bacteria *Thermus aquaticus* that was isolated from a hot spring in Yellowstone National Park, USA (Huber & Stetter, 1998). Thermophiles grow at higher temperatures which gives them several advantages compared to mesophiles in large scale applications. Chemical reactions occur faster due to thermodynamic laws. Thermophiles also often tolerate environmental changes such as pH and temperature fluctuation much better than conventional yeasts and microbial contamination is not a significant problem (Taylor *et al*., 2009).

More than 300 species of thermophilic anaerobes have been described (Wagner & Wiegel, 2008). Thermophilic anaerobic bacteria have gained interest as possible candidates in bioethanol and biohydrogen production due to many qualities they possess. They are commonly able to ferment both C_5 and C_6 sugars and some of them have the ability to produce enzymes to degrade hemicellulose and cellulose (Lynd *et al*., 2008). It is assumed that thermophilic anaerobes have similar properties to those of the early evolutionary life forms on earth when significantly less oxygen was present (Canfield *et al*., 1996).

2.8 Production of hydrogen and ethanol with thermophilic bacteria isolated from hot springs in Iceland

Geothermal areas in Iceland

Iceland is located on the Mid-Atlantic Ridge where two tectonic plates are moving away from each other, making the country one of the largest geothermal areas in the world. Iceland has numerous different geothermal features such as volcanoes, hot springs, geysers, mud pots and fumaroles. Geothermal areas cover in total more than 500 km^2 of the country (0.5% of total surface) and they can be subcategorized in high and low temperature fields (Hreggvidsson & Kristjansson, 2003).

High temperature fields are located within active volcanic zones along the tectonic plate boundary and heat is provided by magma chambers at the depth of 2-5 km. These areas are dominated by sulfuric, clayish hot springs and fumaroles. Very little water is usually present at these areas, but if there is any, it is very acidic (pH 2-4) with high concentrations of dissolved inorganic chemicals. Low temperature fields are located outside these volcanic zones and have large volumes of clear water pools present (20-100°C). The water is alkaline (pH 8-10) and silica deposits can be found along the edges of the pools and hot springs. Vegetation near the water and green algal biomats are typical for low temperature areas (Axelsson *et al.,* 2010). Figure 17 shows the distribution of high- and low-temperature fields in the country.

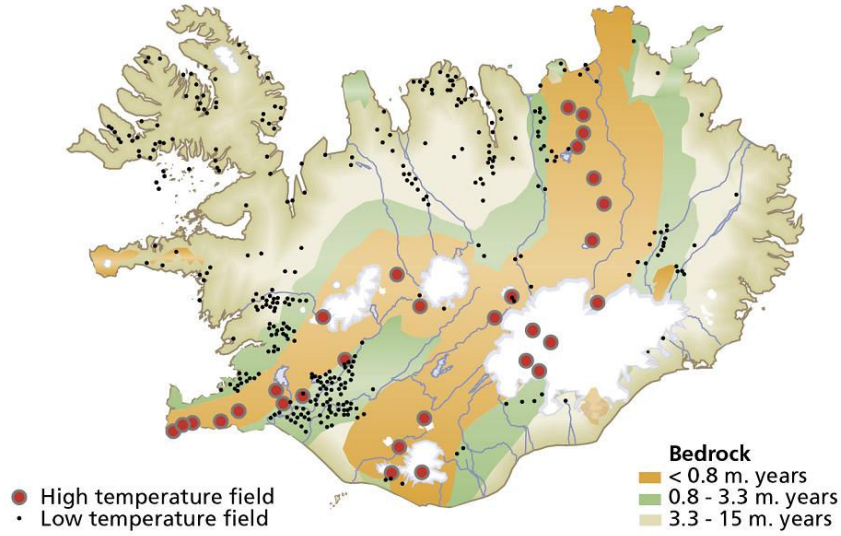

Figure 17. A map of Iceland´s geothermal fields(Orkustofnun, 2012)

The geothermal areas vary greatly in both physical and chemical composition, thus leading to a variety of microbes in these habitats. In the low temperature alkaline hot springs (45-50°C) the phototrophic microbial mats are dominated by the cyanobacteria *Mastigocladus laminosus* or *Phormidium laminosum*. The biomats also contain large number of phototrophic bacteria such as *Cloroflexus* species. Beneath the photosynthetic layer are anaerobic bacteria fermenting the decaying mat (Skirnisdottir *et al.,* 2000; Kristjansson & Alfredsson, 1986). At higher temperatures (60-80°C) species belonging to the family Aquificales are dominating. Hydrogen oxidizing, sulphate reducing and methane producing bacteria have also been isolated from these areas (Kristjansson & Alfredsson, 1986; Marteinsson *et al*., 2004; Vesteinsdottir *et al*., 2011a, 2011b).

Grensdalur

Hot springs are potential environments for the isolation of bacteria that are capable of producing both H_2 and EtOH. The study performed in present investigation was done on several strains isolated from a sampling trip in 2007 from the area Grensdalur which is a part of the Hengill area in the southwest region of Iceland. This geothermal area is known for its broad variety of hot springs with different temperatures, pH, and mineral composition because it is on the boundaries of a high- and low temperature zone. High temperature zones in Iceland can be divided geologically into 36 different types of geothermal phenomena and 15 of

them can be found in Grensdalur (Marteinsson *et al*., 2004). Figure 18 shows different types of geothermal features in Grensdalur. Below is a detailed description of EtOH and H_2 producing strains that have been isolated from Grensdalur and from other geothermal areas in Iceland.

Mud and water with grey and red deposits around (68°C – pH 3.9)

Hot spring run off. Clear water surrounded with vegetation (50°C – pH 7.7)

A clear hot spring with large amount of water and vegetation near edges (88°C – pH 9.7)

Mud pot (80°C – pH 5.7)

Figure 18. Different geothermal features in Grensdalur. Pictures taken in a sampling trip conducted in 2007.

Ethanol producing strains

Thermoanaerobacter mathranii

The earliest investigations on EtOH producing bacteria isolated from Icelandic hot springs are from studies of Ahring and co-workers on *Thermoanaerobacter mathranii* and related bacteria (Ahring *et al*., 1996). This bacterium was isolated from sediment in a slightly alkaline hot spring in Grensdalur area. The bacterium was fully characterized in 1997 (Larsen *et al*., 1997). The cells are gram-variable, straight, rod-shaped and spore-forming. The bacterium can utilize various energy sources and the fermentation end products are ethanol, lactate, acetate, CO_2 and H_2. The optimal conditions for growth are between 70 and 75°C with pH 7.0 (Larsen *et al*., 1997). Since 1997, many studies of the ethanol production capacity of this strain and other related strains, both from pure sugars as well as from hydrolysates made from various lignocellulosic biomass have been done. The bacterium has also been genetically modified to improve EtOH production.

EtOH production from wet oxidized (WO) wheat straw hydrolysates has been investigated using *T. mathranii* (strain A3M1). Different combinations of oxygen pressure, sodium carbohydrate were applied to see the effects on hemicellulose solubilization. Further treatment with acid (H_2SO_4) and enzymes (Celluclast®) was needed to improve EtOH yields from the HLs. The highest EtOH yield achieved was 0.8 g/L (Ahring *et al*., 1999). Higher acid concentration during pretreatment led to lower EtOH yield most likely due to formation of inhibitory compounds (Ahring *et al*., 1999). The following research was aimed at the effects of potential inhibitors on *T. mathranii* (strain A3M4) from wet oxidized wheat straw. The results showed that numerous different inhibitory compounds were present in the HL but at low concentrations and their presence did not reduced EtOH yields by *T. mathranii*. However, when the concentration of these compounds was increased and the HL as well, EtOH production of *T. mathranii* was severely inhibited (Klinke *et al*., 2001).

The first genetic modification performed on the strain was the deletion of lactate dehydrogenase gene to eliminate NADH oxidation pathway (strain BG1L1). To further facilitate NADH regeneration, a heterologous gene (*gldA*) coding for NAD^+-dependent glycerol dehydrogenase was expressed in *T.mathranii*. That resulted in a recombinant strain, BG1G1 which showed increased EtOH yields in the presence of glycerol with xylose as a substrate. Acetate production decreased as well, and shifted towards the production of EtOH to restore redox balance (Yao & Mikkelsen, 2010b). Another strain (BG1E1) was

created by overexpressing one of four alcohol dehydrogenases present in *T. mathranii*. Analysis revealed that NAD(H)-dependent bifunctional aldehyde/alcohol dehydrogenase (AdhE) is particularly important for EtOH production because it is responsible for acetyl-CoA reduction to acetaldehyde in the EtOH formation pathway. This overexpression resulted in increased EtOH yields from xylose (Yao & Mikkelsen, 2010a).

Several other experiments have been performed on strain BG1L1. The strain displays a high tolerance of exogenously added EtOH compared to other thermophilic anaerobic bacteria. BG1L1 can tolerate 8.3% of EtOH and still show a relatively high and stable and EtOH production (Georgieva et al., 2007a). *Thermoanaerobacter* BG1L1 is also a promising EtOH producer from lignocellulosic biomass such as corn stover and wheat straw hydrolysates. When grown on corn stover hydrolysate the sugar efficiency proved to be very high. Xylose was nearly completely utilized (89-98%) compared to 68-76% overall sugar efficiency when grown on wheat straw HL. In both cases the EtOH yield ranged from 0.39-0.42 g/g sugars and a significant resistance of HL occurred when concentrations were increased (Georgieva & Ahring, 2007; Georgieva *et al*., 2008).

Several other strains besides from *T. mathranii* have been isolated by Ahring and coworkers and experiments concerning EtOH tolerance and EtOH production from hydrolysates have been performed (Sommer *et al*., 2004; Georgieva *et al*., 2007b).

Ethanol producing strains of the University of Akureyri

Since 2005, the University of Akureyri (UNAK) has focused on bioprospecting of EtOH producing bacteria. Earliest investigations were published in 2007 when four isolated thermophilic bacteria were phylogenetically investigated but the main emphasis was on H_2 production (Orlygsson & Baldursson, 2007). Two of the strains, AK_1 and AK_{17} were however actually very good EtOH producers. Table 3 shows the most efficient EtOH producing strains that have been isolated so far, site of isolation, their optimal growth conditions, end product formation and phylogenetic relationship. Another sampling trip to Grensdalur was done in 2007 resulting in more than 60 isolations of various EtOH and H_2 producing bacteria from 30 hot springs (Orlygsson *et al*., 2010). Those isolations were made on various substrates, ranging from simple sugars (glucose and xylose) to cellulose and other polymeric carbohydrates at temperatures ranging from 50°C to 75°C. The main outcome from this study was the fact that at low and moderately low temperatures, the majority of the bacteria isolated were good EtOH producers and belong to the genera of *Thermoanaerobacterium*, *Clostridium* and *Caloramator*. At

higher temperatures (70 and 75°C), most isolates were phylogenetically characterized as *Thermoanaerobacter* and *Caldicellulosiruptor*, and main end product formation was towards acetate and H_2. Finally, a sampling trip was conducted in 2009 with the main aim to isolate high temperature tolerant strains. This led to isolation of several interesting isolates, e.g. AK_5 and J_1, discussed below. Figures 19, 20 and 21 are electron microscopy pictures of strains AK_1, AK_{15} and AK_{17}.

Table 3. EtOH producing strains isolated by researchers at the University of Akureyri

Strain	**Sampling sites**	**Isolation year**	**T_{opt} (°C)**	**pH_{opt}**	**Fermentation end products**	**Genus**
AK_1	Grensdalur	2005	45	6.5	EtOH, acetate, H_2, CO_2	*Clostridium*
AK_5	Grensdalur	2009	65	7.0	EtOH, acetate, H_2, CO_2	*Thermoanaero-bacter*
AK_{15}	Viti	2005	60	7.0	EtOH, acetate, lactate, H_2, CO_2	*Thermoanaero-bacter*
AK_{17}	Viti	2005	58-60	6	EtOH, acetate, H_2, CO_2	*Thermoanaero-bacterium*
AK_{54}	Grensdalur	2007	65	5.0-6.0	EtOH, acetate, lactate, H_2, CO_2	*Thermoanaero-bacterium*
J_1	Grensdalur	2009	65	7.0	EtOH, acetate, H_2, CO_2	*Thermoanaero-bacter*

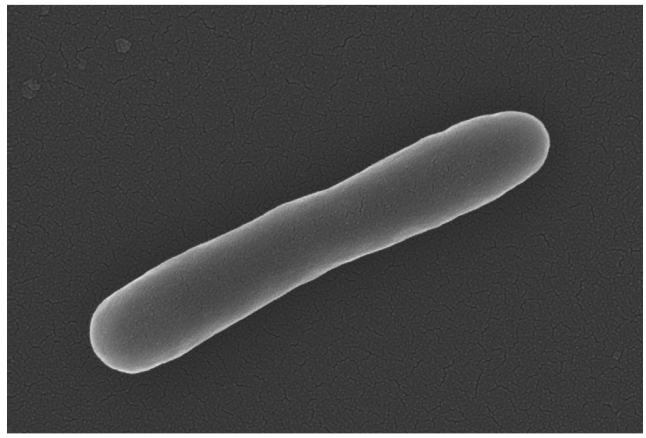

Figure 19. Electron microscopy picture of AK_1.

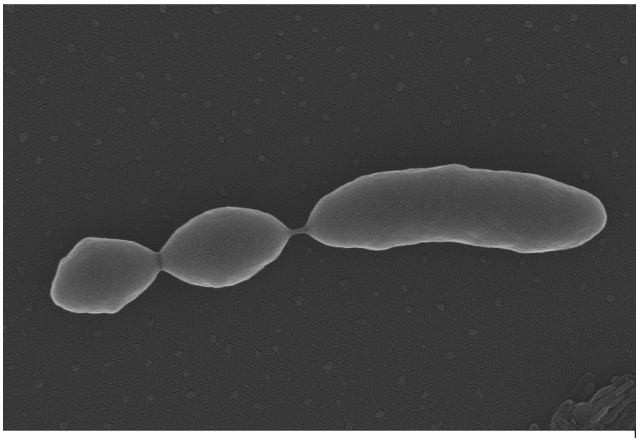

Figure 20. Electron microscopy picture of AK_{15}.

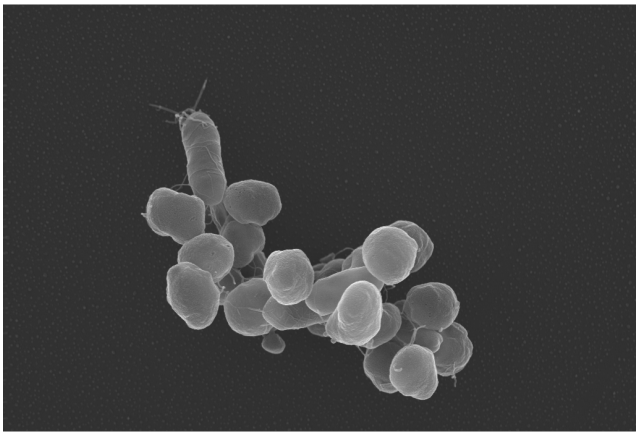

Figure 21. Electron microscopy picture of AK_{17}.

Of the six isolates described in Table 3 there is a great variation in the extent of experiments performed (Almarsdottir *et al*., 2012; Brynjarsdottir *et al*., 2012; Jessen & Orlygsson, 2012; Koskinen *et al*., 2008a; Orlygsson, 2012; Orlygsson & Bakken, 2010; Orlygsson & Baldursson, 2007; Sigurbjornsdottir & Orlygsson, 2012). The investigation of these strains includes experiments on substrate utilization, EtOH tolerance, and the effects of various factors on EtOH yield, such as initial substrate concentration, partial pressure of H_2 and the addition of external electron acceptor and co-culturing with hydrogenotrophic methanogens. Special attention was on EtOH production capacities on hydrolysates from lignocellulosic biomass (cellulose, hemp, straw, grass, paper and sawdust).

These experiments showed that the strains can utilize a variety of carbon sources such as xylose, mannose, galactose, fructose, lactose, pectin, starch, xylan and more. All strains are very sensitive to increased substrate concentration, often severely inhibited at 20 to 30 mM initial glucose concentrations. Exception from this was observed from strain J_1 which completely degrades glucose at concentration up to 100 mM. When tested, the strains tolerate between 1.6 to 3.2% (v/v of exogenously added EtOH in medium (Sveinsdottir *et al*., 2009; Koskinen *et al*., 2008)

A shift in end product formation occurred during several different culture conditions, e.g. by increasing substrate concentration, adding extracellular electron acceptors (thiosulfate) into the medium, and by co-culturing them with hydrogenotrophic methanogens or by directly changing the partial pressure of hydrogen by using different liquid to gas phases in the culture bottles. Most often, EtOH yields could be increased by increasing partial pressure of H_2 or decreased by using external electron acceptors/co-cultures.

Fermentation experiments on different types of pretreated hydrolysates showed that these strains are promising EtOH producers from lignocellulosic biomass. Some experiments concerning the effects of different environmental factors in the pretreatment process were performed, but only on strain AK_{17}. The results showed that little effects were observed in EtOH yields when different concentrations of acid and alkali were used for the pretreatment of cellulose and grass, but changes in enzyme concentration had more pronounced effects, especially on cellulose. Inhibitory experiments with furfural and hydroxymethylfurfural, well known inhibitory compounds on microbial growth, during glucose fermentation revealed that AK_{17} has similar tolerance as most other known thermoanaerobes. Table 4 shows EtOH yield for the six strains when grown on glucose, xylose, cellulose and grass. Yield values on cellulose and grass shown in the table are the highest ones obtained in all experiments.

Table 4. EtOH yield on sugars and lignocellulosic biomass

	Glucose		**Xylose**		**Cellulose**	**Grass**
	mol/mol glu	**mM/g**	**mol/mol xyl**	**mM/g**	**mM/g**	**mM/g**
AK_1	1.5	8.3	0.8	4.5	7.4	3.1
AK_5	1.7	9.4	1.4	7.5	7.7	4.3
AK_{17}	1.5	8.3	1.1	6.1	8.6	5.5
AK_{54}	1.0	5.7	0.6	3.2	3.4	3.3
J_1	1.7	9.4	1.3	7.0	7.5	3.3

Theoretical yield of EtOH from hexose fermentation is 2 mol EtOH/mol sugar, or 11.1 mM/g assuming all the carbon ends up in EtOH and CO_2. This is however never the case with thermophilic bacteria, since some of the carbon ends up in other volatile products, mostly acetate as earlier mentioned (chapter 2.4 - fermentation). Strains AK_5 and J_1 show the highest EtOH yield obtained from glucose of all five strains, or 9.4 mM/g glucose. That is not surprising since they are closely related to *T. thermohydrosulfuricus* and *T. ethanolicus*, both of which are excellent EtOH producers from glucose and xylose (Kannan & Mutharasan, 1986; Lovitt *et al*., 1988). Strain AK_5 also displays the highest EtOH yield on xylose, 7.5 mM/g xylose. AK_{17} displays the highest yields obtained on cellulose and grass. AK_{54} has considerably lower EtOH yields on glucose, xylose and cellulose than the other strains which can be explained by more acetate and H_2 production. The overall yields on cellulose are similar to the glucose yields indicating that the cellulose was almost completely hydrolyzed into glucose. Grass however is a much more complex biomass than cellulose, with wider variety of sugars and other compounds formed when pretreated.

Thus, there seems to be a wide variety of EtOH producing strains from Icelandic hot springs. Strains belonging to the genera of *Clostridium*, *Thermoanaerobacter* and *Thermoanaerobacterium* have been isolated and both phylogenetically and physiologically investigated. The main outcome of these investigations is that it seems to be a common trend among these bacteria that they have very broad substrate spectrum which is an important factor concerning the use of microbes for second generation EtOH production. Also, it is possible to direct their end product formation by regulating culture conditions towards higher concentrations of EtOH. One strain, AK_{17}, has been genetically modified to obtain higher EtOH yields. Genes responsible for both acetate and lactate production have been successfully knocked out resulting in significantly higher EtOH production (Hreggvidsson, 2012).

The main disadvantage of using these microbes is the fact that they are inhibited by relatively low concentrations of both sugars and lignocellulosic HL. Additionally, the relatively low EtOH tolerance might be problematic for scale up processes. Both these factors might however be minimized by using fed-batch or continuous cultures.

Hydrogen producing strains

A few H_2 producing strains have been isolated from Icelandic hot springs. Ahring and co-workers isolated the first three strains; *Caldicellulosiruptor kristjanssonii*, *Caldicellulosiruptor lactoacedicus* and *Caldicellulosiruptor acetigenus* (Mladenovska *et al*., 1995; Bredholt *et al*., 1999; Nielsen *et al*., 1993). In 2006 *C. acetigenus* was reclassified from *Thermoanaerobium acetigenum* based on 16S rRNA sequence analysis and re-examination of physiological properties of the type strain (Onyenwoke *et al*., 2006). The strains have been moderately investigated concerning physiological properties, however a complete genome sequencing has been done for *C. kristjanssonii* and *C. lactoaceticus* and five other strains from the same genus (Blumer-Schuette *et al*., 2011). Despite the lack of research concerning H_2 production on these strains, it is known that within the genus *Caldicellulosiruptor*, some of the best H_2 producers are found. A closely related strain, *C. saccharolyticus* utilizes a wide range of carbon sources including xylose, arabinose, pectin and xylan which are all abundant in lignocellulosic biomass (Rainey *et al*., 1994). This strain has also showed good H_2 yields when grown on pretreated lignocellulosic biomass (Ivanova *et al*., 2009). Continuous H_2 production from glucose and xylose with *C. kristjanssonii* in both pure culture and co-culture with *C. saccharolyticus* has been investigated. The results showed that *C. kristjanssonii* has a very similar performance as *C. saccharolyticus* but together these strains obtained almost maximum theoretical yield (3.8 mol/mol C_6 sugar equivalent), utilizing glucose and xylose simultaneously. The yield was much higher than with each strain individually suggesting specific synergy between the strains (Zeidan & Niel, 2009).

Several H_2 producing strains have been isolated by a research group at the University of Akureyri but most of them are combined EtOH and H_2 producers which have gained attention in recent years. AK_{54}, mentioned in a previous chapter, is one of those strains. This strain produces 1.8 mol H_2/mol glucose (45% of theoretical yield) and 1.96 mol H_2/mol glucose equivalents from cellulose but from lignocellulosic hydrolysates, the yields ranged from 0.34 to 1.47mol H_2/mol glucose equivalent (except for unpretreated hemp leaf which resulted in no H_2 production at all) (Sigurbjornsdottir & Orlygsson, 2012). AK_{14} is a moderate thermophile that belongs to the genus *Clostridium* and was isolated from a sample taken in the 2005 sampling trip. Experiments performed on the strain have shown that it utilizes a variety of carbohydrates and performs typical acetate-butyrate fermentation fermentation (Almarsdottir *et al*., 2010). Both initial substrate concentration and the gas/liquid ratio during fermentation can

dramatically affect H_2 production yields. Maximum H_2 production from glucose was 2.6 mol H_2/mol glucose and 0.2 to 1.2 mol H_2/mol glucose equivalent from various lignocellulosic hydrolysates which is a bit lower but reasonable compared to other strains within the same genus (see table 5 in Manuscript I) (Almarsdottir *et al*., 2010).

Both AK_{54} and AK_{14} have good potential to become efficient H_2 producers from lignocellulosic biomass. More research needs to be done to completely optimize the critical environmental factors that affect the H_2 yield in the fermentation process. To obtain even higher H_2 yields, genetical modification for elimination of undesired end products.

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4 Manuscript I

Ethanol and hydrogen production with thermophilic bacteria from sugars and complex biomass

Ethanol and hydrogen production with thermophilic bacteria from sugars and complex biomass

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Introduction

The increase in carbon dioxide (CO_2) emissions has clearly much more profound effects on global climate than earlier anticipated. The main source of CO_2 is by combustion of fossil fuel but its concentration has increased from 355 ppm in 1990 to 391 ppm in 2011 (Mauna Loa Observatory: NOAA-ASRL, 2011). Production of biofuels from biomass has emerged as a realistic possibility to reduce fossil fuel use and scientists have increasingly searched for new economically feasible ways to produce biofuels. The term biofuel is defined as fuel produced from biomass that has been cultivated for a very short time; the opposite of fuel that is derived from fossil fuel biomass (Demirbas, 2009). Plants and autotrophic microorganisms fix gaseous CO_2 into volatile (sugars) and solid compounds (lignocellulose, starch) during growth. These compounds can thereafter be converted to biofuels which, by combustion, releases CO_2 back to atmosphere. This simplified way of carbon flow is not completely true, because growing, cultivating, harvesting and process conversion to biofuels will, in almost all cases, add more CO_2 to atmosphere although less as compared to fossil fuels.

There are several types of biofuels produced and used worldwide today. The most common are methane, ethanol (EtOH) and biodiesel but also, to a lesser extent, hydrogen (H_2), butanol and propanol. There are also several methods to produce biofuels, ranging from direct oil extraction from fat-rich plants or animal fat (biodiesel) to complex fermentations of various types of carbohydrate rich biomass (H_2, EtOH, butanol). Fermentation processes can be perforemed by both bacteria and yeasts. This overview mainly focuses on the production of EtOH and H_2 from biomass with thermophilic bacteria.

Plants and photoautotrophic microorganisms fix gaseous CO_2 into volatile (sugars) and solid compounds (lignocellulose, starch) during growth. These compounds can thereafter be converted to biofuels which, by combustion, releases CO_2 back to atmosphere.

Production of EtOH and H_2 from biomass

EtOH as a vechicle fuel originated in 1908 when Henry Ford's famous car, Ford Model T was running on gasoline and EtOH or a combination of both (Gottemoeller & Gottemoeller, 2007). Biomass was however not used as a source for EtOH production until in the early thirties of the 20th century when Brazil started to extract sugar from sugarcane for EtOH production. During the World War II, EtOH production peaked at 77 million liters in Brazil (mixed to gasoline at 42%) (Nardon & Aten, 2008). After the war, cheap oil outcompeted the use of EtOH and it was not until the oil crisis in the mid 70's that interest in EtOH rose again. The program "Pro-Alcool" was launched in 1975 to favour EtOH production from sugarcane. In US, there has been a steady increase in EtOH production from starch based plant material, e.g. corn, since the late 1970's (Nass et al., 2007). Perhaps the main reason for the increase in EtOH production is the discovery that methyl *tert*-butyl ether (MTBE), earlier used in gasoline as an additive, was contaminating groundwater, leading to search for alternative and more environmentally friendly source (Vedenov & Wetzsstein, 2008). Today, US and Brazil produce more than 65.3 billion liters of EtOH which corresponds for 89% of the world production (Renewable Fuel Association, 2010).

Production of EtOH from lignocellulose rich biomass has recently been focused upon. The main reason is the fact that EtOH production from starch and sugar based biomasses is in direct competition with food and feed production. This has been criticized extensively lately, because of the resulting rise in the prizes of food and feed products (Cha & Bae, 2011). Production of EtOH from sugars and starch is called first generation production, opposite to second generation production where lignocellulosic biomass is used. Lignocellulose is composed of complex biopolymers (lignin, cellulose and hemicelluloses) that are tightly bound together in plants. The composition of these polymers varies in different plants (cellulose, 36-61%; hemicellulose, 13-39%; lignin 6-29%) (Olsson & Hahn-Hagerdal, 1996). Of these polymers, only cellulose and hemicelluloses can be used for EtOH production. However, before fermentation, the polymers need to be separated by physiological, chemical or biological methods (Alvira et al., 2010). The most common method is to use chemical pretreatment, either weak acids or bases but many other methods are known and used today (see Alvira et al., 2010 and references therein). This extra pretreatment step has been one of the major factor for the fact that EtOH production from complex biomass has not been commercialized to any extent yet compared to first generation

ethanol production. Also, after hydrolysis, expensive enzymes are needed to convert the polymers to monosugars which can only then be fermented to EtOH. Conventionally, most of the EtOH produced today is first generation EtOH but lately, especially after US launched their large scale investment programs (US Department of Energy, 2007), second generation of EtOH seems to becoming a reality within the next few years or decades.

The sugars available for fermentation after the pretreatement and hydrolysis of biomass (when needed) can be either homogenous like sucrose and glucose from sugarcane, and starch, respectively or heterogenous when originating from lignocellulosic biomass. Thus, the main bulk of biomass used for EtOH production today are two types of sugars, the disaccharide sucrose and the monosugar glucose, both of whom can easily be fermented to EtOH by the traditional baker's yeast, *Saccharomyces cerevisae*. This microorgansism has many advantages over other known EtOH producing microorganisms. The most important are high EtOH yields (>1.9 mol EtOH/mol hexose), EtOH tolerance (> 12%), high robustness and high resistance to toxic inhibitors. However, the wild type yeast does not degrade any pentoses (Jeffries, 2006). The use of genetic engineering to express foreign genes associated with xylose and arabinose catabolism have been done with some success (van Maris et al., 2007) and a new industrial strain with xylose and arabinose genes was recently described (Sanchez et al., 2010). Also, no yeast has been reported to have cellulase or hemicellulase activity. The mesophilic bacterium *Zymomonas mobilis* is a highly efficient EtOH producer. The bacterium is homoethanolgenic, tolerates up to 12% EtOH and grows 2.5 times faster compared to yeasts (Rogers et al., 1982). The bacterium utilizes the Entner-Doudoroff pathway with slightly higher EtOH yields than yeasts but lacks the pentose degrading enzymes. Many attempts have however been made to insert arabinose and xylose degrading genes in this bacterium (Deanda et al., 1996; Zhang et al., 1995). The company DuPont has recently started to use a genetically engineered *Z. mobilis* for cellulosic EtOH production (DuPont Danisko Cellulosic Ethanol LLC, 2011).

Especially, the lack of being able to utilize arabinose and xylose, both major components in the hemicellulosic fraction of lignocelluloses, has lead to increased interest in using other bacteria with broader substrate spectrum. Bacteria often possess this ability and are capable of degrading pentoses, hexoses, disaccharides and in some cases even polymers like cellulose, pectin and xylans (Lee et al., 1993; Rainey et al., 1994). The main drawback of using such bacteria is their lower EtOH tolerance and lower yields because of production of other fermentation end products like acetate, butyrate, lactate and alanine (Baskaran et al.,

1995; Klapatch et al., 1994; Taylor et al. 2008). Additionally, most bacteria seem to tolerate much lower substrate concentrations although the use of fed batch or continuous culture may minimize that problem. On the opposite however, many bacteria show good EtOH production rates. The use of thermophilic microorgansims has especially gained increased interest recently. The main reasons are, as previously mentioned, high growth rates but also less contamination risk as well as using bacteria that can grow at temperatures where "self distillation" is possible, thus eliminating low EtOH tolerance and high substrate concentration problems. Also, the possibiltiy to use bacteria with the capacity to hydrolyse lignocellulosic biomass and ferment the resulting sugars to EtOH simultaneously is a promising method for EtOH production.

The production of H_2 is possible in several ways but today the main source of H_2 is from fossil fuels and, to a lesser extent, by electrolysis from water. H_2 is an interesting energy carrier and its combustion, opposite to carbon fuels, does not lead to emission of CO_2. Biological production of H_2 is possible through photosynthetic or fermentative processes (Levin et al., 2004; Rupprecht et al., 2006). This chapter will focus on biological H_2 production by dark fermentation by thermophilic bacteria only. Fermentative production of H_2 has been known for a long time and has the advantage over photosynthetic processes of simple operation and high production rates (Chong et al., 2009). Also, many types of organic material, e.g. wastes, can be used as substrates. Thus, its production possesses the use of waste for the production of renewable energy. H_2 production has though not been commercialized yet but several pilot scale plants have been started (Lee & Chung, 2010; Lin et al., 2010).

Physiology of thermophilic EtOH and H_2 producing bacteria

Thermophilic bacteria can degrade many carbohydrates and produce various end products, among them both EtOH and H_2. Figure 1 shows the carbon flow from glucose by fermentation by the use of Embden-Meyerhof pathway (EMP). The majority of microorganisms degrade hexoses through this pathay or the Entner-Douderoff pathway (ED). The degradation of glucose with EMP generates two NADH, two pyruvates, the key intermediate in most organisms, together with the formation of two ATP by substrate level phosphorylation. The ED pathway, however, is more restricted to Gram-negative bacteria and Archaea and generates only one mol of ATP, which explains its low distribution among anaerobic bacteria. Some bacteria, especially hyperthermophiles, are known to be able to use both pathways simultaneously (Moat et al., 2002; Siebers & Schönheit, 2005).

There are also some variations of the classical EMP among thermophilic microorganisms. Some archaea e.g. *Pyrococcus* and *Thermococcus* use ADP instead of ATP to transfer phosphate groups to hexoses in the preparation steps of the glycolysis. These bacteria also use ferredoxin-dependent glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR) for converting glyceraldehyde-3-phosphate to 3-phosphoglycerate in one step (Chou et al., 2008). Thermophilic bacteria, however, use the common glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and reduce glyceraldehydes-3-phosphate to 1,3-glycerate which is thereafter converted to 3-phosphoglycerate. Thus, both groups produce two molecules of ATP by substrate level phosphorylation but the archaea “sacrifice” one and use it to together with two molecules of AMP to produce two molecules of ADP, needed for hexose phosphorylation. Consequently, the amount of energy conserved in glucose to acetate conversion is 3.2 instead of the expected 4.0 ATP/glucose (Sapra et al., 2003).

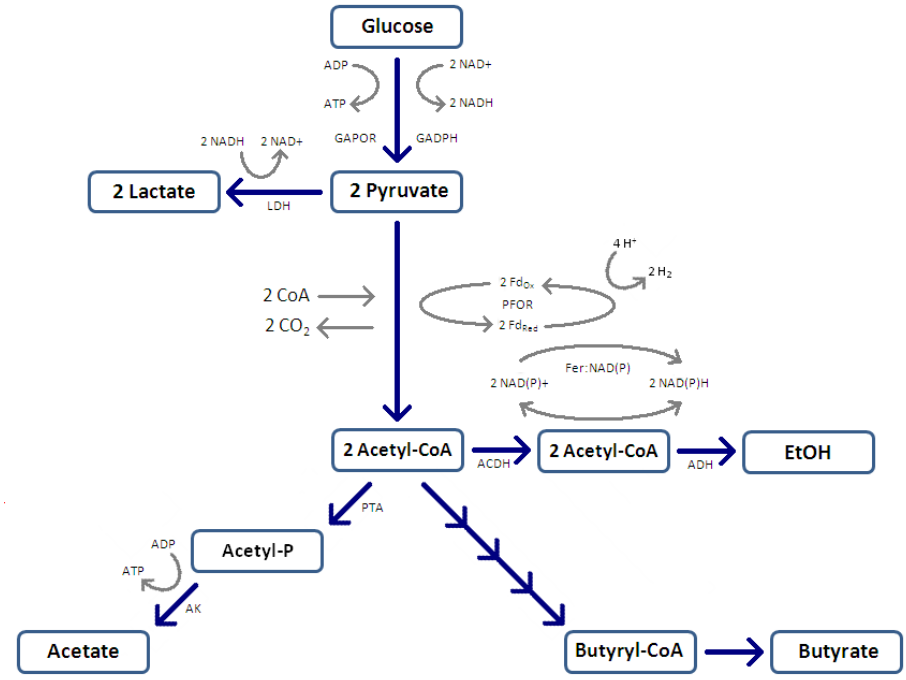

Figure 1. Simplified scheme of glucose degradation to various end products by strict anaerobic bacteria. Enzyme abbreviations: ACDH, acetaldehyde dehydrogenase; ADH, alcohol dehydrogenase; AK, aectate kinase; Fer:NAD(P), ferredoxin:NAD(P) oxidoreductase; H_2-ase, hydrogenase; LDH, lactate dehydrogenase; PFOR, pyruvate:ferredoxin oxidoreductase; PTA, phosphotransacetylase.

Pyruvate is the end product of glycolysis and can be converted to fermentation products like H_2, EtOH and many more (Fig. 1). The carbon flow depends on the microorganisms involved and the environmental conditions. Pyruvate can e.g be reduced to lactate by lactate dehydrogenase (LDH) but the most favourable pathway for anaerobic bacteria is to oxidize pyruvate to acetyl-CoA and CO_2 by using pyruvate:ferredoxin oxidoreductase (PFOR) which can be converted to acetate with concommittent ATP synthesis from the acetyl-phosphate intermediate. Acetate is thus the oxidized product but the main advantage for the microorgansism is the extra ATP produced. The electrons are transported to reduced ferredoxin which acts as an electron donor for hydrogenases and H_2 is produced as the reduced product. There are mainly two types of hydrogenases; NiFe hydrogenases and the FeFe hydrogenases. Recent overview articles have been published on the subject (Chou et al., 2008; Kengen et al., 2009). Acetyl Coenzyme A can

also be converted to acetaldehyde by acetaldehyde dehydrogenase (ACDH) and further to EtOH by alcohol dehydrogenase.

Strict anaerobes can produce H_2 from two major breakpoints during degradation of glucose. Firstly, from a NAD(P)H by GAPDH and from pyruvate ferredoxin oxidoreductase (PFOR) (Jones, 2008). The principal H_2 pathway is through PFOR because of thermodynamics hinderance of reoxidizing NADH (Jones, 2008). It is a well known phenomenon that the low H_2 yields observed by mesophilic and moderate thermophilic bacteria are due to the fact that H_2 production from either ferredoxin or NAD(P)H are thermodynamically unfavourable (Jones, 2008; Hallenbeck, 2009). The redox potential of Fd_{red}/Fe_{ox} couple depends on the microorganism and temperature involved. In nature, high partial pressures of H_2 are relatively uncommon because of the activity of H_2 scavenging microbes, e.g. methanogens or sulfate reducing bacteria (Cord-Ruwisch et al., 1988). This results in a low partial pressure of H_2 which is favourable for a complete oxidation of glucose to acetate and CO_2. At high temperatures, the influence of the partial pressure of H_2 is less on the key enzymes responsible for H_2 production. This is the main reason why extremophilic bacteria have been reported to produce up to 4 moles of H_2 together with 2 moles of acetate in pure cultures and also for the fact that microorganisms growing at lower temperatures direct their end product formation to other reduced products. At lower temperatures, the NADH ferrodoxin oxidoreductase (NOR) that converts NADH to Fd_{red} is strongly inhibited. The E° is – 400 mV for Fd_{red}/Fd_{ox} couple but -320 mV for the $NADH/NAD^+$ couple (Jones, 2008; Hallenbeck, 2009). Therefore, at low temperatures, elevated H_2 concentrations inhibits H_2 evolution at much lower concentrations as compared to extreme temperatures. Mesophilic and moderate thermophilic bacteria respond to this by directing their reducing equivalents to other more favourable electron acceptors and consequently produce reduced products like EtOH, lactate, butyrate and alanine (Fig. 1).

Following are the main stoichiometry equations for the degradation of glucose to various end products by microorganisms with special focus on H_2 and EtOH production.

The amount of H_2 produced depend on the fermentation pathways used and end product formation. For example, if acetic acid is the final product the theoretical yield for one mole of glucose is four moles of H_2:

$$C_6H_{12}O_6 + 4\ H_2O \rightarrow 2CH_3COO^- + 4H_2 + 2HCO_3^- + 4H^+ \qquad (1)$$

If on the other hand the final product is butyric acid, the theoretical yield of H_2 is only two moles of H_2 per mole of glucose:

$$C_6H_{12}O_6 + 2\ H_2O \rightarrow CH_3CH_2CH_2COO^- + 2H_2 + 2HCO_3^- + 3H^+ \quad (2)$$

The production of EtOH by *Saccharomyces cerevisae* and *Zymomonas mobilis* occurs according to:

$$C_6H_{12}O_6 + 4\ H_2O \rightarrow 2CH_3COH^- + 2HCO_3^- + 4H^+ \quad (3)$$

Bacteria however, usually produce a mixture of EtOH together with other end products. This results in lower EtOH yields and, in some cases, production of H_2. If lactate is the only end product, no H_2 is formed:

$$C_6H_{12}O_6 + 4\ H_2O \rightarrow \text{Lactate}^- + 2HCO_3^- + 4H^+ \quad (4)$$

Thermophilic anaerobic bacteria – classification and physiology

In recent years, thermophilic anaerobic bacteria have gained increased attention as potential EtOH and H_2 producing microorganisms. Depending on optimal growth temperatures, thermophilic bacteria can be divided into several categories, e.g. moderate thermophiles (T_{opt} between en 45 to 55°C, true thermophiles (T_{opt} between 55 to 75°C) and extremophiles with optimum temperature above 75°C (Brock, 1986). The ability of thermophiles to live at high temperatures is mainly due to their thermostable proteins; the cell membrane of thermophilic bacteria contains more saturated fatty acids which make it stiffer and more heat resistant as compared to mesophiles (Brock, 1986).

Thermophilic bacteria are capable of adapting to environmental conditions and are able to thrive in geothermal areas although the temperature might be slightly higher than the optimum growth temperature. Geothermal areas offer stability in heat and are thus favorable habitats for thermophilic bacteria (Brock, 1986; Kristjansson & Alfredsson, 1986). Generally, most known thermophilic species are obligate or facultative anaerobes since geothermal areas have low oxygen concentrations (Amend & Shock, 2001). Less variety seems to be of strict anaerobic, heterotrophic thermophilic bacteria (see review of Wagner & Wiegel, 2008 and references therein).

Thermophilic EtOH and H_2 producing bacteria

There are relatively few genera of thermophiles that include bacteria with good H_2 and EtOH producing capacities. Among good EtOH producers are bacteria that belong to the genera of *Clostridium, Thermoanaerobacter* and *Thermoanaerobacterium* but good H_2 producers are the extremophiles like *Caldicellulosiruptor* and *Thermotoga* and the archaeon *Thermococcus* and *Pyrococcus*. It varies to a great extent how much data is available in literature concerning pure culture studies of individual species on biofuel production. Much data is not on the efficiency of these bacteria to produce H_2 and EtOH but merely on phylogenetic status and basic physiological properties. Also, the data on biofuel production properties from these bacteria on hydrolysates from lignocellulosic biomass is scarce but more is known on yields from monosugars. Below, the discussion will be on the major phylogenetic and

physiological characteristics of most of the "good" EtOH and H_2 producing thermophiles known today. Later chapters deal with H_2 and EtOH production rates and yields from both sugars and from complex lignocellulosic biomasses by these bacteria and more.

Clostridium

The genus *Clostridium* belongs to the family Clostridiaceae, order Clostridiales, class Clostridia and phylum Firmicutes. These bacteria are spore forming and often present in environments which are rich in plant decaying material. It is thus not surprising that many species are capable of polymer hydrolyzation and this is one of the main reasons for extensive research on biofuel production from complex biomass by these bacteria (Canganella & Wiegel, 1993; Carreira & Ljungdahl, 1993). Several cellulose-degrading enzymes form a structure called cellulosome, located and embedded on the external surface of the cell membrane (Demain et al., 2005). The genus contains a very diverse group of bacteria as shown by a phylogenetic analysis of Collins and co-workers where *Clostridium* species were compared both within species belonging to the genus and to related taxa (Collins, et al., 1994). This investigation and others lead to the conclusion that more than half of the species currently assigned to the genus *Clostridium* are in fact not closely related to the type species *C. butyricum* and should therefore not be included in the newly defined genus *Clostridium*. The genus contains more than 200 validly described species but only about 15 are thermophilic. Two of those thermophilic Clostridia, *C. thermocellum* and *C. thermohydrosulfuricum* (now *Thermoanaerobacter thermohydrosulfuricum*) have attracted the most attention and the cellulosome of *C. thermocellum* has been characterized extensively (Demain et al., 2005). Among other well known thermophilic Clostridia are *C. thermobutyricum* (Wiegel et al., 1989), *C. thermosucciongenes* (Drent et al., 1991) and *C. clariflavum* (Shiratori et al., 2009) and several others.

Thermoanaerobacterium

Thermoanaerobacterium together with genus *Thermoanaerobacter* falls within clusters V, VI and VII in phylogenetic interrelationships of *Clostridium* species (Collins et al., 1994). The genus was first described in 1993 when two thermophilic, xylan degrading strains were isolated from Frying Pan Springs in Yellowstone National Park (Lee et al., 1993). They were compared with other xylan degrading bacteria and new taxonomic assignments were proposed thereafter. Today the genus

consists of nine validly described species; *T. aciditolerans*, *T. aotearoense*, *T. saccharolyticum*, *T. thermosaccharolyticum*, *T. thermosulfurigenes*, *T. xylanolyticum, T. fijiensis, T. polysaccharolyticum and T. zeae* (German Collection of Microorganisms and Cell Cultures and references therein). Most *Thermoanaerobacterium* species have been isolated from hot springs or leachate of waste from canning factories. *Thermoanaerobacterium* species are known for their abilities to convert carbohydrates to various end products like acetate, EtOH, lactate, H_2 and CO_2. Some species have shown promising EtOH and H_2 production capacity but production of mixed end products limit their use (Ren et al., 2008; 2009; 2010; Romano et al., 2010; Sveinsdottir et al., 2010). *T. saccharolyticum* has however been genetically engineered and both acetate and lactate formation has been knocked out (Shaw et al., 2008). According to the description, members of this genus reduce thiosulfate to elemental sulfur while members of *Thermoanaerobacter* reduce thiosulfate to H_2S (Lee et al., 1993).

Thermoanaerobacter

Bacteria within this genus were originally classified within the genus *Clostridium* because of close phylogenetic relationship and physiological properties. These bacteria use the classical EMP pathway for sugar degradation and produce EtOH, acetate and lactate as major end products (Lee et al., 1993). Most species have broad substrate range and can degrade both pentoses and hexoses. The genus consists of 24 species (subspecies included) originating from various environments like hot springs and oil fields (Collins et al., 1994; Larsen et al., 1997; Lee et al., 1993; German Collection of Microorganisms and Cell Cultures and references therein). Most species produce EtOH and H_2 as well as lactate, and in some cases alanine as end products. The type species, *Thermoanerobacter ethanolicus* and several other species within the genus has been extensively studied for EtOH production (Fardeau et al., 1996; Georgieva & Ahring, 2007; Georgieva et al., 2008a. b; Lacis & Laword 1988a,b; Lamed & Zeikus, 1980a,b). H_2 production is usually low compared to EtOH by *Thermoanerobacter* although *Thermoanaerobacter tengcongensis* has been described to produce up to 4 moles of H_2 from one mole of glucose under nitrogen flushed fermentor systems (Soboh et al., 2004).

Caldicellulosiruptor

The genus *Caldicellulosiruptor* was first proposed in 1994 by Rainey and co-workers on the basis of physiological characteristics and phylogenetic position of a strain they isolated, *Caldicellulosiruptor saccharolyticus* (Tp8T 6331) (Rainey et al., 1995). Today the genus holds nine different species; *C. acetigenus*, *C. bescii*, *C. hydrothermalis*, *C. kristjanssonii*, *C. kronotskyensis*, *C. lactoaceticus*, *C. obsidiansis*, *C. owensensis* and *C. saccharolyticus* (German Collection of Microorganisms and Cell Cultures and referenses therein**)**. All species are extremely thermophilic, cellulolytic, non-spore-forming anaerobes that have been isolated from geothermal environments such as hot springs and lake sediments (Rainey et al., 1994; Yang et al., 2010). *Caldicellulosiruptor* species have a relatively broad substrate spectrum capable to utilize e.g. cellulose, cellobiose, xylan and xylose. Extreme thermophiles, have been shown to have superior H_2 production yields and rates compared to mesophiles and produce few other byproduct besides acetate. This makes *Caldicellulosiruptor* species excellent candidates for H_2 production. *C. saccharolyticus* and *C. owensis* have been extensively studied for H_2 production from sugar and hydrolysates from lignocellulosic biomass (Kadar et al., 2004; Vrije et al., 2007; Zeidan & van Niel, 2010).

Thermotoga

The genus of *Thermotoga* was first described in 1986 when a unique extremely thermophilic bacteria was isolated from geothermally heated sea floors in Italy and the Azores (Huber et al., 1986). Today, nine different species have been identified; *T. elfii*, *T. hyphogea*, *T. lettingae*, *T. maritima* (type species), *T. naphthophila*, *T. neapolitana*, *T. petrophila*, *T. subterranean* and *T. thermarum* **(**German Collection of Microorganisms and Cell Cultures and references therein**)**. These species are extremophiles, growing at temperatures that are highest reported for bacteria. All are strictly anaerobic and the cells are rod-shaped with an outer sheethlike structure called toga. (Huber et al., 1986; Jannasch et al., 1988). Most species have been isolated from deep environments, high temperature and pressure environments like oil reservoirs, often rich of sulfur-compounds. Most of them are thus able to reduce either elemental sulfur, thiosulfate or both. Members of *Thermotoga* ferment sugars to mainly acetate, CO_2 and H_2 like *Caldicellulosiruptor* species. Only three species have been reported producing traces of EtOH. Most strains have shown the property of reducing pyruvate to alanine from sugar fermentation and *T. lettingae* produces alanine from methanol (in the presence of elemental sulfur or thiosulfate) (Balk et al., 2002). Other

special features within the genus is the ability of *T. lettingae* to degrade xylan at 90°C and its property of methanol metabolism (Balk et al., 2002). Hydrogen production has been extensively studied for *T. elfi*, *T. maritima* and *T. neapolitana* (d'Ippolito et al., 2010; Nguyen et al., 2008a,b; van Niel et al., 2002).

Other thermophilic bacteria producing H_2 and EtOH

Apart from the above mentioned genera the capacity to produce EtOH and H_2 has been reported for many other genera. Examples are species within *Caloramator, Caldanaerobacter, Caldanerobius* and the archaeon *Thermococcus* and *Pyrococcus*. Some species within these genera will be discussed in later chapters.

Production of EtOH by thermophilic bacteria

The interest in EtOH production by thermophilic bacteria originates shortly after the oil crisis in the mid 70‘s of the twentieth century. Earliest reports on EtOH production from sugars include work on *Thermoanaerobacter brockii* and *Clostridium thermocellum* (Ben Bassat et al., 1981; Lamed et al., 1980; Lamed & Zeikus, 1980a, 1980b) but later on other *Thermoanaerobacter* species, e.g. *T. finnii,* (Faredau et al., 1996), *T. thermohydrosulfuricus* (Lovitt et al., 1984; Lovitt et al., 1988), *T. mathrani* (Larsen et al., 1997) and *Thermoanaerobacterium* species (Koskinen et al., 2008a; Sveinsdottir et al., 2009; Zhao et al., 2009, 2010). It was however not until recently that the use of thermophilic bacteria for EtOH production from lignocellulosic biomass arises. The earliest reports on EtOH production of more complex nature are from 1981 on starch (Ben Bassat et al., 1981) and 1988 on avicel (Lamed et al., 1988). The first study on lignocellulosic biomass (hemicellulose fraction of birch- and beechwood) 1983 by *Thermoanaerobacter ethanolicus* and several other thermophilic bacteria (Wiegel et al., 1983). Following chapters are divided into two main subchapters; 1) studies of sugar degradation both in batch and continuous cultures with either pure or cocultures of thermophilic bacteria and 2) studies of EtOH production from lignocellulosic biomass by mixed or pure cultures of thermophilic bacteria.

Production of EtOH from sugars

Although it has been known for a long time that thermophilic bacteria produce EtOH from various carbohydrates it was not until 1980 the first papers appeared in literature with the focus on EtOH production. Earlier investigations include work on *Thermoanaerobacter brockii, Thermoanaerobacter thermohydrosulfuricus* and *Clostridium thermocellum* (Ben Bassat et al., 1981; Lamed & Zeikus, 1980a; 1980b; Lovitt et al., 1984). Ethanol yields by *T. brockii* were only moderate or between 0.38 (Lamed & Zeikus, 1980b) to 0.44 mol EtOH mol glucose^{-1} equivalents (Ben Bassat et al., 1981). In the latter investigation the focus was mostly on the effects of additional acetone and H_2 on end product formation. Much higher yields were later observed by *Thermoanaerobacter thermohydrosulfuricus*, or 0.9 to 1.9 mol EtOH mol glucose^{-1}. (Lovitt et al., 1984; 1988), also with the main focus on the effect of solvents on EtOH production, e.g. EtOH tolerance.

Thermoanerobacter ethanolicus was described in 1981 (Wiegel & Ljungdahl., 1981) showing extremely good yields of ethanol from glucose (1.9 mol EtOH mol glucose^{-1}). Later this strain has been extensively studied by Lacis and Lawford more than 20 years ago (Lacis and Lawford 1988a, 1988b, 1989, 1991). Early observation was on high EtOH yields on xylose at low substrate (4.0 g L^{-1}) concentrations. The yields were 1.30 and 1.37 mol EtOH mol xylose^{-1} in batch and continuous cultures, respectively (Lacis & Lawford, 1988a) but only at low substrate concentrations. At higher concentrations (27.5 g L^{-1}) the yields lowered to 0.6 mol EtOH mol xylose^{-1}. Further studies by using xylose limiting continuous cultures, indicated that EtOH yields were more dependent on length of cultivation than upon growth rate and higher yields were presented (1.43 mol mol xylose^{-1}) (Lacis & Lawford, 1988b, 1989). Later data from this strain on glucose showed lower EtOH yields and the direction of the carbon flow was towards lactate formation by increasing substrate concentrations (Lacis & Lawford, 1991). *Thermoanerobacter ethanolicus* JW200 showed also very good EtOH yields from xylose and glucose at low (10 g L^{-1}) substrate concentrations, or 1.45 and 1.95 mol, respectively (Carreira et al., 1982). A mutant strain was later developed (JW200Fe(4)) that showed similar yields but at higher (30 g L^{-1}) substrate concentrations (Carreira et al., 1983). Other investigations on this species on sucrose showed between 1.76 to 3.60 mol EtOH mol sucrose^{-1} with high substrate concentrations (15 to 30 g L^{-1}) (Avci et al., 2006). Recent study on *Thermoanerobacter ethanolicus* strain interestingly shows that the addition of external acetate increases EtOH yields from xylose, glucose and cellobiose (He et al., 2010). EtOH yields on xylose were 1.0 mol EtOH mol glucose^{-1} without any acetate added but increased to 1.17 by adding 150 mM of acetate. Similar increase was observed on glucose, or from 1.16 to 1.34 mol EtOH mol glucose^{-1} without and with added acetate, respectively. It has been suggested that acetate may disrupt energy production through accelerated fermentation (Russel, 1992) which may lead to lower biomass production and higher end product formation. Fardeau et al. (1996) investigated the effect of thiosulfate as electron acceptor on sugar degradation and end product formation by *Thermoanaerobacter finnii*. This strain shows good EtOH yields on xylose or 1.76 mol EtOH mol xylose^{-1} which is actually higher than the theoretical yield (1.67) from this sugar. Yields on glucose were however lower or, 1.45 mol EtOH mol glucose^{-1}. Not surprisingly, the addition of thiosulfate shifted end product formation towards acetate with higher cell yield and lower EtOH production. A study of bacteria isolated from Icelandic hot spring shows that a *Thermoanerobacter* sp. AK33 showed good EtOH yields on monosugars (Sveinsdottir et al., 2009). Glucose and xylose fermentations resulted in 1.5 and 0.8 mol EtOH from one mole of

glucose and xylose, respectively. *Thermoanaerobacterium* AK_{17}, isolated from Icelandic hot spring, has been extensively studied for EtOH production (Koskinen et al., 2008a; Orlygsson & Baldursson, 2007; Sveinsdottir et al., 2009). This strain produces 1.5 and 1.1 mol EtOH from one mole of glucose and xylose, respectively. A moderate thermophile, *Paenibacillus* sp. AK25 has also been shown to produce 1.5 mol EtOH mol glucose^{-1} (Sveinsdottir et al., 2009).

Table 1. EtOH production from sugars by defined and mixed cultures of thermophilic bacteria. Cultivation were either in batch or continuous (con). EtOH yields as well as substrate concentrations and incubation temperature are also shown.

Organisms	Sugar	Cultivation method	Sugar conc. (gL^{-1})	Ethanol yield (mol EtOH mol $sugar^{-1}$)	Temp. (°C)	Reference
T. brockii	Cellobiose	Batch	10.0	0.38	60	Lamed & Zeikus (1980)
T. brockii	Glucose	Batch	5.0	0.44	nd	Ben Bassat et al. (1981)
T. ethanolicus	Glucose	Batch	8.0	1.90	72	Wiegel & Ljungdahl. (1981)
T. ethanolicus	Glucose	Batch	20.0	1.90	68	Carreira et al. (1983)
T. thermohydrosulfuricus	Glucose	Batch	5.0	1.60	60	Lovitt et al. (1984)
T. thermohydrosulfuricus	Glucose	Batch	5.0	0.90	60	Lovitt et al. (1984)
T. thermohydrosulfuricus	Glucose	Batch	10.0	1.40-1.90	60	Lovitt et al. (1988)
T. ethanolicus	Xylose	Batch	4.0-27.5	0.60-1.30	60	Lacis & Lawford (1988a)
T. ethanolicus	Xylose	Con	4.0	1.37	60	Lacis & Lawford (1988a)
T. ethanolicus	Xylose	Con	4.0	1.43	60	Lacis & Lawford (1988b)
T. ethanolicus	Xylose	Con	4.0	1.37	68	Lacis & Lawford (1989)
T. ethanolicus	Xylose	Con	4.0	1.37	67-69	Lacis & Lawford (1991)
T. ethanolicus	Xylose	Con	20.0	1.06	67-69	Lacis & Lawford (1991)
T. finnii	Glucose	Batch	NA	1.45	60	Fardeau et al. (1996)
T. finnii	Xylose	Batch	NA	1.76	60	Fardeau et al. (1996)
C. thermocellum	Cellobiose	Batch	2.6	1.60	60	Knutson et al. (1999)
T. ethanolicus	Xylose	Con	4.0	1.30	69	Hild et al. (2003)
T. ethanolicus	Sucrose	Batch	15-30	1.80-3.60	65	Avci et al. (2006)
T. thermohydrosulfuricus	Sucrose	Batch	15-30	1.10 - 3.00	65	Avci et al. (2006)
Thermoanaerobacter ap 65-2	Sucrose	Batch	15-30	1.30-3.20	65	Avci et al. (2006)
Thermoanaerobacter BG1L1	Xylose	Con	10.0	1.28	70	Georgieva at al. (2008)
Enrichment cultures	Glucose	Batch	18.0	0.10-1.70	50-78	Koskinen et al. (2008)
Coculture	Glucose	Con	12.6-25.2	1.37	60	Koskinen et al. (2008a)
Thermoanaerobacterium AK17	Glucose	Batch	3.6	1.50	60	Sveinsdottir et al. (2009)
Thermoanaerobacterium AK17	Xylose	Batch	3.0	1.10	60	Sveinsdottir et al. (2009)
Thermoanaerobacter Ak33	Glucose	Batch	3.6	1.50	70	Sveinsdottir et al. (2009)
Thermoanaerobacter Ak33	Xylose	Batch	3.0	0.80	70	Sveinsdottir et al. (2009)
Paenibacillus AK25	Glucose	Batch	3.6	1.50	50	Sveinsdottir et al. (2009)
Paenibacillus AK25	Xylose	Batch	3.0	0.90	50	Sveinsdottir et al. (2009)
Mixed culture	Glucose	Batch	5.0	1.53	70	Zhao et al. (2009)
Mixed culture	Xylose	Batch	2.0	1.60	70	Zhao et al. (2010)
Enrichment cultures	Glucose	Batch	9.0	1.34	50-75	Orlygsson et al. (2010)
Enrichment cultures	Xylose	Batch	7.5	1.30	50-75	Orlygsson et al. (2010)
T. ethanolicus	Xylose	Batch	5.0	1.00-1.20	65	He et al. (2010)
T. ethanolicus	Glucose	Batch	5.0	1.20-1.30	65	He et al. (2010)

One of the main drawbacks for the use of thermophilic bacteria for EtOH production from biomass is their low tolerance towards EtOH. Several

studies have been done with *Clostridium thermosaccharolyticum* (Baskaran et al., 1995; Klapatch et al., 1994) and *Thermoanaerobacter* sp. (Georgieva et al., 2008b) to increase EtOH tolerance. The highest EtOH tolerance is by a mutant strain of *Thermoanaerobacter ethanolicus*, or 9% (wt/vol) at 69°C (Carriera & Ljungdahl, 1983) but later studies with JW200 Fe(4), one of its derivatives, show much less tolerance (Hild et al., 2003). Georgieva and co-workers published very high EtOH tolerance (8.3%) for *Thermoanerobacter* BG1L1, a highly efficient xylose degrader in continuous culture studies (Georgieva et al., 2008b). *Thermoanerobacter thermohydrosulfuricus* degrades various pentoses and hexoses as well as starch to high concentrations of EtOH (Ng et al., 1981). By transferring the parent strain (39E) to successively higher concentrations of EtOH, an alcohol tolerant strain (39EA) was obtained (Lovitt et al., 1984). The mutant strain grows at 8% EtOH concentrations (wt/vol) at 45°C but only to 3.3% at 68°C. The parent strain produces 1.5 mol EtOH mol glucose^{-1} without any addition of EtOH but the yield lowered to 0.6 mol at 1.5% initial EtOH concentrations. The mutant strain showed lower EtOH yields without any addition of EtOH, or 0.9 mol EtOH mol glucose^{-1} but the yields did not decrease to any extent by increasing initial EtOH concentrations up to 4%. Further experiments with the wild type also indicated the role of H_2 production and its influence on EtOH production (Lovitt et al., 1988). Thus, by changing the gas phase from nitrogen to H_2 or carbon monoxide, EtOH yields increased from 1.41 mol EtOH mol glucose^{-1} to 1.60 and 1.90 mol, respectively.

Recent studies with mixed cultures (batch) were conducted on glucose (Zhao et al., (2009) and xylose (Zhao et al., 2010) where various environmental parameters were optimized for both EtOH and H_2 production. The main bacterial flora, originating from biohydrogen reactor operated at 70°C and fed with xylose and synthetic medium, was identified as various species of *Thermoanaerobacter, Thermoanaerobacterium* and *Caldanaerobacter*. Highest yields observed to be 1.53 and 1.60 mol EtOH mol glucose^{-1} and xylose^{-1} respectively.

Several efforts have recently been made to enrich for new ethanolgenic thermoanerobes. Two surveys have been done from Icelandic hot springs where several interesting bacteria were isolated with EtOH yields of > 1.0 mol EtOH from one mol glucose and xylose (Koskinen et al., 2008; Orlygsson et al., 2010).

Production of EtOH from complex biomass

Production of EtOH from lignocellulosic biomass has gained increased interest in recent years. The type of biomass used has varied to a great extent, e.g. wheat straw, barley straw, hemp, grass, paper and more. Also, the type of pretreatment used is different from one experiment to another. Most data is on biomass pretreated with dilute sulfuric acid or with alkaline pretreatment. The concentration of hydrolysates made from the biomass is also very broad, mostly varying from 0.2 % (w/v) to 15% (w/v). Finally, either pure or mixed cultures are used and either batch or continuous mode. The maximum yield of EtOH from glucose fermentation is 0.51 g EtOH g glucose^{-1}. This corresponds to 2 mol EtOH/mol hexose or 11.1 mM g^{-1}. Considering the complex structure of lignocellulosic biomass, it is not surprising that EtOH yields are usually considerable lower from such substrates (Table 2). Earliest available data on thermophilic bacteria using polymetric biomass originates from studies on *Thermoanaerobacter ethanolicus* and *Clostridium thermocellum* on hemicellulose from birch- and beechwood (Wiegel et al., 1983). These early reports showed promising results but highest yields were observed from the mutant strain *T. ethanolicus*, 4.5 mM g^{-1} xylose equivalent used. Three strains of *Clostridium thermocellum* produced between 1.40 to 2.60 mM EtOH g avicel^{-1} (Lamed et al., 1988). Higher yields (5.0 mM g^{-1} and 5.5 mM g^{-1}) by this bacterium were shown on the same substrate by others (Ahn et al., 1996; Lynd et al., 1989). Rani and co-workers studied EtOH production from both cellulose and lignocellulosic biomass by *C. thermocellum* (Rani et al., 1998). EtOH yields on avicel and Whatman paper was up to 7.2 and 8.0 mM g^{-1} EtOH, respectively. Similar yields were obtained from paddy straw, sorghum stover and corn stubs, pretreated with alkali. The highest yields of EtOH production from cellulosic biomass by *C. thermocellum* are from filter paper, 8.2 mM g^{-1} substrate (Balusu et al., 2004; 2005). In all studies mentioned above with *C. thermocellum* the concentration of cellulose was below 8.0 g L^{-1}. Lin and co-workers recently investigated degradation of napier grass and cellulose (avicel) by *C. thermocellum* and a mixed enrichment culture (Lin et al., 2010). They used from 2.0 to 40.0 g L^{-1} substrate concentrations. The pure culture produced merely 0.72 mM g^{-1} avicel but up to 3.87 mM g^{-1} Napier grass. The mixed culture produced between 0.7-0.9 mM g^{-1} Napier grass and 0.4–5.7 mM g^{-1} avicel. A dramatic decrease in yields was observed by increasing substrate concentrations.

*Table 2. EtOH production from lignocellulosic biomass by defined and mixed cultures of thermophilic bacteria. Cultivation were either in batch or continuous (con). EtOH yields given in mM/g substrate degraded as well as substrate concentrations and incubation temperature are also shown. * = sugar concentration, ** = 30 to 50% as hydrolysate.*

Organisms	Biomass	Cultivation method	Substr. conc. (gL^{-1})	Ethanol yield (mM g $sugar^{-1}$)	Temp. (°C)	Reference
T. ethanolicus	Wood hydrolysate	Batch	8.0	3.30-4.50	70	Wiegel et al. (1983)
C. thermocellum (3 strains)	Avicel	Batch	20.0	1.40-2.60	60	Lamed et al. (1988)
C. thermocellum	Avicel	Batch	2.5	5.00	60	Lynd et al. (1989)
C. thermocellum	Wood hydrolysate	Batch	4.8	3.10	60	Lynd et al. (1989)
C. thermocellum	Avicel	Con	5.0	5.48	60	Ahn et al. (1996)
C. thermocellum	Avicel	Batch	5.0	3.66	60	Ahn et al. (1996)
C. thermocellum	Whatman paper	Batch	8.0	7.20-8.00	60	Rani et al. (1997)
C. thermocellum	Avicel	Batch	8.0	6.50-7.20	60	Rani et al. (1997)
C. thermocellum	Paddy straw	Batch	8.0	6.10-8.00	60	Rani et al. (1997)
C. thermocellum	Sorghum stover	Batch	8.0	4.80-8.10	60	Rani et al. (1997)
C. thermocellum	Corn stubs	Batch	8.0	4.60-7.80	60	Rani et al. (1997)
Thermophilic strain A3	Xylan	Batch	10.0	5.43	70	Ahring et al. (1996)
T. saccharolyticum	Xylan	Batch	10.0	6.30	60	Ahring et al. (1996)
Thermophilic strain A3	Wheat straw	Batch	60.0 (10.0)*	2.61	70	Ahring et al. (1996)
T. mathranii	Wheat straw	Batch	60.0 (6.7)*	2.61	70	Ahring et al. (1999)
T. mathranii	Wheat straw	Batch	60.0	5.30	70	Klinke et al. (2001)
Several	Wheat straw	Batch	30.0	0.30-0.50	70	Sommer et al. (2004)
Several	Wheat straw	Batch	60.0	0.20-0.40	70	Sommer et al. (2004)
C. thermocellum	Filter paper/Corn steep liq.	Batch	45.0/8.0	8.18	60	Balusu et al. (2005)
T. ethanolicus	Beet molasses	Batch	40.0 (19.5)*	4.81	65	Avci et al. (2006)
T. thermohydrosulfuricus 70-1	Beet molasses	Batch	40.0 (19.5)*	2.95	65	Avci et al. (2006)
Thermoanaerobacter sp. 65-2	Beet molasses	Batch	40.0 (19.5)*	7.25	65	Avci et al. (2006)
Thermoanaerobacter BG1L1	Corn stover	Batch	25.0-150.0	8.50-9.20	70	Georgieva et al. (2007)
Thermoanaerobacter BG1L1	Wheat straw	Batch	30.0-120.0	8.50-9.20	70	Georgieva et al. (2008)
Thermoanaerobacter BG1L1	Corn stover	Con	25.0-150.0	8.50-9.20	70	Georgieva et al. (2008)
Clostridium thermocellum	Avicel	Batch	300-700**	0.70	60	Chinn et al. (2008)
T. ethanolicus	Been card HL	Batch	10.0	1.80	60	Miyazaki et al. (2008)
Clostridium sp.	Been card HL	Batch	10.0	0.85	60	Miyazaki et al. (2008)
Thermoanaerobacterium sp.	Been card HL	Batch	10.0	0.90	60	Miyazaki et al. (2008)
Thermoanaerobacterium AK17	Cellulose	Batch	7.5	5.81	60	Sveinsdottir et al. (2009)
Thermoanaerobacterium AK17	Grass	Batch	7.5	2.91	60	Sveinsdottir et al. (2009)
Thermoanaerobacterium AK17	Paper	Batch	7.5	2.03	60	Sveinsdottir et al. (2009)
Mixed	Napier grass	Batch	2.0-40.0	0.70-0.90	60	Lin et al. (2010)
Mixed	Avicel	Batch	2.0-40.0	0.40-5.70	60	Lin et al. (2010)
C. thermocellum	Napier grass	Batch	2.0-40.0	0.80-3.90	60	Lin et al. (2010)
C. thermocellum	Avicel	Batch	10.0	0.70	60	Lin et al. (2010)
Mixed *(C. thermocellum)*	Banana waste	Batch	10.0-100.0	5.50-9.20	60	Harish et al. (2010)

Ahring and co-workers (Ahring et al., 1996) investigated the potential of five thermoanaerobes for EtOH production from the hemicelluloses fraction of wheat straw hydrolysates. Three of the strains produced only minor amounts of EtOH from xylan but *Thermoanaerobacterium saccharolyticum* HG8 and strain A3 produced 6.30 and 5.43 mM g xylan^{-1}, respectively. Strain A3 was further investigated on hydrolysates made from wheat straw, pretreated with wet oxidation. EtOH yields were lower as compared to xylan, or 2.61 mM g wheat straw^{-1} pretreated without oxygen.

Thermoanerobacter mathranii was isolated in 1993 from Hveragerdi in Iceland (Larsen et al., 1997) and has been adapted by Ahring et al., (1996). The strain has been investigated for EtOH production capacity on wet oxidized wheat straw (Ahring et al., 1999). By using very high substrate concentrations (60 g L^{-1}) and wet oxidation with different amounts of sodium carbonate the amount of total sugars released varied from 3.5 to 9.9 g L^{-1}. A fermentation of the strain on undiluted hydrolysate by the strain resulted in the production of approximately 9 mM of EtOH, or 1.3 mM g sugar^{-1}. This strain was also investigated for the effects of inhibitory compounds and hydrolysate concentration on the fermentation of wheat straw hydrolysates (Klinke et al., 2001). The main outcome was that the addition of hydrolysate to a medium containing 4 g L xylose^{-1} did not inhibit EtOH production and it produced 5.5 mM g xylose^{-1}. Increased concentrations of aromatic compounds and hydrolysates however, severly inhibited EtOH production by the strain. Wheat straw hydrolysates have also been investigated by other thermophilic bacteria (Sommer et al., 2004) but with lower EtOH yields.

Fermentation of beet molasses by three thermophilic *Thermoanaerobacter* species (*T. ethanolicus*, *Thermoanaerobacter* sp. and *T. thermohydrosulfuricus*) were recently investigated (Avci et al., 2006). The concentration of sugars were 19.5 g L^{-1} and and fermentation resulted in yields between 3.0 (*T. thermohydrosulfuricus*) and 7.26 mM g^{-1} (*Thermoanaerobacter* sp.). The highest reported EtOH yields reported from complex biomass are by *Thermoanaerobacter* BG1L1 on corn stover and wheat straw (Georgieva & Ahring, 2007; Georgieva et al., 2008a). The biomass was pretreated with acid or wet oxidation and EtOH yields were up to 9.2 mM g^{-1} for biomass hydrolysates.

Studies on *Thermoanaerobacterium* sp and *Clostridium* sp. on been curd refuse hydrolysates were investigated by Miyazaki and co-workers (Miyazaki et al., 2008) with emphasis on cooperation between aerobic cellulose degrading *Geobacillus* with the anaerobes. EtOH yields in this study were relatively low, or between 0.72 to 1.80 mM g substrate^{-1}. Studies on EtOH production by *Thermoanaerobacterium* sp. AK_{17}, isolated from Icelandic hot spring, on various types of lignocellulosic

biomass were reported recently (Sveinsdottir et al., 2009). Batch culture studies on 7.5 g L^{-1} of cellulose, grass and newspaper, pretreated with heat and enzymes, showed EtOH yields of 2.0 (paper), 2.91 (grass) to 5.81 (cellulose) mM/g biomass. Optimization experiments were recently done on this strain where EtOH yields on grass and cellulose were increased to 4.0 and 8.6 mM g^{-1}, respectively. The main environmental factors concerning increasing EtOH yields were the use of acid/alkali for pretreatment and by lowering the substrate concentration from 7.5 to 2.5 g L^{-1} (manuscript submitted to Bioresource Technology).

Production of H_2 from thermophilic bacteria

H_2 production from various organic materials by fermentation has been known for a long time. Firstly, the focus was mainly on facultative mesophilic bacteria within the genera of e.g. Enterobacter, Citrobacter and strict anaerobes like the typical acetate/butyrate fermentative Clostridia. There are numerous publications which focus on mesophilic bacteria that will not be dealt with in this paper. It has not been until relatively recently that H_2 production by thermophiles has gained increased interest and in the past three years there has been an explosion of number of publications within this field of research. Thermophilic bacteria have many advantages as compared to mesophiles concerning H_2 production, however, have remained less studied. High temperatures favor the stoichiometry of H_2 production resulting in higher H_2 yields compared to mesophilic systems (van Groenestijn et al., 2002; van Niel et al., 2003). Furthermore, thermophilic fermentation results in less variety of end products as compared to those of mesophilic fermentation (van Niel et al., 2003). The discussion below is divided into production of H_2 from sugars and from other biomass.

Production of H_2 from sugars

Pure cultures are, for the most part, used to study effects of environmental factors affecting commercial H_2 production. Several studies on H_2 production on sugars, using pure thermophilic cultures have been reported. The most common are dealing with bacteria belonging to the genera of *Thermoanaerobacterium*, *Caldicellulosiruptor* and *Thermotoga*. Table 3 summarizes studies using pure cultures for H_2 production from sugars.

Table 3. H_2 production from sugars by pure cultures of thermophilic bacteria. Cultivation were either in batch or continuous (con). Volumetric H_2 production rates, H_2 yields as well as substrate concentrations and incubation temperature are also shown.

Organisms	Substrate	Cultivation method	Biomass conc. (g L^{-1})	Volumetric H_2 productivity (mL L^{-1} h^{-1})	H_2 yield (mol H_2 mol glu^{-1} equiv.)	Temp. (°C)	Reference
P. furiosus	Maltose	Con	0.22	5.5-22.0	2.90	98	Schicho et al. (1993)
T. maritima	Glucose	Batch	0.1	6.9	4.00	80	Schroder et al. (1994)
T. elfii	Glucose	Con	10.0	0.6	3.30	65	van Niel et al. (2002)
C. saccharolyticus	Sucrose	Con	10.0	0.6	3.30	70	van Niel et al. (2002)
T. neapolitana	Glucose	Batch	5.0	0.6	N/A	70	Van Ooteghem et al. (2002)
T. tengcongensis	Glucose	Con	4.5	N/A	4.00	75	Soboh et al. (2004)
C. saccharolyticus	Glucose	Batch	1.7	N/A	2.50	70	Kadar et al. (2004)
C. saccharolyticus	Xylose	Batch	1.6	11.3	2.70	70	Kadar et al. (2004)
C. saccharolyticus	Xyl/Glu	Batch	1.0	9.2	2.40	70	Kadar et al. (2004)
C. saccharolyticus	Glucose	Con	4.0	2.5	3.60	70	Vrije et al. (2007)
T. thermosaccharolyticum	sucrose	Batch	20.0	3.0	2.53	60	O-Thong et al. (2008)
T. thermosaccharolyticum	Glucose	Batch	10.0	1.6	2.42	60	Ren et al. (2008)
T. thermosaccharolyticum	Xylose	Batch	10.0	1.6	2.19	60	Ren et al. (2008)
T. neapolitana	Glucose	Batch	5.0	N/A	2.40	80	Eriksen et al. (2008)
T. neapolitana	Glucose	Batch	7.5	N/A	1.84	80	Nguyen et al. (2008a)
T. maritima	Glucose	Batch	7.5	N/A	1.67	80	Nguyen et al. (2008a)
T. neapolitana	Glucose	Batch	2.5	0.1	3.85	77	Munro et al. (2009)
C. thermocellum	Cellobiose	Batch	1.1	N/A	1.73	60	Levin et al. (2006)
C. saccharolyticus	Glucose	Con	10.0	N/A	3.00	70	Willquist et al. (2009)
T. neapolitana	Glucose	Batch	7.0	N/A	3.24	77	Nguyen et al. (2010b)
T. neapolitana	Xylose	Batch	4.0	N/A	2.20	77	Nguyen et al. (2010b)
T. thermosaccharolyticum	Xylose	Batch	12.2	N/A	2.37	60	Cao et al. (2010)
T. neapolitana	Glucose	Con	5.0	6.3	3.85	80	d'Ippolito et al. (2010)
C. owensensis	Glucose	Con	10.0	1.9	3.80	70	Zeidan & van Niel (2010)
C. owensensis	Xylose	Con	10.0	1.4	2.70	70	Zeidan & van Niel (2010)
C. thermolacticum	Lactose	Batch	10.0	N/A	1.80	58	Collet et al. (2003)
Clostridium AK14	Glucose	Batch	3.6	N/A	2.21	50	Almarsdottir et al. (2010)
Clostridium AK14	Xylose	Batch	3.0	N/A	2.55	50	Almarsdottir et al. (2010)

Thermotoga *neopolitana* was first described by Jannasch and co-workers (1988) but erliest data of H_2 production is from 2002 where the bacterium produced 2.0 ml L^{-1} h^{-1} on glucose in batch cultures (van Ootegehem et al., 2002). H_2 production capacity from glucose by this species has since then been investigated in detail by others (Eriksen et al., 2008; d'Ippolito et al., 2008; Nguyen et al., 2008, 2010; Munro et al., 2009) showing yields between 1.84 to 3.85 mol H_2 mol $glucose^{-1}$. Xylose can also be used by the bacterium with good yields, or 2.20 mol H_2 mol $xylose^{-1}$ (Nguyen et al., 2010b). Most studies reported on H_2 production by *T. neopolitana* have been conducted in batch experiments with relatively low sugar concentrations (5 to 7 g L^{-1}). The only experiment in continuous culture is reported by d'Ippolito et al., (2010) on glucose but very high yields were reported (3.85 mol H_2 mol $glucose^{-1}$). Other studies on species within the genus have been on *T. elfii* (van Niel et al., 2002)

and *T. maritima* (Nguyen et al., 2008; Schröder et al., 1994) with H_2 yields varying from 1.67 to 4.00 (maximum) mol H_2 mol glucose^{-1}.

Species belonging to genus *Caldicellulosiruptor* have been intensively investigated for H_2 production. *C. saccharolyticus* grown on sucrose showed good yields in continuous culture, or 6.6 mol H_2 mol sucrose^{-1} (= 3.3 mol H_2 mol hexose^{-1}) (van Niel et al., 2002) and between 2.5 and 3.0 mol H_2 for one mole of xylose and glucose in batch (Kadar et al., 2004; Willquist et al., 2009). Higher yields were observed in continuous culture, or 3.6 as well as high H_2 production rates (Vrije et al., 2007). Recently *C. owensis* has also been shown to be a good H_2 producer both in continuous culture with H_2 yields of 3.8 and 2.7 from glucose and xylose, respectively (Zeidan & van Niel, 2010). Hydrogen production from glucose (4.5 g L^{-1}) in batch by *Thermoanaerobacter tengcongensis* has been investigated (Soboh et al., 2009). The culture was continuously flushed with N_2 to keep the partial pressure of H_2 low. This resulted in higher growth rates but due to high N_2 flushing rates H_2 could not be quantified. However, glucose was almost completely converted to acetate and since no external electron acceptor was added, it was assumed that 4.0 mol H_2 were formed per mol glucose degraded. Other thermophilic bacteria that have been investigated for H_2 production capacity are e.g. *Clostridium* sp. (Almarsdottir et al., 2010 ; Levin et al., 2006), *Thermoanerobacterium saccharolyticum* (Cao et al., 2010; Kadar et al., 2004) and *Pyrococcus furiosus* (Schicho et al., 1993).

In practice it may not be feasible to use pure cultures for H_2 production in large scale production facilities. Therefore, a more attention has recently been upon the use of mixed culture studies for H_2 production, often with sugars as model substrates.

Table 4. H_2 production from sugars by mixed cultures of thermophilic bacteria. Cultivation were either in batch or continuous (con). Volumetric H_2 production rates, H_2 yields as well as substrate concentrations and incubation temperature are also shown.

Origin	Substrate	Cultivation method	Biomass conc. (g L^{-1})	Volumetric H_2 productivity (mL L^{-1} h^{-1})	H_2 yield (mol H_2 mol glu^{-1} equiv.)	Temp. (°C)	Reference
Mixed	Glucose	Con	4.9	N/A	2.47	70	Kotsopoulus et al. (2005)
Compost	Lactose	Fed-batch	2.0	N/A	3.70	55	Calli et al. (2008)
Compost	Xylose	Fed-batch	2.0	N/A	1.70	55	Calli et al. (2008)
Natural anaerobic mixed culture	Xylose	Batch	20.0	N/A	0.80	55	Lin et al. (2008)
Anaerobic culture fromhot spring	Glucose	Batch	4.5	N/A	1.16	52	Karadag et al. (2009)
Household solid waste	Xylose	Batch	0.5	N/A	1.62	70	Kongjan et al. (2009)
Household solid waste	Xylose	Con	1.0	2.6	1.61	70	Kongjan et al. (2009)
Cow manure	Glucose	Con	5.0	50.8	3.32	75	Yokoyama et al. (2009)
Mixed	Xylose	Con	6.0	3.4	2.60	70	Zeidan et al. (2010)
Mixed	Glucose	Batch	2.0	N/A	1.58	70	Zhao et al. (2009)
Mixed	Xylose	Batch	2.0	N/A	1.84	70	Zhao et al. (2010)
Sediments-rich samples from hot springs	Glucose	Batch	10.0	N/A	1.71	60	Hniman et al. (2010)
Sediments-rich samples from hot springs	Xylose	Batch	10.0	N/A	1.57	60	Hniman et al. (2010)
Anaerobic culture from hot spring	Glucose	Con	9.0	N/A	1.10	37	Karadag & Puhakka (2010)
Enrichment cultures from hot springs	Glucose	Batch	18.0	N/A	2.10	59	Koskinen et al. (2008a)
Mixed	Glucose	Con	3.6	6.1	0.80	60	Koskinen et al. (2008b)
Enichment culture from hot spring	Glucose	Batch	5.9	N/A	3.20	60	Koskinen et al. (2008c)
Enichment culture from hot spring	Glucose	Con	18.0	N/A	2.74	58	Koskinen et al. (2008c)

The origin of bacteria used in such studies are from e.g. compost, hot springs, manure or anaerobic digestion systems (Calli et al., 2008; Hniman et al., 2010; Karadag et al., 2009; Karadag & Puhakka, 2010; Lin et al., 2008; Zhao et al., 2009; Zhao et al., 2010). Available data from such experiments are presented in Table 4. Although the yields of H_2 production are usually lower as compared to pure culture studies, very high yields have indeed been obtained. An example of this is from the study of xylose and lactose, fed batch fermentation with bacteria from compost. Yields on lactose were 3.7 mol H_2 mol $lactose^{-1}$ (Calli et al., 2008). Glucose fermentation in continuous culture with bacteria from manure resulted in 3.3 mol H_2 mol $glucose^{-1}$ (Yokoyama et al., 2009). Enrichment culture from Icelandic geothermal hot spring produced H_2 of up to 3.2 mol H_2 mol $glucose^{-1}$ in batch assay (Koskinen et al., 2008c). A continuous culture study showed H_2 yields of 2.74 mol H_2 mol $glucose^{-1}$. The enrichment culture was dominated by strains closely affiliated with *Thermobrachium celere.*

Production of H_2 from complex biomass

Available data on H_2 production from complex biomass has exploded in the last three years. Complex biomass, such as food waste and lignocellulosic agricultural residues have been used for thermophilic biohydrogen production in both laboratory and pilot scale. The discussion

below will be divided according to H_2 production from different types of biomass.

Agricultural wastes and energy crops

Several studies have been done with various corn straw as substrate both in pure (Ivanova et al., 2009) and mixed (Kongjan & Angelidaki, 2010; Kongjan et al., 2010) cultures. Mixed cultures, originating from methanogenic sludge from a potato factory were used in continuous cultures (UASB, CSTR, AF) with hemicellulose rich wheat straw (Kongjan & Angelidaki, 2010). The highest H_2 production yields of 9.5 mmol H_2 g sugar^{-1} (1.7 mol H_2 mol glucose^{-1}) was achieved in the UASB reactor. The reactors were fed with hydrolysates that contained 4.4% (TS), mainly xylose. The hydrolysate prepared with hydrothermal pretreatment was diluted prior to inoculation to 25% (v/v). The main conclusion from this study was that reactor configuration is of great importance for enhancing and stabilizing H_2 production. In another study on this substrate the focus was on the importance of hydrolysate concentrations (Kongjan et al., 2010). High hydrolysate concentrations strongly inhibited H_2 production. Batch culture trials on 5% hydrolysate concentrations showed highest yield or 14.1 mmol H_2 g sugar^{-1} (2.55 mol H_2 mol hexose^{-1} equivalent) but CSTR-reactor that ran on 20% HL showed considerable lower yields or 7.9 mmol H_2 mol sugar^{-1} (1.43 mol H_2 mol glucose^{-1} equivalent). Phylogenetic analysis of the mixed cultures showed presence of *Caldanaerobacter subterraneus*, *Thermoanaerobacter subterraneus* and *Thermoanaerobacterium thermosaccharolyticum*.

*Table 5. H_2 production from agricultural wastes and energy crops. Cultivation were either in batch or continuous (con). Volumetric H_2 production rates, H_2 yields as well as substrate concentrations and incubation temperature are also shown. * = concentrations of sugars.*

Culture	Feedstock	Cultivation method	Biomass conc. ($g\ L^{-1}$)	Volumetric H_2 productivity ($mL\ L^{-1}\ h^{-1}$)	H_2 yield ($mol\ H_2\ mol\ glu^{-1}$ equiv.)	Temp. (°C)	Reference
Mixed	Cellulose wastewater	Batch	5.0	ND	0.82	55	Liu et al. (2003)
C. saccharolyticus	Paper sludge	Batch	8.4	91.8	3.70	70	Kadar et al. (2004)
C. thermocellum	Delignified wood fibers	Batch	0.1-4.5	ND	1.00-2.30	60	Levin et al. (2006)
C. thermocellum 27405	Cellulose	Batch	0.1-4.5	ND	0.80-2.00	60	Levin et al. (2006)
C. thermocellum 27405	Whatman paper	Batch	0.1-4.5	ND	0.80-1.90	60	Levin et al. (2006)
Thermotoga neapolitana	Microcrystalline cellulose	Batch	5.0	ND	1.00-2.20	80	Nguyen et al. (2008b)
C. thermocellum	Dried distillers grain	Batch	5.0	5.1	1.27	60	Magnusson et al. (2008)
C. thermocellum	Barley hulls	Batch	5.0	2.0	1.24	60	Magnusson et al. (2008)
C. thermocellum	Cellulose	Batch	1.1	5.1	0.76	60	Magnusson et al. (2008)
C. thermocellum	Contaminated barley hulls	Batch	5.0	5.4	1.18	60	Magnusson et al. (2008)
Coculture	Cellulose	Batch	5.0	ND	1.80	60	Liu et al. (2008b)
T. thermosaccharolyticum	Corn stover	Batch	6.4-12.2	ND	2.24	60	Cao et al. (2009b)
T. thermosaccharolyticum	Miscanthus hydrolysate	Batch	10.0	282.2	3.40	72	Vrije et al. (2009)
Thermotoga neapolitana	Miscanthus hydrolysate	Batch	14.0	275.5	3.20	80	Vrije et al. (2009)
Mixed	Napier grass	Batch	10.0	ND	1.20	55	Lo et al. (2009)
Coculture	Cellulose (filter paper)	Batch	9.0	ND	1.36	55	Geng et al. (2010)
C. saccharolyticus	Wheat straw	Batch	20.0	ND	3.71	70	Ivanova et al. (2009)
C. saccharolyticus	Sweet sorghum plant	Batch	30.0	ND	1.80	70	Ivanova et al. (2009)
C. saccharolyticus	Sugarcane bagasse	Batch	15.0	ND	2.30	70	Ivanova et al. (2009)
C. saccharolyticus	Maize leaves	Batch	8.0	ND	3.70	70	Ivanova et al. (2009)
Mixed	Oil palm trunk hydrolysate	Batch	10.0	ND	1.94	60	Hniman et al. (2010)
Mixed	Corn stover	Batch	13.3	ND	1.53	55	Liu & Cheng (2010)
Clostridium AK14	Cellulose	Batch	5.0	ND	1.10-1.20	50	Almarsdottir et al. (2010)
Clostridium AK14	Hemp stem	Batch	5.0	ND	0.60-0.70	50	Almarsdottir et al. (2010)
Clostridium AK14	Hemp leaf	Batch	5.0	ND	0.20-0.40	50	Almarsdottir et al. (2010)
Clostridium AK14	Grass	Batch	5.0	ND	0.80-0.90	50	Almarsdottir et al. (2010)
Clostridium AK14	Paper	Batch	5.0	ND	0.10-0.40	50	Almarsdottir et al. (2010)
Clostridium AK14	Barley straw	Batch	5.0	ND	0.70-0.80	50	Almarsdottir et al. (2010)
Mixed	Wheat straw	Con	3.9*	34.2	1.70	70	Kongjan & Angelidaki (2010)
Mixed	Wheat straw	Con	3.9*	10.1	1.51	70	Kongjan & Angelidaki (2010)
Mixed	Wheat straw	Con	3.9*	20.6	1.00	70	Kongjan & Angelidaki (2010)
Mixed	Wheat straw	Batch	0.8-3.9*	ND	1.20-2.60	70	Kongjan et al. (2010)
Mixed	Wheat straw	Con	3.1*	7.7	1.42	70	Kongjan et al. (2010)
Mixed	Wheat straw	Batch	50.0	ND	2.54	70	Kongjan et al. (2010)
T. thermosaccharolyticum W16	Corn stover	Batch	10*	250.9	2.70	60	Ren et al. (2010)
Thermotoga neapolitana	Korean rice straw	Batch	10*	31.8	0.41	75	Nguyen et al. (2010a)
Thermotoga neapolitana	Korean rice straw	Batch	10.0	112.4	0.49	75	Nguyen et al. (2010a)

Caldicellulosiruptor saccharolyticus has been used for H_2 production from hemicellulose-rich pine tree wood shavings, maize leaves, wheat straw, sugarcane bagasse and the sweet sorghum bagasse without chemical pretreatment in batch (Ivanova et al., 2009). From wheat straw, the strain produced 2.04 mmol H_2 g $(dw)^{-1}$ (0.36 mol H_2 mol $glucose^{-1}$), i.e. less than 10% of theoretical yields of sugars present in the biomass. Yields calculated on the basis of glucose consumed were however extremely high, or 3.8 mol H_2 mol $glucose^{-1}$. Lower yields were obtained from other biomass. *Thermotoga neapolitana* produced 2.3 to 2.7 mmol H_2 g korean rice $straw^{-1}$ (0.4 to 0.5 mol H_2 mol $hexose^{-1}$ equivalent) from untreated and thermally ammonia or dilute sulfuric acid pretreatment, respectively (Nguyen et al., 2010b). Fermentation of hydrolysates from Miscanthus hydrolysates by *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfi*, pretreated by alkali, resulted in 3.4 and 3.2 mol H_2 mol $glucose^{-1}$ equivalent, respectively (de Vrije et al., 2009).

Corn stover and corn stover cornstalk have been investigated for H_2 production capacity by many (Cao et al., 2009; Datar et al., 2007; Liu et al., 2008b; Liu & Cheng, 2010; Ren et al., 2010). Pure culture studies on *Thermoanaerobacterium thermosaccharolyticum* on corn stover hydrolysates showed maximum of 2.7 mol H_2 mol $glucose^{-1}$ equivalent diluted corn stover hydrolysates that contained a mixture of glucose, xylose and arabinose (total sugar concentration, 10 g L^{-1}) (Ren et al., 2010). Pretreatment consisted of mincing with hammer mill, drying and enzymatic hydrolysis. The bacterium showed classical acetate/butyrate fermentation and yields were similar as on equal amounts of pure sugars. Earlier reports on the production capacity of this bacterium on corn stover pretreated with acid showed similar yields, or 2.24 mol H_2 mol $glucose^{-1}$ (Cao et al., 2009). From a study of Liu and Cheng (2010), corn stover was pretreated with microwave assisted strategy and the resulting biomass hydrolysate fermented with mixed thermophilic microflora from a anaerobic digester. H_2 production capacity was however modest, or 1.53 mol H_2 mol $glucose^{-1}$ equivalents

A co-culture of *Clostridium thermocellum* and *Thermoanaerobacterium thermosaccharolyticum* grown on hydrolysate made from 5 g L^{-1} of corn stalk and corn cob powder (no pretreatment), resulted in 1.8 mol H_2 mol $glucose^{-1}$ (Liu et al., 2008b). *Clostridium* AK14 was used to degrade hemp (both stem and leaf), grass, paper and straw (Almarsdottir et al., 2010). Highest yields were observed on grass pretreted with 0.75% sulfuric acid and enzymes, or 6.23 mol H_2 g VS^{-1}. Pretreatment with either alkali or acid increased H_2 in most cases substantially.

Several studies of H_2 production form cellulose have been conducted (Almarsdottir et al., 2010; Geng et al., 2010; Levin et al., 2006;

Liu et al., 2003; Liu et al., 2008b; Nguyen et al., 2008). Various sources of cellulose have been used, e.g. wastewater (Liu et al., 2003) Whatman filter paper (Almarsdottir et al., 2010; Geng et al., 2008), microcrystalline cellulose (Liu et al., 2008b; Nguyen et al., 2008b). Hydrogen yields from these studies (all batch) varied from 0.95 to 2.32 mol H_2 mol glucose^{-1} equivalent. In some studies the focus was on different pretreatment methods used. Studies with pure cultures of *Clostridium* AK14, a moderate thermophilic bacterium showed similar results (1.17 mol H_2 mol glucose^{-1} equivalent) from Whatman paper whether it was only enzymatically pretreated or pretreated with both enzymes and weak acid or alkali (Almarsdottir et al., 2010). Hydrogen production from microcrystalline cellulose by *Thermotoga neapolitana* increased however from 1.59 to 2.2 mol H_2 mol glucose^{-1} equivalent by using ionic liquid pretreatment (Nguyen et al., 2008b). The influence of substrate concentrations on H_2 yields from degradation of cellulosic substrates by *Clostridium thermocellum* were investigated by Levin et al., (2006). Highest yields were observed on delignified wood fibers at 0.1 g L^{-1}, 2.32 mol H_2 mol glucose^{-1} equivalents. At 4.5 g L^{-1} yields dramatically decreased to less than 1 mol H_2 mol glucose^{-1}. Other reports on H_2 production from ligncocellulosic biomass presented in Table 5 include studies on paper sludge (Kadar et al., 2004), oil palm trunk hydrolysate (Hniman et al., 2010), Napier grass (Lo et al., 2009) and barley hulls (Magnusson et al., 2008) and are not discussed in detail in this overview.

Starch and mixed biomass

Several studies of H_2 production from starch have been done, both with pure soluble starch and a starch based biomass. Akutsu and co-workers used mixed cultures from five different kinds of sludge as inocula to produce hydrogen from starch in CSTR-reactors without any pretreatment (Akutsu et al., 2008). The highest H_2 production yields (2.3 mol H_2 mol glucose^{-1} equivalent) was obtained with thermophilically digested waste activated sludge as inocula. Phylogenetic analysis showed the presence of *Thermoanaerobacterium* in all reactors. *Janthinobacterium* and aerobic bacteria of the genus *Flavobacterium* were also detected . Two other studies by Akutsu and co-workers focused on the effects of different factors on H_2 production from starch (Akutsu et al., 2009a, 2009b). In the first study (Akutsu et al., 2009b) the effects of substrate concentrations (10-70 g L^{-1}) on H_2 production were investigated in continuous cultures using a mixed culture originating from thermophilic acidogenic sludge treating potato waste. The H_2 yields varied from 1.84 to 2.82 mol H_2 mol glucose^{-1} at 70 and 20 g L^{-1} substrate concentrations, respectively. The maximum H_2 production rate was 182

ml $L^{-1}h^{-1}$. In the other study (Akutsu et al., 2009a), the effects of hydrolic retention time, pH and substrate concentrations were further investigated. Hydrogen production rate was gradually increased from 62 to 167 ml H_2 $L^{-1}h^{-1}$ by lowering the HRT from 40 h to 6h but on the other hand, maximum H_2 yields were obtained at 48 h HRT, or 1.7 mol H_2 mol $glucose^{-1}$ equivalent. Additionally, H_2 production diminished greatly when pH was higher than 6.0 or lower than 4.7 indicating the importance of pH for H_2 production (Akutsu et al., 2009a). Study of starch degradation and H_2 production in repeated batch by extreme mixed cultures, originating from cow manure showed H_2 yields of 1.73 mol H_2 mol $glucose^{-1}$ (Yokoyama et al., 2007). The main emphasis was on the phylogenetic analysis of the microbiological community and presence of various *Caldanaerobacter* species were observed.

*Table 6. H_2 production from starch and mixed biomass. Cultivation were either in batch or continuous (con). Volumetric H_2 production rates, hydrogen yields as well as substrate concentrations and incubation temperature are also shown. * = Repeated batch, ** = Semicontinuous*

Culture	Feedstock	Cultivation method	Biomass conc. (g L^{-1})	Volumetric H_2 productivity (mL L^{-1} h^{-1})	H_2 yield (mol H_2 mol glu^{-1} equiv.)	Temp. (°C)	Reference
T. kodakaraensis	Starch	Con	0.5	6.7	3.30	85	Kanai et al. (2005)
Mixed	Food waste	Con	6.9	ND	2.50-2.80	55	Chu et al. (2008)
Mixed	Soluble starch	Batch*	6.3	ND	1.73	75	Yokoyama et al. (2007)
Mixed	Starch	Con	10.0	42.4-70.8	1.40-2.30	55	Akutsu et al. (2008)
C. saccharolyticus	Sweet sorghum	Batch	2.0	nd	2.63	72	Ivanova et al. (2009)
Mixed	Starch	Con	60.0	nd	1.68	55	Akutsu et al. (2009a)
Mixed	Starch	Con	15.0-70.0	nd	1.84-2.82	55	Akutsu et al. (2009b)
Mixed	Wheat starch	Batch	20.0	7.4	2.40	55	Cakir et al. (2010)
T. neapolitana	Algal starch	Batch	5.0	44.6-227.0	1.80-2.50	75	Nguyen et al. (2010c)
C. saccharolyticus	Carrot pulp	Batch	10.0	351.7	2.80	72	Vrije et al. (2010)
T. neapolitana	Carrot pulp	Batch	10.0	280.0	2.70	80	Vrije et al. (2010)
Mixed	Rice winery wastewater	Con	10.0 (COD)	158.3	2.14	55	Yu et al. (2002b)
Mixed	Food waste	Con	25.0 (sugars)	ND	0.60-1.80	55	Shin et al. (2004)
Mixed	Food waste	Con	14.1 (VSS)	16.7-41.7	1.00-2.40	55	Shin & Youn (2005)
Mixed	POME	Batch	85.0 (COD)	24.2	2.53	60	O-Thong et al. (2008)
Mixed	Household solid waste	Batch	0.5	ND	0.30-2.00	70	Liu et al. (2008a)
Mixed	Household solid waste	Batch*	10.0 (VS)	ND	0.82	70	Liu et al. (2008b)
Mixed	Kitchen waste	Batch	23.7 (VSS)	ND	0.88	55	Lee et al. (2008)
Mixed	Cheese whey (lactose rich)	Con	Variable	12.5 - 329.1	ND	55	Azbar et al. (2009)
Mixed	Cheese whey wastewater	Batch	21.3	ND	1.55	55	Azbar et al. (2009)
Mixed	Pig slurry	Con	45.0 (TS)	3.8	ND	70	Kotsopoulos et al. (2009)
Mixed	Kitchen waste	Con	60.5	66.7	0.23	55	Wang et al. (2009)
Mixed	POME	Con	7.0-8.4 (VSS)	379.2	2.17	60	Prasertsan et al. (2009)
Mixed	Crude Palm Oil + sucrose	Batch	24.0	ND	2.50	55	Ismail et al. (2009)
Mixed	Vegetable kitchen waste	Con*	10.0	41.7	1.70	55	Lee et al. (2010)

Cakir and co-workers compared hydrogen production from ground wheat starch under mesophilic (37°C) and thermophilic conditions (55°C)with mixed microflora from a heat-treated anaerobic sludge (Cakir et al., 2010). The starch was pretreated with sulfuric acid and heat in order to

convert it to soluble sugars. The highest H_2 yield was 2.40 mol H_2 mol glucose^{-1}, obtained under the thermophilic conditions. The hyperthermophilic *Thermotoga neapolitana* was used by to produce hydrogen from green algal biomass (Nguyen et al., 2010c). Starch is a major accumulated constituent of algal biomass and therefore makes a good potential feedstock for both EtOH and H_2 production. Two different pretreatments were used to disrupt the algal cell wall (sonication and MeOH exposure) and two other to improve starch conversion to H_2 (HCl+heat and enzyme). All methods gave good effect on H_2 production but the highest H_2 yield (2.5 mol H_2 mol glucose^{-1}) was obtained with enzymatic hydrolysis (Nguyen et al., 2010c). Ivanova and co-workers used maize leaves to produce H_2, both pretreated and unpretreated (Ivanova et al., 2009). The biomass was treated with a cellulase-producing aerobic bacteria, *Bacillus amyloliquefaciens*. The pretreatment greatly improved the H_2 yields. Unpretreated maize leaves yielded 1.53 mol H_2 mol glucose^{-1} while pretreated leaves yielded 3.71 mol H_2 mol glucose^{-1}.

Many types of different food waste biomass have been used to produce H_2, almost exclusively with mixed cultures from various seed sludge. Lee and co-workers have done two different studies on H_2 production from high vegetable kitchen waste (Lee et al., 2008 and Lee et al., 2010). No pretreatment was used in either study. In the first study, a series of batch fermentation tests were conducted at four different pH levels to observe the effects of pH on the H_2 production. Hydrogen yields from different pH levels were all similar, the highest obtained at pH 7.0 (0.49 mmol H_2 g COD^{-1}) except for pH 5.5 (the lowest pH level), where there was no H_2 production at all (Lee et al., 2008). The main bacteria present belong to the genus *Clostridium*. In the other investigation much higher yields were obtained, or 1.7 mmol H_2 g COD^{-1} and the predominant species was closely affiliated to *Thermoanaerobacterium thermosaccharolyticum* (Lee et al., 2010). Recent study of H_2 production from kitchen waste with mixed cultures from various sources showed good production rates (66.7 ml L^{-1} h^{-1}) but much lower yields (0.23 mol H_2 mol glucose^{-1} equivalent) (Wang et al., 2009). A continuous culture study on H_2 production from food waste by the use of mixed culture originating from anaerobic waste water treatment plant resulted in maximum of 2.8 mol H_2 mol hexose^{-1} (Chu et al., 2008). Other studies with food waste include e.g. continuous culture (CSTR) studies by Shin et al., (2004) and Shin &Youn (2005) at sugar concentration of 25 g L^{-1}. Clearly the effects of substrate concentrations are important buth higest yields (1.8 mol H_2 mol hexose^{-1}) were obtained at 8 g VS/L (Shin et al., 2004). Maximum H_2 production rate and yield occurred at 8 g VSL^{-1} d^{-1}, 5 days HRT and pH 5.5 (Shin & Youn, 2005). Hydrogen production from

household solid waste by using extreme-thermophilic (70°C) mixed culture resulted in 2 mol H_2 mol hexose^{-1} (Liu et al., 2008a) and 0.82 mol H_2 mol hexose^{-1} (Liu et al., 2008b).

Other studies on various mixed substrates include pig slurry (Kotsopoulous et al., 2009), rice winery wastewater (Yu et al., 2002), palm oil effluent (POME) (Ismail et al., 2010; O'Thong et al., 2008; Prasertsan et al., 2009), and cheese whey (Azbar et al., 2009a, 2009b), and are presented in Table 6. Fewer studies have been done using pure microbial cultures producing H_2 from complex biomass. *Caldicellulosiruptor saccharolyticus* and *Thermotoga neapolitana* showed good H_2 yields from carrot pulp hydrolysate, or 2.8 and 2.7 mol H_2 mol hexose^{-1}, respectively (de Vrije et al., 2010). *Thermococcus kodakaraensis* KOD1 showed very high H_2 yields on starch (3.3 mol H_2 mol hexose^{-1}) in continuous culture in a gas lift fermentor with dilution rate of 0.2 h^{-1} (Kanai et al., 2005).

Pros and cons of using thermophiles for biofuel production

The use of thermophilic bacteria for production of H_2 and EtOH has several pros and cons compared to the use of mesophilic bacteria, phototrophic bacteria and yeasts. It is possible to compare the use of different microorganisms by looking at several factors of both practical and economical point of view. Historically, yeasts have been and still are, the microorganisms most widly used for EtOH production from homogenous material like sucrose and glucose. The main reason for this are e.g. very high yields, few end products and high EtOH tolerance. However, wild type yeasts do not have degradation genes for pentose and polymer degradation and genetic engieering studies have not yet delivered stable organisms for large scale production. The main benefits of using bacteria for biofuel production is their broad substrate spectrum and they may therefore be a better choice for EtOH production from more complex biomass e.g. agricultural wastes (Taylor et al., 2008). The main drawback of the use bacteria for biofuel production is their low EtOH tolerance and more diverse end product formation. This is the main reason for no commercialized large scale plants have been build yet. Thermophilic bacteria are often very tolerant towards various environmental extremes. Apart from growing at higher temperatures, often with higher growth rates, many are acid and salt tolerant which may be of importance when various mixed substrates are used. In general bacteria tolerate lower EtOH concentrations as compared to yeasts and elevated substrate concentrations may inhibit growth. This may possible be solved by either using fed batch or continuous cultures or by „self distillation“ of EtOH.

H_2 production by mesophilic bacteria has been known for a long time. The main drawback of using mesophilic bacteria is the fact that H_2 production is inhibited at relatively low partial pressures of H_2 resulting in a change of carbon flow away from acetate (and H_2) towards e.g. EtOH and lactate. Extremophilic bacteria are less phroned towards this inhibition and much higher H_2 concentrations are needed before a change in the carbon flow occurs. H_2 production by photosynthesis has gained increased interest lately but H_2 production rates are much slower as compared to bacteria and a need for large and expensive reactors inhibit its practical use. Additionally, fermentation is not dependend on light and can be runned continuously.

Furfural and hydroxymethylfurfural (HMF) are furan derivatives from pentoses and hexoses, respectively and are among the most potent inhibitory compounds generated from acid hydrolysis of lignocellulosic biomass. Most microorganisms are more sensitive to furfural than HMF

but usually inhibition occurs at concentrations above 1 g L^{-1}. Sensitivity of thermophilic bacteria towards these compounds seems to be similar as compared to yeast (de Vrije et al., 2009; Cao et al., 2010).

Genetic engineering of thermophiles – state of the art

The main hindrance of using thermophilic bacteria are low tolerance to EtOH and the production of other end products like acetate and lactate. Several efforts have been done to enhance EtOH tolerance for thermophiles. Most of these studies were performed by mutations and adaptation to increased EtOH concentrations (Lovitt et al., 1984,1988; Georgieva et al., 1988) and has already been discussed. Elimination of catabolic pathways leading to other end products by genetic engineering has only got attention in the past few years.

The first report on genetic engineering on thermophilic bacteria to increase biofuel production is on *Thermoanaerbacterium saccharolyticum* (Desai et al., 2004). The L-lactate dehydrogenase (LDH) was knocked out leading to increased EtOH and acetate production on both glucose and xylose and total elimination of lactate production. The wild type strain produced 8.1 and 1.8 mM of lactate from 5 g L^{-1} of glucose and xylose, respectively. Later study of the same species resulted in elimination of all acid formation and generation of homoethanolic strain. This strain uses pyruvate:ferredoxin oxidoreductase to convert pyruvate to EtOH with electron transfer from ferredoxin to NAD(P) but this is unknown by any other homoethanolgenic microbes who use pyruvate decarboxylase. The strain produces 37g L^{-1} of EtOH which is the highest yields reported so far for a thermophilic anaerobe (Shaw et al., 2008).

Two *Geobacillus thermoglucosidasius* strains producing mixed acids from sugar fermentation with relatively low EtOH yields were recently genetically engineered to increase yields (Cripps et al., 2009). The authors developed an integration vector system that led to the generation of stable gene knockouts but the wild type strains had shown problems of genetic instability. They inactivated *ldh* and to deal with the excess carbon flux they upregulated the expression of PDH (pyruvate dehydrogenase) to make it the sole fermentation pathway. One of their mutants (TM242) produced EtOH from glucose at more than 90% of the maximum theoretical yields (Cripps et al., 2009).

Yao and Mikkelsen (2010) metabolically engineered a strain of *Thermoanaerobacter mathranii* was genetically engineered to improve the EtOH production (Yao & Mikkelsen, 2010). A strain that had already had the *ldh* gene deleted to eliminate an NADH oxidation pathway (Yao & Mikkelsen, 2010) was used. The results obtained indicated that using a more reduced substrate such as mannitol, shifted the carbon balance towards more reduced end products like EtOH. In order to do that without

having to use mannitol as a substrate they expressed an NAD^+-dependent GLDH (glycerol dehydrogenase) in this bacterium.

A possible approach to increase H_2 yields is to convert more of the substrate to H_2 by altering metabolism by genetic engineering. Studies on either maximizing yields of existing pathways or metabolic engineering of new pathways have been published (Hallenbeck & Gosh, 2010). Genetic manipulation and metabolic flux analysis are well developed and have been suggested to be applied to biohydrogen (Hallenbeck & Benemann, 2002; Vignais et al., 2006). However, no study on genetic engineering on thermophilic bacteria considering H_2 production have been published to our knowledge. So far, the main emphasis has been on the mesophilic bacteria *E.coli* and *Clostridium* species.

Fermentative bacteria often possess several different hydrogenases that can operate in either proton reduction and H_2 oxidation (Hallenbeck & Benemann, 2002). Logically, inactivation of H_2 oxidation would increase H_2 yields. This has been shown for *E. coli* where elimination of *hyd*1 and *hyd*2 led to a 37% increase in H_2 yield compared to the wild type strain (Bisaillon et al., 2006).

Studies on metabolically engineering Clostridia to increse H_2 production have been published. One study showed that by decreasing acetate formation by inactivate *ack* in *Clostridium tyrobutyricum*, 1.5-fold enhancment in H_2 production was observed; yields from glucose increased from 1.4 mol H_2-mol $glucose^{-1}$ to 2.2 mol H_2-mol $glucose^{-1}$ (Liu et al., 2006).

Conclusion

Many bacteria within the genera *Clostridium, Thermoanaerobacter, Thermoanaerobacterium, Caldicellulosiruptor* and *Thermotoga* are good H_2 and/or EtOH producers. Species within *Clostridium* and *Caldicellulosiruptor* are of special interest because of their ability to degrade cellulose and hemicelluloses. Highest EtOH yields on sugars and lignocelluloses hydrolysates are 1.9 mol EtOH mol glucose^{-1} and 9.2 mM g biomass^{-1} (corn stover and wheat straw) by *Thermoanaerobacter thermohydrosulfuricus* and *Thermoanaerobacter* species, respectively. Highest H_2 yields on sugars and lignocelluloses hydrolysates are 4 mol H_2 mol glucose^{-1} and 3.7 mol H_2 mol glucose^{-1} equivalent (from wheat straw) by *Thermotoga maritima* and *Caldicellulosiruptor saccharolyticus*, respectively. Clearly many bacteria within these genera have great potential for EtOH and hydrogen production, especially from complex lignocellulosic biomass. Recent information in genome studies of thermoanaerobes has led to experiments where *Thermonanaerobacterium* and *Thermoanaerobacter* species have been genetically engineered to make them homoethanolgenic. Thus, the greatest drawback of using thermophilic bacteria for biofuel production, their mixed end product formation, can be eliminated but it remains to see if these strains will be stable for upscaling processes.

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5 Manuscript II

Ethanol production from monosugars and lignocellulosic biomass by thermophilic bacteria isolated from Icelandic hot springs

Ethanol production from monosugars and lignocellulosic biomass by thermophilic bacteria isolated from Icelandic hot springs

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Abstract

Seven strains of thermophilic bacteria were isolated from several Icelandic geothermal areas on various carbohydrates (glucose, xylose, xylan, pectin, cellulose). Phylogenetic studies (16S rRNA) revealed that four of the isolates belong to the genus *Thermoanaerobacterium*, two to *Thermoanaerobacter* and one to *Paenibacillus*. The *Thermoanaerobacterium* strains had pH optima at low pH's (pH 5.0 – 6.0), the *Thermoanarobacter* at slightly acidic to neutral pH's (pH 6.0 – 7.0) and the *Paenibacillus* strain at pH 8.0. Similarly there was a clear distinction of temperature optima between the various genera; *Thermoanerobacterium* strains had temperature optima close to 60°C, *Thermoanaerobacter* at 70°C and the Paenibacillus at 50°C. Ethanol tolerance was from low (MIC = 1.6% v/v) for *Thermoanaerobacter* to moderately high (MIC = 3.2% v/v) for the *Thermoanaerobacterium* and *Paenibacillus* strains. Ethanol production capacity on 20 mM of glucose and xylose showed that six of the strains produced between 1.0 to 1.5 mol-EtOH mol-l glucose and 0.4 to 1.3 mol- EtOH mol-l xylose, respectively. One strain showed much lower yields. Strain AK_{17} gave the best yields on glucose and xylose with 1.5 mol-EtOH mol-l glucose and 1.1 mol-EtOH mol-l xylose, respectively. Other end products analyzed in the culture broth were acetate and hydrogen but in lower amounts. Growth on 0.75 % (w/v) hydrolysates made from cellulose (Whatman paper), non inked paper, inked paper, glossy paper, saw dust and grass (*Phleum pratense*) resulted in good ethanol production yields for most of the strains. Strain AK_{17} produced 43.4 mM of ethanol from cellulose, 21.2 mM from grass, between 14.4 to 23.3 mM from the three types of paper hydrolysates and 3.2 mM from sawdust. Other strains produced less ethanol from biomass hydrolysates but its production was in correlation to lower ethanol production yields from monosugars fermentation. Other end products from hydrolysates were, as in the case of monosugar fermentation, acetate and hydrogen, but in lower amounts.

Introduction

Biofuels have gained increased interest in recent years due to environmental, economic and natural security concerns (Qureshi et al. 2006). Biofuels are made from renewable energy sources since they are produced from biomass and their production is based on CO_2 neutral concepts. Today, most of the energy demands are met by non-renewable energy sources, resulting in resource depletion, environmental deterioration and public health problems (Sanches & Cardona, 2008). Therefore, a demand to develop novel renewable energy harvesting technologies and to introduce sustainable energy carriers exists. Bioethanol as an alternative to fossil fuels has been extensively studied and is already produced in scale of 51 ML (Renewable Fuels Association, 2008) worldwide. About 90% of all ethanol is derived from sugar or starch based crops by fermentation (first generation ethanol); the rest is produced chemically. The world's largest ethanol producers are Brazil and the USA, which together account for about 87 % of global ethanol production. Fuel ethanol is produced in Brazil mainly from sugar cane and in the USA from corn (Turkenburg et al. 2000; Renewable Fuels Association, 2008).

Fermentation technologies for sugar and starch based crops are well developed, but have been strongly debated since the biomass used is of high value for the food and feed applications. Therefore second generation ethanol production has been an interesting alternative because it is made from non-edible sources such as lignocellulosic material, which comprises mainly cellulose, hemicelluloses and lignin. Except for lignin, these long-chain polysaccharides can be hydrolysed to produce a mixture of hexoses (C_6) and pentoses (C_5) (Badger, 2007; Winters, 2007). Although, there is an extra step in the hydrolysis of lignocelluloses to monosugars, lignocelluloses are highly abundant and diverse in the terms of availability. Additionally, the cost of feedstock is lower for lignocelluloses compared to agricultural crops (Mann, 2004).

The most common way of bioethanol production today is by fermentation using the yeast *Saccharomyces cerevisiae* with high ethanol yields from starch based substrates (almost 2 moles/ mol of glucose). In the past decades thermophilic bacteria have gained more attention because of fast growth rates and their ability to degrade a broad variety of both hexoses and pentoses (Sommer et al. 2004; Georgieva & Ahring 2007; Orlygsson & Baldursson, 2007; Koskinen et al. 2008). Although, ethanol tolerance of thermophiles is generally less than those of *S. cerevisiae* and the well known mesophilic bacterium *Zymomonas mobilis*, they have several advantages like lower risk of contamination, increased bioconversion rates and product recovery (Lynd, 1989). A variety of

thermophilic microorganisms capable of producing ethanol have been isolated and characterized in the past two decades from different environments, including farm soils, sewage plants, riverbanks, thermal springs, sediments, as well as waste composts, with the intention of their evaluation and development for large-scale ethanol production. These bacteria include *Thermoanaerobacter ethanolicus* (Kannan & Mutharasan 1985; Wiegel & Ljungdahl 1986), *Thermoanaerobacter thermohydrosulfuricus* (Wiegel et al. 1979), *Thermoanaerobacter mathranii* (Larsen et al. 1997), *Thermoanaerobacter brockii* (Zeikus et al. 1979; Lamed & Zeikus 1980; Lee et al. 1993), *Clostridium thermosaccharolyticum* (renamed *Thermoanaerobacterium thermosaccharolyticum* (Vancanneyt et al. 1987) and *Clostridium thermocellum* (Herrero & Gomez 1980; Lamed & Zeikus 1980).

Hot springs are a potential source for thermophilic, H_2 and EtOH producing microorganisms. The aim of this research was to use newly isolated ethanol producing microorganisms from hot springs in Iceland for production of EtOH from selected waste/biomass material.

Materials and methods

Sampling sites

The seven strains investigated in this study were isolated from various hot springs in Graendalur in the Hengill area in SW-Icelandand from the Krafla area in NE-Iceland. The temperature and pH of the hot springs where the strains were collected is shown in Table 1as well as the initial temperature and pH used for enrichment from the samples. Isolation and characterization of strain AK_{17} has already been described earlier (Orlygsson & Baldursson, 2007). Temperatures were measured directly from the hot springs but the pH was measured from experimental bottles upon arrival at the laboratory.

Table 1. Strain identification number of samples and the temperature and pH of environmental samples. Also shown are the temperature and pH used for original incubation.

Strain	Temperature (site)	pH (site)	Temperature (isolation)	pH (isolation)
AK_{17}	70°C	6.5	65°C	7.0
20-07-X	72°C	6.8	70°C	7.0
25-07-C	50°C	7.4	50°C	7.0
33-07-Xo	71°C	8.0	70°C	7.0
64-07-X	59°C	7.0	60°C	7.0
66-07-G	62°C	7.4	60°C	7.0
66-07-P	62°C	7.4	60°C	7.0

Media

The medium (per liter) consisted of: KH_2PO_4 1.5 g, Na_2HPO_4 2.3 g, NH_4Cl 2.2 g, NaCl 3.0 g, $CaCl_2$ 8.8 g, $MgCl_2$ x $6H_2O$ 0.8 g, yeast extract 2.0 g, resarzurine 1 mg, trace element solution 1 ml, vitamin solution 1 ml and $NaHCO_3$ 0.8 g. Carbon and energy sources were 20 mM or in the case of polymers, 3 g l^{-1}. The vitamin solution was according to DSM141. The trace element was as described earlier (Orlygsson and Baldursson, 2007). The medium was prepared by adding the buffer to distilled water, which was then boiled for 5-10 min and cooled while flushing with nitrogen. The mixture was then transferred to cultivation bottles using the

Hungate technique (Hungate 1969) and then autoclaved. All other components of the medium were added separately through filter sterilized solutions.

Isolations and enrichments

Samples were collected using an extended pole equipped with grip arms placed at the end of it. Serum bottles (120 ml) were fixed at the end, opened and completely filled with geothermal liquid/mud samples and closed with butyl rubber and aluminum caps. A five ml aliquot from each sample was inoculated into120 ml serum bottles containing 45 ml medium with 2 g l^{-1} YE and either 20 mM monosugars (glucose or xylose) or 3 g l^{-1} of xylan, pectin or cellulose. The samples were incubated at temperatures slightly below the experimental site temperatures. In most cases, because of the dense inoculum, it was not possible to follow growth with increased absorbance by using a spectrophotometer. Therefore, after seven days, an aliquot of 5 ml of each enrichment culture was transferred into a new fresh carbon-containing media. This was repeated three times. Positive samples from the third enrichment series were diluted (tenfold dilutions) and inoculated in the same medium with 20 g l^{-1} of Gelrite® in Hungate roll tubes. Visible colonies were picked up with sterile Pasteur pipettes and inoculated into fresh media. Six isolates were obtained and analyzed for full 16S rRNA sequence analysis.

Determination of minimum inhibition concentration

MIC (*minimum inhibitory concentration)* determination was performed for the seven strains in order to determine their maximum ethanol tolerance. The experiment was carried out in 23 ml serum bottles containing 10 ml medium and different concentrations of ethanol (0%, 0.2%, 0.4%, 0.8%, 1.6%, 3.2%, 6.4% and 8%). The initial glucose concentration was 20 mM of glucose and 2 g/L of yeast extract but control samples did not contain any ethanol or glucose. Optical density was measured (OD_{600}) in the beginning and at the end of the incubation period (120 h) to determine the MIC's for each strain.

Determination of pH_{opt} and T_{opt}

Determination of a rough temperature and pH optimum were done at four different temperatures (50°C, 60°C, 70°C and 75°C) and six different pH levels (pH 4.0 – 9.0). Determination of growth was performed on spectrophotometer at 600 nm. Log-phase growth rates (μ) were derived from the absorbance (OD) data using the standard equation $\ln(X/X_0) = (\mu)(t)$, where X is the measured culture OD, X_0 is the initial culture OD, and t is the elapsed time.

Preparation and pretreatment of hydrolysates from complex biomass substrates

Hydrolysates (HL) were made from a number of different biomass: Whatman filter paper (cellulose), white glossy paper (WGP), newspaper with (NPi) and without ink (NP), sawdust and grass (*Phleum pratense*). The Whatman paper consists of 99% cellulose and was used as a control. The grass was dried overnight at 50°C and cut into small pieces (< 3 mm). All paper was shredded and thereafter cut with scissors. Ten grams of each biomass was weighed into separate Waring blenders and water was added until the total mass of 400 g (2.5% dry weight) was reached. Water and raw biomass were mixed together thoroughly for one minute or until homogenized. After that, each mixture was put in 500 ml flasks which were autoclaved for 90 minutes. After cooling, the pH was measured and adjusted if needed with either HCl or NaOH to pH 5.0. Thereafter, 1 ml of Celluclast® and 1 ml of Novozymes 188 were added into each flask and they were placed in 45°C water bath for 68 hours. Finally, the pH level of the HL´s was adjusted to pH´s that suited the optimum of each strain.

Physiological experiments – fermentation of monosugars and hydrolysates

End product formation from monosugars and from lignocellulosic HL´s was done by inoculating 1 ml of a fresh culture into 49 ml of medium containing glucose, xylose or HL's from various lignocellulosic materials. The concentration of glucose and xylose was 20 mM and the concentrations of HL's were 20, 30, 50 and 70% v/v). Experimental bottles containing various concentrations of HL´s always contained the

same amount of all other components as described earlier; the medium was never diluted of other components like salts, trace elements or vitamins. Fermentation time was one week and samples for volatile fatty acids, ethanol and hydrogen were taken and analyzed at the beginning and in the end of the experimental time.

Strain identification

For 16S rRNA analysis, 16S rRNA genes were amplified from DNA with primers F9 and R1544, specific for bacterial genes (Skirnisdottir et al., 2000) with PCR. In most cases 6-700 bp was used for analysis (strains were both fully and partially sequenced). The PCR products were sequenced with universal 16S rRNA primers: F9, F515, F1392, R357, F1195 and R1544 by using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Subsequently the DNA was analyzed with 3730 DNA analyzer from Applied Biosystems. The nucleotide sequence was displayed and analyzed with Sequencer (Gene Code Corporation) (Skirnisdottir et al. 2000). Sequences from 16S rRNA gene analysis were uploaded to the NCBI database using the nucleotide-nucleotide BLAST (BLASTn). Ribosomal Database Project was also used to obtain sequences of related strains. The most similar sequences obtained from the databases were aligned with the results from the sequencing in the program BioEdit and ClustalX where final alignments were done to generate phylogenetic trees. The program TreeCon was used to view the trees. *Escherichia coli* (AE000406) was selected as out-group.

Analytical methods

Ethanol, acetate and hydrogen were measured by gas chromatograph as previously described (Orlygsson and Baldursson, 2007).

Results

Isolations of bacterial strains – phylogenetic studies

Originally, more than sixty strains were isolated from various carbon substrates from several hot springs in Graendalur in SW-Iceland and from the Krafla area in NE-Iceland. Twelve of these strains were selected for further studies based on good growth rates and high ethanol yields from monosugar fermentations. In this study, seven strains (Table 1) were characterized both phylogenetically and physiologically, by studying fermentation spectrum on monosugars (glucose and xylose) as well as on HL´s made from various lignocellulosic biomasses. Additionally, strain AK_{17} was included in present study, an isolate already isolated at our laboratory (Orlygsson & Baldursson, 2007). Three of the seven strains were isolated on monosugars, two on xylan, one on pectin and one on cellulose (Table 2). Six of the strains belonged either to *Thermoanaerobacterium* or *Thermoanaerobacter*. The four *Thermoanaerobacterium* strains (AK_{17}, 64-07-X, 66-07-G and 66-07-P) are phylogenetically very close when compared to each other (Figure 1). Their closest relatives are *Clostridium thermoamylolyticum* and *Thermoanaerobacterium aciditolerans*. The two strains (20-07-X and 33-07-Xo) that belonged to *Thermoanarobacter* showed 99.4% homology to each other and their closest relatives are *Thermonaerobacter thermohydrosulfuricus* and *Thermoanerobacter* sp. Strain 25-07-C was the only strain isolated on cellulose and is phylogenetically far away from the other strains. 16S rRNA analysis reveals that this strain belongs to the genus *Paenibacillus* (Figure 1).

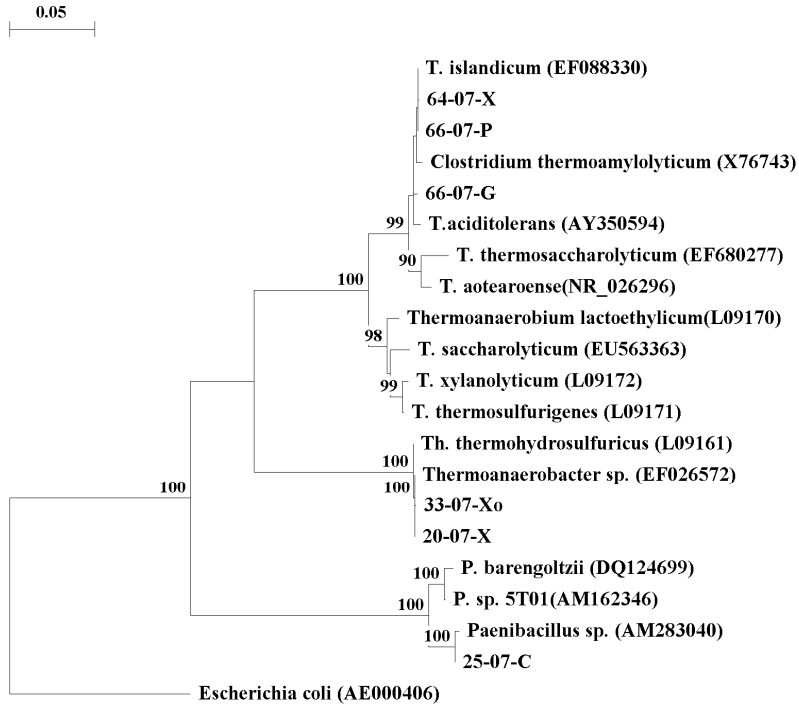


Figure 1. Phylogeny of the strains in this study based on the 16S rRNA gene partial sequences (600-700 bp). The phylogenetic tree was generated using a distance matrix and neighbouring joining algorithms with 300 bootstraps. Only supported bootstrap values (>95%) are shown. Escherichia coli (AE000406) was selected as an out-group. The scale bar indicates 0.05 substitutions per nucleotide position.

Table 2. Carbon substrates used for isolation of seven thermophilic bacteria strains. Also shown are the environmental factors used during enrichment and isolations and the closest phylogenetic genus.

Strain	Genus	Carbon source	T (°C)	pH
AK_{17}	*Thermoanaerobacterium*	Glucose	60	6
20-07-X	*Thermoanaerobacter*	Xylan	70	7
25-07-C	*Paenibacillus*	Cellulose	50	7
33-07-Xo	*Thermoanaerobacter*	Xylose	70	7
64-07-X	*Thermoanaerobacterium*	Xylan	60	7
66-07-G	*Thermoanaerobacterium*	Glucose	60	7
66-07-P	*Thermoanaerobacterium*	Pectine	60	7

Physiological properties of strains

The strains were isolated at temperatures between 50 to 70°C. During isolations they were incubated at temperatures slightly below their natural environmental temperatures. Thus, it was not surprising that most of the strains had similar temperature optimum as their isolation temperature (Table 3). The generation time for the strains varied from 0.71 h (strain 25-07-C) to 2.00 h (strain 20-07-X). There is a correlation between the phylogenetic relationship of the strains and the temperature optimum. *Paenibacillus* (25-07-C) has the lowest T_{opt} (50°); the four *Thermanaerobacterium* strains grew best at 60°C and the two *Thermoanerobacter* at 70°C. Similarly pH optimum was determined for all strains (Table 4). Strikingly, all strains grew best at different pH than the environmental pH's they were isolated from. Again, the fastest growing strain was 25-07-C but strain 66-07-P had the slowest growth rate. As for the temperature optimum there is a clear relationship between the phylogenetic status and the pH optimum. All *Thermoanaerbacterium* strains have low pH optimum (pH 5.0 – 6.0) whereas *Thermoanaerobacter* species grow best at pH between 6.0 and 7.0 and the *Paenibacillus* strain had pH optimum at pH 8.0.

Table 3. Determination of T_{opt} for seven thermophilic bacterial strains. Generation time and maximum optical density of strains are shown as well as isolation temperature.

Strain	Genus	Isolation T (°C)	T_{opt} (°C)	Generation time (h)	$OD_{max(600nm)}$
AK_{17}	*Thermoanaerobacterium*	65	58	1.24	1.27
20-07-X	*Thermoanaerobacter*	70	70	2.00	0.94
25-07-C	*Paenibacillus*	50	50	0.71	1.82
33-07-Xo	*Thermoanaerobacter*	70	70	1.22	1.46
64-07-X	*Thermoanaerobacterium*	60	60	1.01	1.24
66-07-G	*Thermoanaerobacterium*	60	60	1.95	1.63
66-07-P	*Thermoanaerobacterium*	60	60	0.96	1.47

Table 4. Determination of pH_{opt} for seven thermophilic bacterial strains. Generation time and maximum optical density of strains are shown as well as the isolation pH.

Strain	Genus	Isolation pH	pH_{opt}	Generation time (h)	$OD_{max(600nm)}$
AK_{17}	*Thermoanaerobacterium*	6	6	0.40	1.24
20-07-X	*Thermoanaerobacter*	7	6	0.93	1.14
25-07-C	*Paenibacillus*	7	8	0.39	1.59
33-07-Xo	*Thermoanaerobacter*	7	7	0.83	1.35
64-07-X	*Thermoanaerobacterium*	7	5	0.90	1.07
66-07-G	*Thermoanaerobacterium*	7	5	1.33	1.78
66-07-P	*Thermoanaerobacterium*	7	5	1.99	1.37

Ethanol tolerance

Table 5 shows the ethanol tolerance of the seven strains. The four *Thermoanaerobacterium* species (64-07-X, 66-07-P, 66-07-G, AK_{17}) and the *Paenibacillus* strain (25-07-C) showed ethanol tolerance up to 3.2% (v/v) but the two *Thermoanaerobacter* species (20-07-X, 33-07-Xo) had lower ethanol tolerance (1.6%).

Table 5. Minimum inhibitory concentrations of ethanol for seven thermophilic bacterial strains. The final optical density (OD) was used as indicator of growth; ++++ = OD > 1.0; +++ = OD between 0.7 and 1.0; ++ = OD between 0.3 and 0.7; + OD below 0.3 but above control.

		% vol / vol							
Strain	**Genus**	**0.0**	**0.2**	**0.4**	**0.8**	**1.6**	**3.2**	**6.2**	**8.0**
AK_{17}	*Thermoanaero-bacterium*	++++	++++	++++	++++	+++	++	-	-
20-07-X	*Thermoanaero-bacter*	++++	++++	++++	++++	++	-	-	-
25-07-C	*Paenibacillus*	++++	++++	++++	++++	+++	+	-	-
33-07-Xo	*Thermoanaero-bacter*	++++	++++	++++	++++	++	-	-	-
64-07-X	*Thermoanaero-bacterium*	++++	++++	++++	++++	+++	++	-	-
66-07-G	*Thermoanaero-bacterium*	++++	++++	++++	++++	+++	+	-	-
66-07-P	*Thermoanaero-bacterium*	++++	++++	++++	++++	+++	++	-	-

End product formation from glucose and xylose

Before determining the ability of the strains to produce ethanol from lignocellulosic biomass, growth characteristics on monosugars (glucose and xylose) was performed. The four *Thermoanarobacterium* strains all showed similar end product formation on glucose fermentation, i.e. high ethanol yields and low acetate formation (Table 6).

Table 6. End product formation (in mM) from glucose (20 mM) and xylose (20 mM) by seven thermophilic bacterial strains.

		Glucose (20mM)			Xylose (20mM)		
Strain	**Genus**	**EtOH (mM)**	**Acetate (mM)**	**H_2 (mmol/L)**	**EtOH (mM)**	**Acetate (mM)**	**H_2 (mmol/L)**
AK_{17}	*Thermoanaero-bacterium*	29.9	7.5	11.8	21.3	8.3	0.0
20-07-X	*Thermoanaero-bacter*	3.2	3.1	2.0	5.1	6.5	4.7
25-07-C	*Paenibacillus*	26.1	12.5	0.0	18.8	11.4	0.0
33-07-Xo	*Thermoanaero-bacter*	29.5	6.3	3.8	15.3	7.5	3.8
64-07-X	*Thermoanaero-bacterium*	22.9	9.9	13.4	19.2	10.4	17.3
66-07-G	*Thermoanaero-bacterium*	20.8	7.1	9.9	8.9	3.7	3.2
66-07-P	*Thermoanaero-bacterium*	25.1	10	12.7	25.4	8.1	8.0

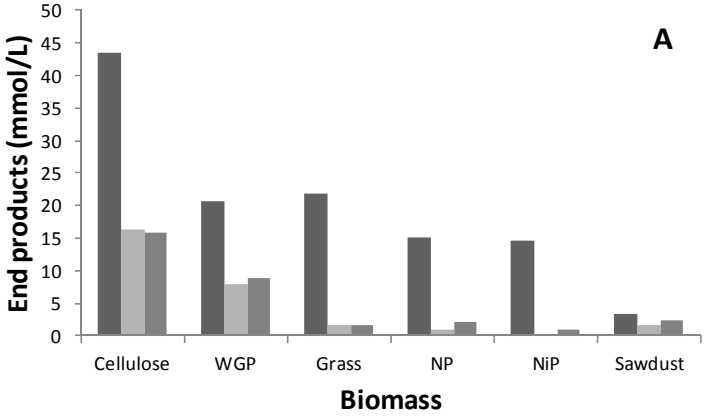
The ratio between ethanol and acetate varies between 2.3 (64-07-X) to 3.5 (AK_{17}) and the ethanol yield (mol ethanol per mol glucose) varies from 1.2 (66-07-P) to 1.70 (AK_{17}) which corresponds to 60 – 85% of theoretical yield. All *Thermoanaerobacterium* strains produced similar amounts of acetate (7.1 to 10.7 mM) and hydrogen (9.9 to 13.4 mmol l^{-1} from glucose. On xylose, similar fermentation spectrum was observed between three of the four *Thermoanarobacterium* strains; high ethanol concentrations and the ratio between ethanol and acetate was between 2.0 and 2.7. The ethanol yield for these three strains varied from 63 (64-07-X) to 83% (66-07-P) but much lower yields were observed for strain 66-07-G (35%). Acetate production was in good correlation with ethanol (30- 50% compared to ethanol) but a great variation was observed in the amount of hydrogen produced by the four strains.

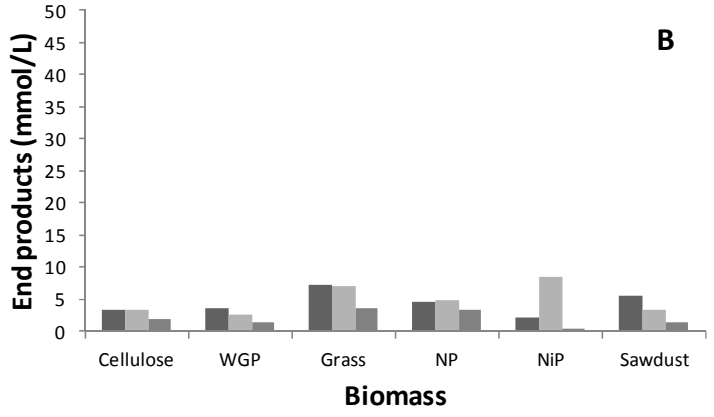
The two *Thermoanarobacter* strains (20-07-X and 33-07-Xo) showed a great variation in fermentation end product formation. Strain 33-07-Xo showed similar fermentation pattern as the "good ethanol producing" *Thermoanaerobacterium* strains with high ethanol concentrations analyzed in the fermentation broth both on glucose and xylose. Strain 20-07-X, however, was a poor ethanol and acetate producer.

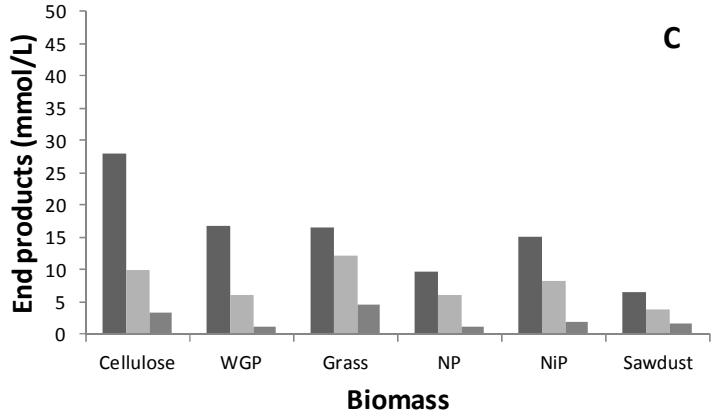
Finally, strain 25-07-C (*Paenibacillus*) was a good ethanol producer, both on glucose and xylose, but no hydrogen was detected during its fermentation on either of the monosugars tested.

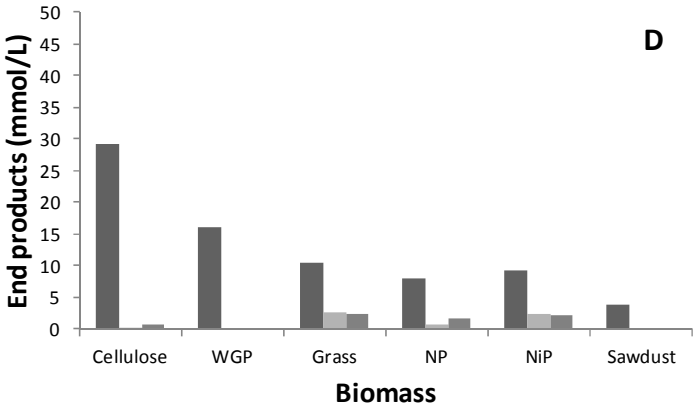
End product formation from hydrolysates

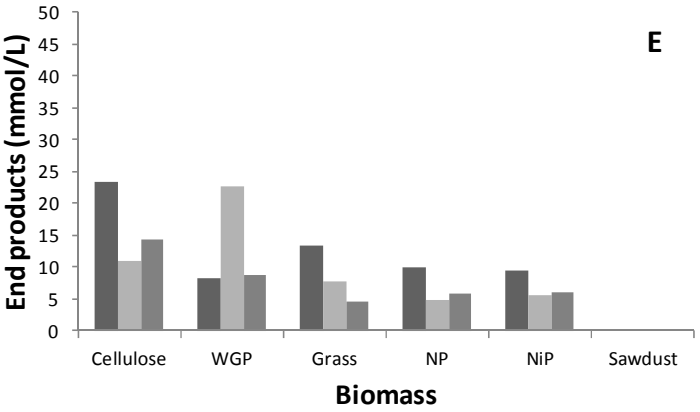
All seven strains were inoculated into medium containing 30% HL´s (0.75 g (w/v) from six different types of biomass cellulose, WGP, NPi, NP, sawdust and grass). In general, ethanol production from the HL´s were high to low in the following order; cellulose > WGP > grass > NP > NPi > saw dust (Table 6). Highest ethanol production was observed on cellulose for all strains except for the poor ethanol producer 20-07-X. Strain AK_{17} produced 43.6 mM of ethanol from cellulose HL whereas strain 20-07-X produced only 4.8 mM. The amount of end products produced from cellulose HL was in good correlation with end product formation observed on glucose alone (Table 5). The amount of ethanol produced from grass was usually less than 30% as compared to cellulose HL except for strain 20-07-X which showed slightly higher amounts. The degradation of sugars from the three different types of HL's papers used in this study resulted in most cases in somewhat lower ethanol values as compared to grass. An exception from this was though observed for strain 66-07-G on WGP which did not produce any end products from this substrate. Sawdust HL degradation resulted in lowest ethanol production for all strains.

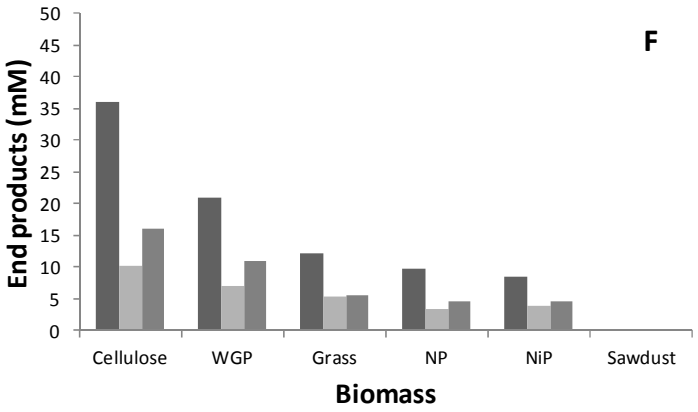

A
End products (mmol/L)
50
45
40
35
30
25
20
15
10
5
0
Cellulose
WGP
Grass
NP
NiP
Sawdust
Biomass


B
End products (mmol/L)
50
45
40
35
30
25
20
15
10
5
0
Cellulose
WGP
Grass
NP
NiP
Sawdust
Biomass


C
End products (mmol/L)
50
45
40
35
30
25
20
15
10
5
0
Cellulose
WGP
Grass
NP
NiP
Sawdust
Biomass


D
End products (mmol/L)
50
45
40
35
30
25
20
15
10
5
0
Cellulose
WGP
Grass
NP
NiP
Sawdust
Biomass


E
End products (mmol/L)
50
45
40
35
30
25
20
15
10
5
0
Cellulose
WGP
Grass
NP
NiP
Sawdust
Biomass


F
End products (mM)
50
45
40
35
30
25
20
15
10
5
0
Cellulose
WGP
Grass
NP
NiP
Sawdust
Biomass

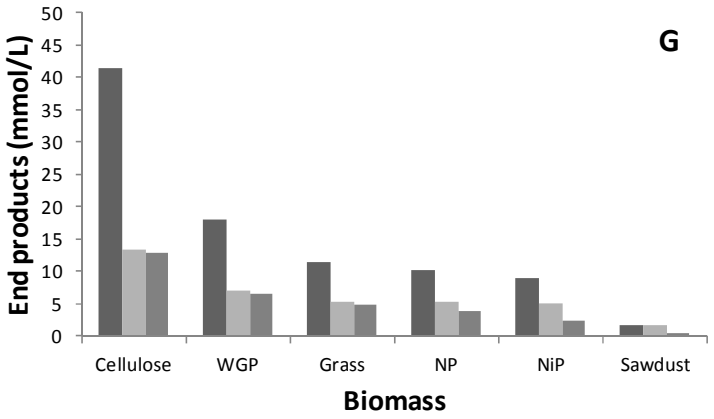


Figure 2. End product formation from biomass types tested for (A) AK_{17}, (B) 20-07-X, (C) 25-07-C (D) 33-07-Xo (E) 64-07-X (F) 66-07-G (G) 66-07-P. From left to right for biomass types are ethanol, acetate and hydrogen.

End product formation from hydrolysates

All seven strains were inoculated into a medium containing hydrolysates containing 7.5 g L^{-1} (0.75 w/v) from six different types of biomass (cellulose, WGP, NPi, NP, sawdust and grass). In general, ethanol production from the HL's ranged from high to low in the following order: cellulose > WGP > grass > NP > NPi > sawdust (Figure 2). The highest ethanol production was observed on cellulose for all strains except for the poor ethanol producer 20-07-X. Strain AK_{17} produced 43.6 mM of ethanol from cellulose HL whereas strain 20-07-X produced only 3.4 mM. The amount of end products produced from cellulose HL correlated well with end product formation observed on glucose alone (Table 6). The amount of ethanol produced from grass was between 33 to 60% of that from cellulose HL except for the poor ethanol producer 20-07-X, where it was slightly higher. Sawdust HL gave the lowest ethanol production by all strains.

Discussion

From previous studies on thermophilic, saccharolytic and anaerobic bacteria a substantial collection of ethanol and hydrogen producing bacteria have been obtained. Many of these bacteria can degrade both five and six carbon monosugars. For a viable second generation bioethanol production we need strains that can convert all the main carbohydrates constituents of lignocellulosic material to ethanol (Wright, 1988; Lynd 1996; von Sivers & Zacchi 1995).

In the present study the potential of some of these isolates to produce ethanol from monosugars (glucose and xylose) as well as from HL's made from various lignocellulosic biomasses was tested. The seven strains were subjected to a screening programme based on ethanol tolerance and yield of ethanol from monosugars. Earlier experiments had showed that increased concentration of sulfuric acid (0, 0.75 and 1.5%) used in pretreatment of lignocellulosic biomass had little effect (results not showed) and it was therefore decided to use only heat (121°C) and no acid on the biomass for 90 min.

Phylogenetic studies on strain AK_{17} revealed that this bacterium belongs to the genus *Thermoanaerobacterium*. The phylogeny of the strain and its capability of hydrogen and ethanol production have already been thoroughly investigated (Orlygsson & Baldursson, 2007, Koskinen et al. 2008). Three other strains that were isolated in the present study were closely related (less than 1.0 % difference was found within the four strains) to strain AK_{17} as well as with *Clostridium thermoamylolyticum* and *Thermoanaerobacterium aciditolerans*, the latter a well known ethanol and hydrogen producer (Kublanov et al. 2007). Two strains belong to the genus *Thermoanaerobacter* and were closely related to *Thermoanaerobacter thermohydrosulfuricus,* also known as a good ethanol producer (Vancanneyt et al. 1987, Kannan & Mutharasan 1985, Wiegel et al. 1979). Strain 25-07-C belongs to *Paenibacillus* with its closest relationship to *Paenibacillus* (AM283040), and several species within this genus are known for their ethanol production (Marwoto et al. 2004).

Ethanol production capacity among thermoanaerobes has been well known for many years now and is potentially thought a possible future renewable energy source (Cook et al. 1991, Sommer et al. 2004). *Thermoanarobacter ethanolicus* has been reported to have the highest ethanol yields from glucose and xylose. Ethanol yields from glucose were reported to be 1.9 mol-EtOH/mol glucose in batch (Wiegel & Ljundahl 1981) and in continuous cultures (Lacis & Lawford 1988; Lacis & Lawford 1991). Another well known ethanol producing thermoanaerobe

is *Thermoanaerobacter thermohydrosulfuricus,* which produces 1.5 mol-EtOH/mol glucose (Wiegel et al. 1979). The four *Thermoanaerobacterium* strains in the present study produced more than 1.0 mol-EtOH and three strains more than 0.4 mol-EtOH from glucose and xylose, respectively. The best ethanol producer on glucose was strain AK_{17} with 1.5 mol-EtOH/mol glucose (75% of theoretical yield) whereas strain 66-07-P produced most ethanol from xylose (1.27 mol-EtOH/mol xylose; 76% of theoretical yield). The two *Thermoanaerobacter* strains showed very different ethanol production yields; strain 33-07-Xo produced 29.5 mM ethanol from 20 mM of glucose (73.8% of theoretical yield) and 15.3 mM from xylose whereas strain 20-07-X produced nine times less from glucose and three times less from xylose. This could have been caused by either insufficient glucose degradation or production of other end products that were not analysed in the culture broth, e.g. lactate. The *Paenibacillus* strain (25-07-C) was a good ethanol producer with 65% and 56% yields from glucose and xylose, respectively. Ethanol production has been reported with *Paenibacillus polymyxa* yielding up to 0.74 mol-EtOH/mol-glucose (Nakashimada et al. 2000, Marwoto et al. 2004) and *Paenibacillus* sp. strain JDR-2 under oxygen limitations (Chow et al. 2007). Members of the genus *Paenibacillus* are facultative anaerobic, spore-forming organisms and thermophilic strains have been reported (Wang et al. 2008). Some of these bacteria excrete diverse assortments of extracellular polysaccharide hydrolyzing enzymes, including xylanases, cellulases, amylases, gelatinases, ureases and β-galactosidases (Velázquez et al. 2004).

Other end products produced were hydrogen and acetate, both well known end products from sugar fermentation by species within *Thermoanaerobacter*, *Thermoanaerobacterium* and *Paenibacillus* (Nakashimada et al. 2000, Marwoto et al. 2004, Wiegel & Ljundahl 1981, Vancanneyt et al. 1987).

To compare ethanol yields from cellulosic and hemicellulosic material HL's (7.5 g L^{-1}) were made from paper, grass and sawdust as well as from pure cellulose (Whatman paper). High ethanol yields were obtained from the cellulose but due to the differing lignin content of some of other biomass types, lower yields were observed (Figure 2). Whatman paper consists of 99% glucose. This means that if all the glucose that is bound in the paper cellulose is released during hydrolysis and enzyme treatment, a glucose concentration of 41.7 mM would have been present in the final media mixed with the HL's. The end product stochiometry of glucose fermentation by strain AK_{17} is (data derived from Table 6):

$$1.00\ \text{Glucose} \rightarrow 1.50\ \text{EtOH} + 0.38\ \text{Acetate} + 1.88\ CO_2 + 0.30\ H_2 \quad \text{(I)}$$

Thus, from the cellulose HL's 41.7 mM of glucose would theoretically give 62.5 mM of ethanol. The actual ethanol concentration found in the fermentation broth of strain AK_{17} was however 43.6 mM (control subtracted) or 70% the theoretical yield according to equation I. The most probable reason for this low yield was because of the high initial glucose concentrations causing substrate inhibition (Sommer et al., 2004). Indeed, different loadings of cellulose HL's have shown that ethanol yields for strain AK_{17} ranged from 97% at 5.0 g L^{-1} hydrolysate to 26% at 17.5 g L^{-1} hydrolysate HL caused by undegraded glucose residues in the culture broth (results not shown).

The fermentation of strain AK_{17} on other HL's showed lower ethanol production. Similar values were obtained for the WGP and grass: 20.6 to 21.9 mM. The other paper types (both NP and NPi) gave slightly lower ethanol production (14.6 to 15.2 mM) but much lower yields were observed from sawdust. The other strains produced less ethanol from the various HL's but production was generally proportional to the lower yields from monosugars (Table 6 and Figure 2).

The yields of ethanol produced in the present study can be regarded as relatively good when compared to other studies. Sommer and co-workers (Sommer et al. 2004) showed that thermophilic bacteria produced between 9.8 – 25.7 mM of ethanol from undiluted wheat straw hydrolysate (60 g L^{-1}). *Thermoanaerobacter ethanolicus* produced 24 mM of ethanol when cultivated in steam-exploited birch wood hemicelluloses hydrolysate (0.8 w/v) (Wiegel et al. 1983) and *Clostridium thermosaccharolyticum* produced 40 mM of ethanol in oak sawdust pretreated with 1% sulfuric acid (Liu et al. 1988). Clearly, at high hydrolysate concentrations, the yield of ethanol decreases dramatically. Microorganisms producing promising yields on pure glucose and xylose do not necessarily do well in pretreated hydrolysate that contains inhibitory compounds like acetate, furfural and lignin degradation products (Watson et al. 1984, Palmqvist and Hahn-Hagerdal 2000).

Some investigation of ethanol production in pretreated lignocellulosic biomass is an important screening criterion when considering a microorganism for real-life applications (Hahn-Hagerdal et al. 1993, Zacchi et al. 1988, Wyman 1999, Hinman et al. 1989). Grass or waste paper is likely to be the future substrates in Icelandic ethanol production, based on the conversion of both the cellulose and the hemicellulose fractions into ethanol. The strains isolated here are therefore promising candidates for such applications.

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